Islet Coordinately Regulates Motor Axon Guidance and Dendrite Targeting through the Frazzled/DCC Receptor

Graphical Abstract

Highlights

- Islet is required for fra expression in a subset of motor neurons
- Islet overexpression induces fra expression and a fra-dependent phenotype
- Isl and fra are required for axon and dendrite targeting in motor neurons
- Overexpression of Fra rescues motor axon and dendrite targeting in isl mutants

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In Brief

How individual transcription factors establish multiple aspects of neuronal identity remains poorly understood. Santiago and Bashaw find that the Islet transcription factor coordinately regulates two essential aspects of motor neuron morphology, axon and dendrite position, through a single downstream effector, the guidance receptor Frazzled/DCC.
Islet Coordinately Regulates Motor Axon Guidance and Dendrite Targeting through the Frazzled/DCC Receptor

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SUMMARY

Motor neuron axon targeting in the periphery is correlated with the positions of motor neuron inputs in the CNS, but how these processes are coordinated to form a myotopic map remains poorly understood. We show that the LIM homeodomain factor Islet (Isl) controls targeting of both axons and dendrites in Drosophila motor neurons through regulation of the Frazzled (Fra)/DCC receptor. Isl is required for fra expression in ventrally projecting motor neurons, and isl and fra mutants have similar axon guidance defects. Single-cell labeling indicates that isl and fra are also required for dendrite targeting in a subset of motor neurons. Finally, overexpression of Fra rescues axon and dendrite targeting defects in isl mutants. These results indicate that Fra acts downstream of Isl in both the periphery and the CNS, demonstrating how a single regulatory relationship is used in multiple cellular compartments to coordinate neural circuit wiring.

INTRODUCTION

The diversity of cell types is one of the nervous system’s most remarkable features, and understanding how this diversity is achieved remains a major challenge. Many studies have shown that combinations of transcription factors act in a cell-type-specific manner to specify a neuron’s morphological and functional properties (Corty et al., 2009; Hobert, 2015). The regulation of axon and dendrite targeting is key to determining a neuron’s connectivity and is controlled through the combined action of guidance receptors, adhesion molecules, and cytoskeletal regulators (Lefebvre et al., 2015; O’Donnell et al., 2009). Although recent studies have begun to delineate relationships between these cellular effectors and the transcription factors that control their expression, it remains unclear to what extent individual transcription factors regulate multiple aspects of neural morphogenesis (Santiago and Bashaw, 2014). In particular, although several factors have been shown to control both axon and dendrite development, whether they do so through shared or distinct targets is unknown. In mice, the target-induced E26 transformation-specific (ETS) factor Pea3 (Etv4) is required for axonal branching in a subset of limb-innervating neurons and for the position and connectivity of motor neuron dendrites in the spinal cord (Livet et al., 2002; Vrieseling and Arber, 2006). In the Drosophila olfactory system, the Pit-Oct-Unc (POU) factor Acj6 is required in a subset of projection neurons both for axonal branching in the lateral horn and for dendrite targeting in the antennal lobe (Komiyama et al., 2003). However, in these and other examples, the downstream programs that mediate the effects of these transcription factors on axon and dendrite targeting remain unidentified.

In the Drosophila larval and adult nervous systems, motor neuron dendrites form within stereotyped medio-lateral regions in the CNS that correlate with cell identity and with the position of motor axons in the periphery (Baek and Mann, 2009; Brierley et al., 2009; Mauss et al., 2009). Slit-Roundabout (Robo) and Netrin-Fra signaling are key regulators of dendrite targeting, and manipulating the levels of Robo1 or Fra causes shifts in dendrite position, suggesting that these receptors act in a cell-autonomous manner (Brierley et al., 2009; Mauss et al., 2009).

In adult motor neurons, birth order correlates with dendrite position, suggesting the involvement of a temporal code of transcription factors (Brierley et al., 2009). There is no indication that birth order plays a role in dendrite targeting in the embryo (Mauss et al., 2009); instead, the correlation between the dorsal-ventral position of axons and the medio-lateral position of dendrites suggests the intriguing hypothesis that the same factors that specify dorsal-ventral axon guidance decisions may also regulate dendrite position. Taken together, these data suggest that subset-specific transcription factors regulate dendrite targeting through their effects on fra, robo1, or genes in those pathways in Drosophila motor neurons. However, this model remains untested.

The well-conserved transcription factors Even-skipped (Eve), Hb9, Islet (Isl), and Lim3 are expressed in restricted subsets of embryonic motor neurons and have been extensively studied in the context of axon guidance, but whether the same transcriptional regulators control dendrite development is not known (Broihier and Skeath, 2002; Fujikoa et al., 2003; Labrador et al., 2005; Landgraf et al., 1999; Odden et al., 2002; Thor and Thomas, 1997; Thor et al., 1999). We have shown previously that Hb9 acts through the Robo2 receptor to regulate axon guidance in RP3 neurons, a subset of ventrally projecting motor neurons.
neurons (Santiago et al., 2014). Here we describe a parallel pathway by which Isl regulates fra expression in the same neurons and demonstrate that this pathway is important for muscle target selection. We also characterize a requirement for both isl and fra in regulating the medio-lateral position of RP3 dendrites and show that the dendrite targeting defects in isl mutants can be rescued by cell-type-specific overexpression of Fra. These results provide an example of how a single transcription factor contributes to neural map formation by coordinately regulating the guidance of axons to their peripheral targets, and of dendrites to their positions in the central nervous system, through its effect on a single downstream effector.

