The Homeodomain Transcription Factor Hb9 Controls Axon Guidance in Drosophila through the Regulation of Robo Receptors

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http://dx.doi.org/10.1016/j.celrep.2014.02.037
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SUMMARY

Transcription factors establish neural diversity and wiring specificity; however, how they orchestrate changes in cell morphology remains poorly understood. The Drosophila Roundabout (Robo) receptors regulate connectivity in the CNS, but how their precise expression domains are established is unknown. Here, we show that the homeodomain transcription factor Hb9 acts upstream of Robo2 and Robo3 to regulate axon guidance in the Drosophila embryo. In ventrally projecting motor neurons, hb9 is required for robo2 expression, and restoring Robo2 activity in hb9 mutants rescues motor axon defects. Hb9 requires its conserved repressor domain and functions in parallel with Nkx6 to regulate robo2. Moreover, hb9 can regulate the medio-lateral position of axons through robo2 and robo3, and restoring robo3 expression in hb9 mutants rescues the lateral position defects of a subset of neurons. Altogether, these data identify Robo2 and Robo3 as key effectors of Hb9 in regulating nervous system development.

INTRODUCTION

Combinations of transcription factors specify the tremendous diversity of cell types in the nervous system (Dasen, 2009; Hobert, 2011; Shirasaki and Pfaff, 2002). Many studies have identified requirements for transcription factors in regulating specific events in circuit formation as neurons migrate, form dendritic and axonal extensions, and select their final synaptic targets (reviewed in Polleux et al., 2007; Zarin et al., 2014). In most cases, the downstream effectors through which transcription factors control changes in neuronal morphology and connectivity remain unknown, although several functional relationships have been demonstrated (van den Berghe et al., 2013; Jinushi-Nakao et al., 2007; Labrador et al., 2005; Luria et al., 2008; Marinos-Mondéjar et al., 2012; Nóbrega-Pereira et al., 2008; Wilson et al., 2009).

Conserved homeodomain transcription factors regulate motor neuron development across phyla. Studies in vertebrates and invertebrates have shown that motor neurons that project to common target areas often express common sets of transcription factors, which act instructively to direct motor axon guidance (Kania and Jessell, 2003; Kania et al., 2000; Landgraf et al., 1999; Thor and Thomas, 1997). In mouse and chick, Nkx6.1/ Nkx6.2 and MNR2/Hb9 are required for the specification of spinal cord motor neurons, and for axon pathfinding and muscle targeting in specific motor nerves (Arber et al., 1999; De Marco Garcia and Jessell, 2008; Sander et al., 2000; Thaler et al., 1999; Vallstedt et al., 2001). In Drosophila, Nkx6 and Hb9 are expressed in embryonic motor neurons that project to ventral or lateral body wall muscles, and although they are not individually required for specification, they are essential for the pathfinding of ventrally projecting motor axons (Broihier and Skeath, 2002; Broihier et al., 2004; Odden et al., 2002). Axons that project to dorsal muscles express the homeodomain transcription factor Even-skipped (Eve), which regulates guidance in part through the Netrin receptor Unc5 (Fujikawa et al., 2003; Labrador et al., 2005; Landgraf et al., 1999). Eve exhibits cross-repressive interactions with hb9 and nkk6, which function in parallel to repress eve and promote islet and lim3 expression (Broihier and Skeath, 2002; Broihier et al., 2004). HB9 and Nkx6 act as repressors to regulate transcription factors in the spinal cord (Lee et al., 2008; Muhr et al., 2001; William et al., 2003); however, guidance receptors that act downstream of Hb9 and Nkx6 have not been characterized. Interestingly, in both flies and vertebrates, Hb9 and Nkx6 are also expressed in a subset of interneurons, and knockdown experiments in Drosophila have suggested a role for hb9 in regulating midline crossing (Broihier et al., 2004; Odden et al., 2002; Sander et al., 2000; Vallstedt et al., 2001; Wilson et al., 2005).

Roundabout (Robo) receptors regulate midline crossing and lateral position within the developing CNS of invertebrates and vertebrates (Jaworski et al., 2010; Kastenhuber et al., 2009; Kidd et al., 1998; Long et al., 2004; Rajagopalan et al., 2000a, 2000b; Sabatier et al., 2004; Simpson et al., 2000a, 2000b). Two recent studies in mice have also identified a role for Robos in regulating motor axon guidance in specific motor neuron populations (Bravo-Ambrosio et al., 2012; Jaworski and Tessier-Lavigne, 2012). The three Drosophila Robo receptors have diversified in their expression patterns and functions. Robo,
hereafter referred to as Robo1, is broadly expressed in the ventral nerve cord and prevents inappropriate midline crossing by signaling repulsion in response to midline-derived Slit (Kidd et al., 1998, 1999). Robo2 is initially expressed in many ipsilateral pioneers and also contributes to Slit-mediated repulsion (Rajagopalan et al., 2000a; Simpson et al., 2000b). Subsequently, robo2 expression is more restricted, and it is required to specify the medio-lateral position of axons (Rajagopalan et al., 2000b; Simpson et al., 2000a). Robo3 is expressed in a subset of CNS neurons and also regulates lateral position (Rajagopalan et al., 2000b; Simpson et al., 2000a).