RESULTS

**Isl Is Required for fra Expression in RP Motor Neurons**

The RP3 motor neurons innervate the NetrinB-expressing muscles 6 and 7 and are enriched for fra mRNA during the late stages of embryonic development, and it was reported previously that, in the absence of fra or Netrin, there are significant defects in the innervation of muscles 6 and 7 (Kолодziej et al., 1996; Labrador et al., 2005; Mitchell et al., 1996). This phenotype is also detected in the absence of hb9/exex or isl/tailup, two transcription factors expressed in RP3 as well as in other ventrally projecting motor neurons, suggesting that hb9 or isl may regulate fra (Broihier and Skeath, 2002; Thor and Thomas, 1997). Interestingly, Hb9, Isl, and the LIM homeodomain factor Lim3 were all recently shown to bind directly to the fra locus in vivo, as determined by a genome-wide DNA adenine methyltransferase identification (DAM-ID) analysis performed in Drosophila embryos (Wolfram et al., 2014; Figure S1). However, DAM-ID results do not provide information about the functional significance of the detected binding events or about the cell types in which they occur. To determine whether Hb9, Isl, or Lim3 regulate the expression of fra in embryonic motor neurons, we performed in situ hybridization experiments and analyzed fra mRNA expression with single-cell resolution in embryos mutant for these factors (Figure 1; Figure S2). We found that only isl is required for fra expression in the RP3 motor neurons at stage 15, when RP axons have reached the ventral muscle field but have not yet selected their final targets. 80% of RP3 neurons in abdominal segments A2–A7 in isl/+ embryos are positive for fra mRNA versus 38% in isl mutant embryos (p < 0.001; Figure 1D). We also observed a significant difference in fra mRNA levels in RP3 neurons between isl mutants and heterozygotes when quantifying pixel intensity from the fra in situ, whereas we detect no difference in the signal of the isl-H-tau-myc transgene (p < 0.01; Figure 1B). We detect no change in the number or position of RP3 neurons in isl mutants, consistent with previous data demonstrating that Isl is not required for the generation or survival of Drosophila motor neurons (Thor and Thomas, 1997). Importantly, we did not find a requirement for either hb9 or lim3 in regulating fra mRNA expression in any RP motor neurons (Figure S2), demonstrating that Isl’s effect on fra is specific and could not have been predicted simply from similarities in loss of function phenotypes or from transcription factor binding data. We previously found that Hb9 is required for robo2 expression in RP3 (Santiago et al., 2014). Interestingly, just as hb9 is not required for fra expression in RP neurons, isl is not required for robo2 expression (Figure S2). A previous study reported that isl: hb9 double mutants have a stronger intersegmental nerve b (ISNb) phenotype than either single mutant, but muscle 6/7 innervation defects were not quantified (Broihier and Skeath, 2002). We scored motor axon guidance defects in isl: hb9 double
mutants and found that the double mutants display significantly more muscle 6/7 innervation defects than either single mutant (38% of hemisegments with defects in isl; hb9 double mutants compared with 20% in isl mutants and 17% in hb9 mutants, p < 0.01 in both cases; Figure 2C). Similarly, embryonic mutant for both robo2 and fra have a stronger motor axon phenotype than either robo2 or fra single mutants (44% in robo2ex123, fra+/robo2ex123, fra−/+ robo2ex123, fra−/− mutants versus 20% in fra−/+fra−/− mutants and 21% in robo2ex123/robo2ex123 mutants; p < 0.001 in both cases; Figure S2). Note that, because robo2, fra double mutants have severe defects in midline crossing, motor axon phenotypes should be interpreted with caution (Evans et al., 2015). These results show that Hb9 and Isl act in parallel to regulate distinct downstream programs in RP3 neurons, demonstrating how combinations of transcription factors result in specific cell surface receptor profiles and axon trajectories.

**Restoring fra Expression in isl Mutants Rescues Ventral Muscle Innervation**

To determine whether isl and fra act in the same genetic pathway during RP3 guidance, we examined embryonic mutant for both genes. In isl-null mutants, 20% of hemisegments lack muscle 6/7 innervation, whereas fra-null mutants have a significantly stronger phenotype (34% of hemisegments, p < 0.01; Figure 2C). isl, fra double mutants do not have more muscle 6/7 innervation defects than fra single mutants (40%, p = 0.2; Figure 2C), consistent with isl and fra acting in the same pathway. If fra acts downstream of Isl during motor axon targeting, then we reasoned that restoring Fra expression in isl mutant neurons might rescue muscle 6/7 innervation. Indeed, we found that pan-neural overexpression of Fra in isl mutants partially but significantly rescues these defects (Figure 2D). The difference between genotypes was most noticeable when we counted hemisegments in which a growth cone stalls at the 6/7 cleft as well as those in which it fails to reach it (all embryos were scored blind to genotype; see Experimental Procedures). In isl mutants, a growth cone stalls at or fails to reach the 6/7 cleft in 27% of hemisegments compared with 15% of hemisegments in sibling mutants overexpressing Fra (p = 0.003; Figure 2D). We also analyzed the data by comparing the number of embryos with 6/7 innervation defects. In isl mutants, 0% of embryos have no 6/7 innervation defects in A2–A6, 44% have one defect, and 56% have two or more defects (n = 16 embryos). In contrast, in isl mutants overexpressing Fra, 29% of embryos have no innervation defects, 29% have one defect, and 41% have two or more defects (n = 24 embryos, p = 0.03 by Fisher’s exact test when comparing the number of embryos with no defects). The incomplete rescue could be due to differences in the timing or levels of GAL4/UAS-mediated expression of Fra compared with its endogenous regulation or could indicate that Isl regulates additional downstream effectors important in this process. Nevertheless, these data strongly suggest that Fra is an essential downstream effector of Isl during the guidance of the RP3 axon to its target muscles (Figure 2E).

**Overexpression of Isl in Ipsilateral Neurons Induces fra Expression and fra-Dependent Midline Crossing**

To further investigate the relationship between isl and fra, we tested whether ectopic expression of isl is sufficient to induce fra expression. For these experiments, we used the apterous (ap) neurons, a subset of interneurons whose axons form a single fascicle on either side of the midline that are labeled by ap-Gal4 (Lundgren et al., 1995). The ap neurons express low levels of fra (see below), do not express isl (Thor and Thomas, 1997; data not shown), and do not cross the midline. Fra overexpression causes ectopic midline crossing of ap axons (Neuhaus-Folli and Bashaw, 2015; O’Donnell and Bashaw, 2013). We found that overexpression of Isl with ap-Gal4 produces high levels of midline crossing, phenocopying the effect of Fra overexpression (Figures 2F and 2H). In stage 17 control embryos, ap axons cross the midline in 12% of segments, whereas in embryos overexpressing UAS-Isl with ap-Gal4, ap axons cross the midline in 60% of segments (Figure 2H). This phenotype is dose-dependent because embryos with two copies of an UAS-Isl insertion display significantly more ectopic midline crossing than embryos with one insert (84%, p < 0.001, data not shown).

To determine whether Isl overexpression results in fra induction, we analyzed the expression of fra mRNA in situ in ap neurons (Figure 2G). We found that, in stage 15 wild-type embryos, a low percentage of ap neurons express fra (25% of ventral ap clusters were fra+). In contrast, in embryos overexpressing isl from two UAS-Isl insertions, 37% of the ventral ap clusters were fra+ (p < 0.01 compared with controls) (Figure 2G). To test whether the ectopic crossing phenotype depends on fra function, we overexpressed Isl in embryos homozygous for a null allele of fra. Strikingly, removing fra completely suppresses the crossing phenotype, indicating that fra is required for Isl to produce its gain-of-function effect (15% of segments with ap midline crossing in fra−/fra− embryos overexpressing UAS-Isl, p < 0.0001 compared with 1× GOF (gain of function) in controls, not significant (n.s.) compared with fra−/fra−; Figure 2H). Although we cannot rule out that Isl affects the expression of other genes in the Fra pathway to cause midline crossing, these results demonstrate that ectopically expressing Isl causes an increase in fra expression and a fra-dependent axon guidance phenotype and suggest that the functional relationship between isl and fra may be used in multiple contexts.

**Isl Is Not Required for Early fra Expression or for RP Axon Midline Crossing**

Fra mutants have defects in RP axon midline crossing, as shown by retrograde labeling of single motor neurons (Furrer et al., 2003). In addition, Netrin-Fra signaling controls the medio-lateral position of dendrites in several groups of motor neurons (Mauss et al., 2009). Therefore, we asked whether isl regulates midline crossing or RP3 dendrite development through fra. We used a genetic strategy to label single motor neurons by mosaic expression of a membrane-tethered GFP transgene under the control of lim3b-GAL4, which labels RP motor neurons, sensory neurons, and several other motor and interneuron populations (Cerlet and Thor, 2004). We identified RP3 neurons by the stereotyped position of the RP3 cell body and by the targeting of its axon to muscles 6 and 7. Because of the axon targeting defects observed in isl and fra mutants, we relied on cell body position to identify RP3 neurons in mutants. By this approach, we detect significant midline crossing defects in RP3 axons in fra mutants, as reported previously (18 of 23 axons fail to cross the midline in...
In (C), fra/fra versus 0/16 axons in fra/+). To our surprise, however, we observed no defects in RP axon midline crossing in isl mutants (33 of 33 RP3 axons cross the midline in isl/isl).

Isl and fra expression both initiate earlier than stage 13, the time at which RP axons cross the midline (Broadie and Bate, 1993; Thor and Thomas, 1997). Therefore, we examined whether isl is required for fra expression during the early stages of commissural axon guidance. Interestingly, we found that isl is not required for fra expression at stage 13 in any of the ventrally projecting RPs (Figure S3). In contrast, in stage 15 isl mutant embryos from the same collection, we observed a decrease in fra expression in RP1 and RP3 (Figures 1 and 6). The temporal pattern of fra expression in RP motor neurons is dynamic, so that a larger proportion of RP1 and RP3 neurons express fra mRNA during late embryogenesis than during the stages of midline crossing. We detect a requirement for isl in regulating fra in RP1 and RP3 as early as stage 14, when the RP motor axons have exited the CNS (Figure S3). Taken together, these results suggest that isl is not essential for early fra expression but required for fra expression during the late stages of motor neuron differentiation. The stages at which we detect a requirement for isl in regulating fra correspond to when RP3 axons are exploring their ventral muscle targets, consistent with a model in which Isl instructs the final stages of RP3 axon targeting through Fra.

A Difference in Dendritic Targeting between RP3 and RP5 Neurons Correlates with a Difference in fra Expression

Figure 2. Isl Acts through fra to Regulate RP3 Motor Axon Guidance, and Isl Overexpression in Interneurons Induces fra Expression and a Midline-Crossing Phenotype

(A) Schematic of two hemisegments; dorsal is up and anterior is left. The asterisk indicates the absence of muscle 6/7 innervation by RP3.

(B) isl or fra mutant embryos stained for FasII, which labels all motor axons. Asterisks indicate an absence of muscle 6/7 innervation. (C) isl/hb9 double mutants have an additive phenotype compared with the single mutants (**p < 0.001, Student’s t test). (D) isl mutants overexpressing Fra pan-neurally have fewer defects than sibling mutants (*p < 0.01, Student’s t test).

(E) Model for how Isl and Hb9 regulate RP3 axon guidance (see also Santiago et al., 2014).

(F) Stage 17 embryos in which the ap neurons are labeled with apGFP > UAS-TauMycGFP. Isl overexpression causes ectopic midline crossing (asterisks), which is suppressed when isl is overexpressed in fra mutants.

(G) Quantification of fra+ ventral ap neurons in wild-type embryos and embryos overexpressing Isl, following in situ hybridization for fra mRNA. Overexpression of Isl causes upregulation of fra in ventrally expressed ap neurons (*p < 0.005, Student’s t test).

(H) Quantification of fra axon midline crossing. Isl gain of function causes ectopic crossing (**p < 0.001, Student’s t test) but not when Isl is overexpressed in fra-null mutants.

We used the FLP-out genetic labeling strategy to visualize individual late-stage RP motor neurons and analyze their dendrites. We focused on the large contralateral arbor of the RP motor neurons that spans one side of the nerve cord in wild-type embryos and forms branches that extend into several medio-lateral zones (Mauss et al., 2009). Analyses using isl-tau-myc and lim3a-tau-myc transgenes confirmed that the RP cell bodies retain their stereotyped positions in isl mutants and that the relative dorsal-ventral positions of RPs 1/4, 3, and 5 are preserved, allowing us to identify distinct classes of RP motor neurons (Landgraf et al., 1997; data not shown).

We found that most RP3 neurons in late-stage isl/+ embryos form contralateral dendritic arbors that send projections into the zone between the medial FasII+ axon pathways and the intermediate FasII+ pathways, hereafter referred to as the “intermediate zone,” consistent with previously published images of RP3 neurons from wild-type embryos (89%, n = 18; Figure S1; Movie S1). Interestingly, the dendritic morphology of RP3 was distinct from that of a related neuron, RP5, that also expresses Isl and Lim3b-Gal4 and that can be unambiguously identified in both wild-type and mutant embryos because its cell body is found in a more ventral position than the other RP neurons (Landgraf et al., 1997; Movie S2). In wild-type embryos, the RP5 axon targets muscles 12 and 13 (VL1 and VL2) as well as other ventral muscles (Landgraf et al., 1997; Mauss et al., 2009). Most RP5 neurons in isl/+ embryos exclusively target their dendrites to the lateral zone of the neuropleurite (80%, n = 20; Figure 3A). Furthermore, the difference we observe in the dendritic targeting of RP3 and RP5 neurons correlates with a difference in fra expression. Although fra expression in RP3 and RP5 neurons in control embryos is comparable when RP axons are crossing the midline (Figure S3), by stage 15, significantly fewer RP3 than RP5 neurons express fra (Figure 3B). Interestingly, isl is not required for the low levels of fra expression in late-stage RP5 neurons, in contrast to its role in promoting high levels of fra in late-stage RP3 neurons (Figure 3B).

Finally, we monitored endogenous Netrin expression in late-stage nerve cords using a Myc-tagged NetB knockin allele (Brankatschk and Dickson, 2006) and detected significant
enrichment of Netrin protein in the area between the intermediate and medial FasII+ axon bundles (Figures 3C and 3D). This area corresponds to the zone where we detect contralateral dendritic projections from RP3 neurons, suggesting that high levels of Fra in RP3 may instruct the formation of dendritic arbors in this region in response to Netrin.

**isl and fra Regulate the Targeting of RP3 Motor Neuron Dendrites in the CNS**

We next analyzed RP motor neuron dendrites in *isl* mutant embryos to determine whether Isl regulates dendritic position or morphogenesis through Fra or other effectors. We did not observe a significant difference in the morphology or medio-lateral position of RP5 dendrites between heterozygous and mutant embryos (data not shown). In striking contrast, many RP3 neurons in *isl* mutants fail to extend contralateral dendrites into the intermediate zone (48%, n = 33, p = 0.01 compared with *isl/+* embryos, Fisher’s exact test; Figures 4A and 4B). Instead, the dendrites of these RP3 neurons remain fasciculated with the intermediate FasII+ axon pathways and do not send medial extensions toward the midline (Figure 4A). To more quantitatively measure medio-lateral position and to address the possibility that defects in targeting are secondary to defects in outgrowth, we traced RP3 neurons using Imaris software and measured total contralateral dendrite lengths and the total number of dendrite tips (Figures 4C–4F; see Figure S4 for additional examples of dendritic traces and Movies S3 and S4 for examples of z stacks that were used for tracing). We also measured the total length of contralateral dendrites in the intermediate zone of the neuropile, defined as the area between the medio-FasII+ and the intermediate FasII+ axon pathways (Figures 4C and 4D). Although RP3 neurons displayed increased variability in the size of their dendritic arbors in *isl* mutants, there was no significant difference in the total length or tip number of RP3 dendrites between *isl* mutants and heterozygotes, suggesting that targeting defects in *isl* mutants are not likely due to reduced outgrowth (Figures 4E and 4F). However, the ratio of RP3 dendrites in the intermediate zone over total RP3 dendrite length was significantly reduced in *isl* mutants, confirming that *isl* mutant RP3 dendrites are shifted laterally relative to controls (p = 0.01; Figures 4C and 4D).

We next analyzed the dendrites of RP3 neurons in *fra/+* and *fra* mutant embryos (Figure 5). As with *isl* mutants, we relied upon cell body position to identify RP3 neurons and excluded neurons with ambiguous positions (Supplemental Experimental Procedures). In *fra* mutant RP3 neurons whose axons fail to cross the midline, a single dendritic arm forms off the ipsilateral primary neurite, and we traced this arbor. We observed a significant lateral shift in the position of RP3 dendrites in *fra* mutants both by scoring for the presence of dendrites in the intermediate zone and by quantitative analysis of the dendrites of traced neurons (Figures 5B and 5C). The lateral shift in *fra* mutants was more pronounced than in *isl* mutants, consistent with our observation that some RP3 neurons retain *fra* expression in the absence of *isl* (Figure 1). Of note, the lateral shift phenotype did not correlate with whether the RP3 axon had crossed the midline because we detected it at similar frequencies in both contralateral and ipsilateral arbors (Figure 5A). Curiously, several RP3 contralateral dendritic arbors appeared reduced in size in
**Figure 4. Isl Regulates the Medio-lateral Targeting of RP3 Dendrites in the CNS**

(A) RP3 neurons from stage 17 embryos of the indicated genotypes are labeled with anti-GFP (green); FasII+ axons are stained (magenta). Arrows point to the intermediate zone; arrows with asterisks point to dendrites that fail to target the intermediate zone. Higher-magnification panels of the selected areas are shown. Asterisks indicate background fluorescence from a cell in another plane. Right: contralateral dendrites were traced on Imaris; skeletons of traces are shown against FasII+ axons.

(B) Percentage of RP3 neurons that target their contralateral dendrites to intermediate and lateral regions of the nerve cord. Fewer RP3 neurons extend dendrites into the intermediate zone in isl/isl embryos (*p < 0.05, Fisher's exact test).

(C) Examples of dendrite traces in isl/+ and isl/isl embryos in which intermediate and lateral dendrites are color-coded in cyan and yellow, respectively. FasII+ axons are shown in red.

(D) Box and whisker plots of the length of RP3 contralateral dendrites in the intermediate zone divided by the total length of RP3 contralateral dendrites. isl/isl neurons have a reduction in the fraction of dendrites in the intermediate zone (p = 0.01, Student's t test).

(E) Box and whisker plots of the total length of contralateral RP3 dendrites. There is no significant difference between isl/+ and isl/isl neurons (p = 0.75).

(F) Box and whisker plots of the number of contralateral dendrite tip endings. There is no significant difference between isl/+ and isl/isl embryos (p = 0.67).

(G) Summary of the frequency of axon and dendrite defects detected in isl/+ and isl/isl RP3 neurons. n, number of neurons.

In (D)–(F), the mean is indicated by x. Inner points and outlier points are displayed. An exclusive median method was used to calculate quartiles. isl/+ denotes tup^+/+, lim3b-Gal4/Cyo, elav^+/ig. isl/isl denotes tup^/+, lim3b-Gal4/Dif2L)Exel7072. Scale bars, 5 μm. See also Figure S4 and Movies S3 and S4.
fra mutants (Figure 5A, sixth panel), whereas this phenotype was not seen in control embryos. However, as in isl mutants, there was no significant change in the total dendrite length or tip number in fra mutants compared with their sibling controls, although there was increased variability in the sizes of dendritic arbors in the mutants (Figures 5D and 5F). These findings are consistent with previous reports that Netrin-Fra signaling does not play a major role in regulating the outgrowth of motor neuron dendrites in the nerve cord (Brierley et al., 2009; Mauss et al., 2009).

**Axon and Dendrite Targeting Defects Are Not Correlated in Individual RP3 Neurons**

Our single-cell labeling method allows us to precisely describe the axon targeting defects in isl and fra mutants and determine whether they correlate with defects in dendrite position. Axon and dendrite targeting occur at approximately the same developmental stage, and there is no evidence that one process depends on the other (Kim and Chiba 2004; Landgraf et al., 2003). Importantly, previous studies using retrograde labeling of motor neurons in mutant embryos were not able to address this question because they relied upon motor axons reaching the correct muscles to be visualized (Mauss et al., 2009).

To determine whether defects in dendrite position correlate with defects in axon targeting, we scored both phenotypes in single labeled RP3 neurons in embryos with muscles fully preserved following dissection. All of the RP3 axons that we were able to score in isl heterozygous embryos innervated the muscle 6/7 cleft (n = 14; Figure 4G). In contrast, 18 of 26 isl mutant RP3
axons innervated muscles 6/7, and eight stalled at the 6/7 cleft or earlier along RP3’s trajectory or bypassed the choice point (31% have defects, n = 26; Figure 5G). In fra mutant embryos, 10 of 22 RP3 neurons failed to innervate the muscle 6/7 cleft and stalled at or bypassed the choice point (45% have defects, data not shown). This phenotype is stronger than the frequency at which we detect a complete loss of muscle 6/7 innervation in isl or fra mutants by scoring with anti-FasII (Figure 2). To determine whether this enhancement was due to the heat shock (H.S.) step that is required for genetic labeling, we scored defects using anti-FasII in embryos heat-shocked for either 5 min or 1 hr (Experimental Procedures) and found that the 1-hr H.S. mildly enhances muscle innervation defects in isl mutants (to 30.4%) whereas a 5-min H.S. does not (to 24.7%, data not shown). Importantly, the two H.S. protocols did not result in any difference in the frequency of dendrite targeting defects observed in isl mutants because 7 of 17 RP3 dendrites in isl mutants are shifted laterally in embryos treated with 1-hr H.S. and 9 of 16 dendrites are shifted after 5-min H.S (data not shown).

To our surprise, we did not detect a correlation between axon and dendrite defects in isl mutants (Figure 4G). Although 5 of 26 RP3 neurons displayed defects in both axons and dendrites in isl mutants, 12 of 26 neurons showed defects in one process but not the other (Figure 4G). A similar analysis in fra mutants revealed that 8 of 22 RP3 neurons displayed defects in both muscle 6/7 innervation and dendrite position, whereas 8 of 22 displayed normal targeting in one process but not the other (data not shown). These data suggest that axon and dendrite targeting can occur independently within an individual RP3 neuron and that the central targeting defects we observe in isl mutants are not likely to be secondary to defects in muscle innervation.

isl and fra Regulate the Targeting of RP1 and RP4 Motor Neuron Dendrites

We next asked whether isl and fra regulate dendrite development in other classes of motor neurons. RP1 and RP4 also express isl, fra, and lim3b-Gal4. We detect a requirement for isl in regulating fra expression in RP1, but not in RP4, at stage 15 (Figure 6C).
Interestingly, most RP1 neurons, like RP3 neurons, retain high levels of fra at this stage, whereas few RP4 neurons express fra in late-stage control embryos (Figure 6C). Previous descriptions of RP1 and RP4 neurons indicate that they form contralateral dendritic arbors of distinct morphologies; RP1’s dendritic arbor is taller and found more medially (Mauss et al., 2009). However, because the axons of RP1 and RP4 target adjacent muscles external to muscles 6 and 13, and their cell bodies are found close to the midline at a similar dorsal-ventral position, we could not unambiguously distinguish between them in our single-cell labeling experiments. Nevertheless, when we scored RP1 and RP4 neurons together, we observed a significant lateral shift in the position of RP1 and RP4 dendrites in isl mutants compared with heterozygous siblings: 3 of 22 RP1 and RP4 dendritic arbors were excluded from the intermediate zone in isl heterozygous embryos (14%) compared with 16 of 22 in mutant embryos (73%; p = 0.0002, Fisher’s exact test; Figures 6A and 6D). We detected a similar phenotype in RP1 and RP4 dendrites in fra mutants. Specifically, 6 of 24 RP1 and RP4 dendritic arbors were excluded from the intermediate zone in heterozygotes (25%) compared with 17/19 in fra mutants (93%; p < 0.0001, Fisher’s exact test; Figures 6B and 6E). Although additional work will be necessary to determine whether the defects in dendrite position that we detect in RP1 and RP4 neurons in isl mutants correlate with changes in fra expression, these data demonstrate that isl is required for high levels of fra expression in at least two classes of motor neurons (RP1 and RP3), both of which require isl and fra for dendritic targeting.

**isl Regulates Dendrite Development in RP3 Neurons through fra**

To directly test whether isl regulates RP3 dendrite position through its effect on fra expression, we overexpressed a UAS-HA-Fra transgene using lim3b-GAL4 in isl mutants and used the hsFLP technique to sparsely label RP motor neurons (Figure 7). Strikingly, in isl mutants overexpressing Fra, 0 of 21 RP3 contralateral dendritic arbors were excluded from the intermediate zone compared with 8 of 22 (36%) in siblings lacking the UAS-Fra transgene (p < 0.01, Fisher’s exact test; Figure 7B). To quantitatively measure dendrite position, we obtained traces of RP3 dendrites. We detected a robust rescue of the lateral shift phenotype in isl mutants, as measured by the length of dendrites in the intermediate zone over the total dendrite length (p < 0.001, Student’s t test; Figure 7C). Indeed, the ratio of dendrites in the intermediate zone in rescued mutants was higher than in heterozygous controls, perhaps reflecting a gain-of-function effect caused by artificially high levels of Fra from transgenic overexpression. Importantly, Fra overexpression did not have any effect on total dendritic arbor lengths or tip numbers (Figures 7D and 7E), strongly arguing that the rescue we observe is not caused by an increase in the total size of the arbors. Although we cannot rule out that Isl regulates dendrite position in part through additional effectors, our observation that cell-type-specific overexpression of Fra in isl mutants rescues dendrite targeting provides compelling support for the model that fra acts downstream of isl to control RP3 dendrite morphogenesis. Together with our demonstration that isl directs...
RP3 motor axon targeting through the regulation of fra, we conclude that isl coordinates regulates the targeting of axons in the periphery and of dendrites in the CNS through a common downstream effector.

**DISCUSSION**

It is well established that transcription factors play subset-specific roles in regulating neural morphogenesis, but identifying the cellular effectors that act downstream of these factors remains a major challenge, as does understanding how individual transcription factors coordinate to establish multiple aspects of cell fate. In this study, we show that isl is required for fra/DCC expression in a subset of *Drosophila* motor neurons and that this regulatory relationship contributes to two key aspects of motor neuron morphology. Loss of function and genetic rescue experiments indicate that fra acts downstream of isl in motor neurons both during axon guidance in the periphery and dendrite targeting in the CNS. These data describe a mechanism by which a single transcription factor contributes to neural map formation by controlling the position of both the inputs and outputs of a neuron through an identified downstream effector.

**A Role for a Cell-Type-Specific Transcription Factor in Controlling Myotopic Map Formation**

In the vertebrate spinal cord, the position of motor neuron cell bodies correlates with the targeting of their axons in the periphery (Catela et al., 2015). This myotopic map may be established through the action of transcription factors that coordinate control cell migration and axon guidance. In particular, Lhx1 and Is1 are expressed in limb-innervating lateral motor column (LMC) motor neurons and regulate the trajectory of their axons as well as the medio-lateral settling position of their cell bodies (Kania and Jessell, 2003; Luria et al., 2008; Palmesino et al., 2010). Lhx1 and Is1 regulate axon guidance through EphA4 and EphB receptors, respectively, and a recent study suggests that Lhx1 regulates cell body position through a distinct effector, the Reelin signaling protein Dab-1 (Palmesino et al., 2010).

In *Drosophila*, unlike in vertebrates, the position of motor neuron cell bodies does not necessarily correlate with the targeting of their axons in the periphery because neurons that innervate adjacent muscles can be found far apart within a segment (Landgraf et al., 2003; Mauss et al., 2009). Instead, recent studies have shown that both the larval and the adult *Drosophila* nervous systems use a myotopic map in which the position of motor neuron dendrites, rather than their cell bodies, correlates with the position of their target muscles (Brierley et al., 2009; Mauss et al., 2009). This may be a conserved feature of motor systems across phyla because the dendritic patterning of at least four motor neuron pools in the spinal cord correlates with muscle target identity in the mouse (Vrieseling and Arber, 2006).

Slit-Robo, Netrin-Fra, and Sema-Plexin signaling have been shown to control motor neuron dendrite targeting in *Drosophila*, and rescue experiments suggest that these guidance receptors act cell-autonomously in this process (Brierley et al., 2009; Mauss et al., 2009; Syed et al., 2016). In addition, the initial targeting of motor neuron dendrites in the embryo is largely unaffected by manipulations that affect the position or the activity of pre-synaptic axons or the presence of muscles, suggesting that this process is likely under the control of cell-autonomous factors, although these remain unidentified (Landgraf et al., 2003; Mauss et al., 2009).

We address several key questions about how motor neuron dendrite targeting is specified in *Drosophila*. First, we show that fra expression in two classes of motor neurons (RP3 and RP5) correlates with the medio-lateral position of their dendrites. Previous studies suggested that different classes of motor neurons express different levels of guidance receptors to direct the position of their dendrites, but this had not been demonstrated. Second, we find that Isl, which was shown previously to regulate axon targeting in a subset-specific way, also regulates dendrite targeting. Third, we find that Isl regulates both processes through fra. Surprisingly, we did not detect a correlation between axon and dendrite phenotypes in isl mutants. The absence of a correlation suggests that the dendrite positioning defects are not secondary to defects in target selection, consistent with a previous study in which the general patterning of motor neuron dendrites was not disrupted in muscle-less embryos (Landgraf et al., 2003). However, additional experiments that disrupt axon targeting and monitor the medio-lateral position of dendrites will be necessary to confirm that the two occur independently.

Future work will also be necessary to identify additional transcription factors that specify motor neuron dendrite development. We previously identified a role for Hb9 in regulating robo2 and robo3 expression, but it is not known whether these receptors regulate motor neuron dendrite development (Sanfilippo et al., 2014). We detect no change in robo1 mRNA levels in RP3 neurons in either *hb9* or *isl* mutants (data not shown). Robo signaling could be regulated post-translationally. Comm is required for midline crossing of motor neuron dendrites and may endogenously regulate their medio-lateral position (Furrier et al., 2003, 2007). The temporal pattern of *comm* expression does not support a role in dendrite targeting, however, because *comm* is not expressed in RP motor neurons at late stages of embryogenesis (Keleman et al., 2002; C.S. and G.J.B., unpublished data).

The functional consequences of dendrite targeting defects remain to be explored. It is likely that shifting the position of motor neuron dendrites alters their connectivity, but testing this hypothesis will require identifying the pre-synaptic neurons that impinge on the RPs during locomotive behavior. Forcing a shift in the position of dendrites dorsally projecting motor neurons does not abolish their connectivity with known pre-synaptic partners but does change the number of contacts established (Couton et al., 2015). In mice, the ETS factor Pea3/Etv4 is required for the dendritic patterning of a subset of motor neurons, and electrophysiological recordings reveal changes in connectivity in *Pea3* mutant spinal cords (Vrieseling and Arber, 2006). It will be of high interest to investigate whether analogous defects are detected in *isl* or *fra* mutant embryos.

**Isl Is an Essential Regulator of Multiple Features of RP3 Identity**

*Drosophila* Isl was initially described as a subset-specific regulator of axon guidance (Thor and Thomas, 1997). More recently,
Wolfram et al. (2012) demonstrated that Isl also acts instructively to establish the electrophysiological properties of RP motor neurons through repression of the potassium ion channel Shaker. Our data show that, in addition to regulating the axonal trajectory and the electrophysiological properties of the RP3 neuron, Isl also establishes its dendritic position. Hobert (2015) has defined terminal selectors as transcription factors that coordinately regulate gene programs conferring multiple aspects of a neuron’s identity, including its neurotransmitter phenotype, ion channel profile, and connectivity. Unlike the early-acting factors that function transiently to specify cell fate, terminal selectors are expressed throughout the life of an animal and are required for the maintenance of neuronal identity. Although there are several examples of transcription factors that act this way, it remains unclear how widespread a phenomenon it is (Allan et al., 2005; Eade et al., 2012; Flames and Hobert, 2009; Kratsios et al., 2011; Lodato et al., 2014). Does Isl fit the criteria for a terminal selector? Isl is not required for all aspects of RP3 identity because RP neurons retain expression of other motor neuron transcription factors in isl mutants, and their axons exit the nerve cord (Broihier and Skeath, 2002; Thor and Thomas, 1997). Future work will be necessary to determine whether Isl is required throughout larval life for the maintenance of RP3’s physiological and morphological features and to what extent Isl coordinate establishes multiple features of RP neuron identity.

**Hb9 and isl Act in Parallel to Regulate Axon Guidance through Distinct Downstream Effectors**

Co-expressed transcription factors could act synergistically to regulate specific downstream programs, in parallel through completely distinct effectors, or by some combination of the two mechanisms. Indeed, examples of all of these scenarios have been described. Both in vitro and in vivo studies demonstrate that, in vertebrate spinal motor neurons, Isl1 forms a complex with Lhx3 and that the Isl1-Lhx3 complex binds to and regulates different genes than Lhx3 alone or than a complex composed of Isl1 and Phox2b, a factor expressed in hindbrain motor neurons (Cho et al., 2014; Mazzoni et al., 2013; Thaler and others, 2002). In a subset of spinal commissural neurons, Lhx2 and Lhx9 act in parallel to promote midline crossing through upregulation of Rig-1/Robo3 (Wilson et al., 2008). In Drosophila dorsally projecting motor neurons, Eve, Zfh1, and Grain act in parallel to promote the expression of unc5, beat1a, and fas2, although Eve also regulates additional targets important for axon guidance that are not shared by Zfh1 or Grain (Zarin et al., 2012, 2014).

Here we show that Isl and Hb9 act in parallel through at least two distinct effectors and propose that they regulate their targets by different mechanisms. Hb9 likely indirectly promotes robo2 expression by repressing one or multiple intermediate targets because its conserved Engrailed homology repressor domain is required for its function in motor axon guidance and for robo2 regulation (Santiago et al., 2014). In vertebrate motor neurons, Isl1 forms a complex with Lhx3 to directly activate several of its known targets (Cho et al., 2014; Lee et al., 2016; Thaler et al., 2003). A recent genome-wide DAM-ID analysis found that Isl binds to multiple regions within and near the fra locus in Drosophila embryos, suggesting that it may directly activate fra (Wolfram et al., 2014; Figure S1). Our finding that lim3 is not required for fra expression in RP motor neurons, together with evidence that Isl can alter the electrical properties of muscle cells independently of Lim3, suggest that Drosophila Isl does not need to form a complex with Lim3 for all of its functions (Wolfram et al., 2014). Future research will be necessary to detect Isl binding events in embryonic motor neurons, although these experiments are challenging when binding occurs transiently or in a small number of cells (Agelopoulos et al., 2014). Interestingly, overexpression of Isl using ap-Gal4 or hbo9-Gal4 induces fra only in certain subsets of these neurons, consistent with a model in which Isl binds to the fra locus in a cell-type-specific manner (Figure 2; data not shown). The generation of many large-scale datasets for transcription factor binding sites presents the field with the task of reconciling these data with clearly defined genetic relationships during specific biological processes (Lacin et al., 2014; Mazzoni et al., 2013; Wolfram et al., 2014). Our study and others have initiated this effort, but it will be important to investigate the functional significance of other putative transcription factor-effector relationships to achieve a better understanding of how transcriptional regulators control cell fate (Cho et al., 2014; Hattori et al., 2013; Lodato et al., 2014; Wolfram et al., 2012).

**EXPERIMENTAL PROCEDURES**

**Genetics**

All crosses were carried out at 25 °C. Embryos were genotyped using balancer chromosomes carrying lacZ markers or by the presence of epitope-tagged transgenes. Transgenic UAS-Isl1SmMyc flies were generated by BestGene using pC31-directed site-specific integration into landing sites at cytological position 86Bf. See Supplemental Experimental Procedures for a list of mutant alleles used in this study.

**Immunofluorescence and Imaging**

Dechorionated, formaldehyde-fixed, methanol-devitellinized embryos were fluorescently stained using standard methods (see Supplemental Experimental Procedures for a list of antibodies used). Embryos were mounted in 70% glycerol/PBS. Fluorescent mRNA in situ hybridization was performed as described previously with a digoxigenin-labeled probe (Santiago et al., 2014). The fra antisense probe was transcribed from linearized cDNA cloned into pBluescript. Fluorescence quantification was performed as described previously (Santiago et al., 2014). Images were acquired using a spinning disk confocal system (PerkinElmer) built on a Nikon Ti-U inverted microscope using a Nikon OFN25 60x objective with a Hamamatsu C10600-10B charge-coupled device (CCD) camera and Yokogawa CSU-10 scanner head with Volocity imaging software. Max projections were generated, cropped, and processed using ImageJ.

**Single-Cell Labeling**

Embryos containing hsFLP, UAS-FRTstopFRT-mCD8-GFP, and lim3b-Gal4 transgenes were collected overnight at 25 °C in standard caged. Embryos were heat-shocked at 37 °C the next morning for 3–5 min (FLP.122) or 45–60 min (FLP.12), Embryos were fixed 9 hr after H.S. using standard procedures. See Supplemental Experimental Procedures for additional details on phenotypic quantification.

**Statistics**

For statistical analysis, comparisons were made between genotypes using Student's t test or Fisher's exact test, as appropriate. For multiple comparisons, a post hoc Bonferroni correction was applied. Outliers were not excluded from statistical analyses. Sample sizes are indicated in the figures or figure legends.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.01.041.

AUTHOR CONTRIBUTIONS

Conception and Design, C.S. and G.J.B.; Investigation, Analysis, and Writing – Original Draft, C.S.; Writing – Review and Editing, C.S. and G.J.B.; Resources and Supervision, G.J.B.

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