Characterization of the expression domains of the Drosophila Robos revealed an intriguing pattern, in which Robo1 is expressed on axons throughout the width of the CNS, Robo3 is found on axons in intermediate and lateral zones, and Robo2 is enriched on the most lateral axons (Rajagopalan et al., 2000b; Simpson et al., 2000a). These patterns are transcriptional in origin, as replacing any robo gene with the coding sequence of another Robo receptor results in a protein distribution that matches the endogenous expression of the replaced gene (Spitzweck et al., 2010) (C.S., T. Evans, and G.J.B., unpublished data). A phenotypic analysis of these gene-swaps alleles revealed the importance of transcriptional regulation for the diversification of robo gene function (Spitzweck et al., 2010). Robo2 and robo3’s roles in regulating lateral position are largely dependent on their expression patterns, although unique structures within the Robo2 receptor are also important for its function in lateral position (Evans and Bashaw, 2010; Spitzweck et al., 2010). In the peripheral nervous system, the atonal transcription factor regulates robo3 in chordotonal sensory neurons, directing the position of their axon terminals (Zlatic et al., 2003). In the CNS, the transcription factors lola and midline contribute to the induction of robo1 (Crowner et al., 2002; Liu et al., 2009). However, how the expression patterns of robo2 and robo3 are established to direct axons to specific medio-lateral zones within the CNS remains unknown.

This study identifies a functional relationship between hb9 and the Robo2 and Robo3 receptors in multiple contexts. We show that Hb9 acts through Robo2 to regulate motor axon guidance and can direct the medio-lateral position of axons in the nerve cord through its effects on robo2 and robo3. Furthermore, hb9 interacts genetically with nkx6 and requires its conserved repressor domain to regulate robo2. Together, these data establish a link between transcriptional regulators and cell surface guidance receptors, providing an example of how upstream factors act through specific guidance receptors to direct circuit formation.

RESULTS

Robo2 Is Required in Neurons for Motor Axon Pathfinding

Hb9 regulates motor axon pathfinding across species, but its downstream effectors remain unknown. In Drosophila, hb9 is required for the formation of the ISNb nerve, which innervates a group of ventral muscles (Broihier and Skeath, 2002). In our hands, approximately 20% of hemisegments in hb9 mutant embryos lack innervation at the muscle 6/7 cleft, whereas these defects are rarely observed in wild-type animals or hb9 heterozygotes (Figure 1). To identify potential targets of hb9, we examined the expression patterns of axon guidance genes by in situ hybridization. We found that during the stages when motor axons navigate the muscle field, robo2 mRNA is enriched in ventrally projecting motor neurons (Figure S1).

To determine whether robo2 regulates motor axon guidance, we examined robo2 mutant embryos for innervation defects. In 20% of hemisegments in robo2 mutants, the axon that normally innervates the muscle 6/7 cleft is either absent or stalled at the main ISNb trunk (Figure 1). This phenotype is similar to that of hb9 mutants and is observed using multiple robo2 alleles (Figure 1; data not shown). Robo2 heterozygotes and robo2/+; hb9/+ double heterozygotes do not have significant defects (Figure 1; data not shown). Robo2 mutants have no defects in axons forming the ISN, SNa, SNe, TN, or ISNd nerves. Importantly, restoring one copy of an 83.9 kb bacterial artificial chromosome (BAC) transgene that contains the robo2 locus and its flanking genomic sequence fully rescues the 6/7 innervation defects of robo2 mutants (Figure 1B).

Robo2 is expressed in ventral muscles and in motor neurons (Figure S1). To determine if robo2 acts in neurons to regulate motor axon pathfinding, we expressed a UAS-Robo2RNAi transgene using ftzng-GAL4, which drives expression in many motor neurons and their precursors (Thor et al., 1999). Expressing UAS-Robo2RNAi with ftzng-GAL4 in an otherwise wild-type background produces no effect but causes significant 6/7 innervation defects when expressed in robo2 heterozygotes (Figure 1B). Conversely, expressing UAS-Robo2 RNAi in robo2 heterozygotes using the pan-muscle driver 24bGAL4 has no effect (Figure 1B). Together, these data suggest that robo2 is required neuronally to regulate ISNb pathfinding.

Hb9 Is Required for robo2 Expression in the RP Motor Neurons

To test if hb9 regulates robo2 in ventrally projecting motor neurons, we examined robo2’s expression pattern in hb9 mutants. In stage 16 wild-type or hb9 heterozygote embryos, robo2 mRNA is readily detected in the raw prawn (RP) motor neurons (Figures S1 and 1C). In particular, robo2 transcript is enriched in RP3, the neuron that innervates the muscle 6/7 cleft (Figure 1C). In hb9 mutants, robo2 mRNA is significantly decreased in the RP motor neurons (Figure 1D). An average of 83% of RP3 neurons in hb9kk30/+ heterozygous embryos, but only 49% of RP3 neurons in hb9kk30/hb9154e mutants, express detectable robo2 at stage 16 (p < 0.001, Student’s t test) (Figure 1D). This difference is observed as early as stage 14, when robo2 mRNA begins to accumulate in RP3, and is detected using multiple hb9 alleles (Figures 1 and 3; data not shown). Interestingly, hb9 mutants show no change in the expression of robo1, which is broadly expressed in many motor neurons including the RPs (data not shown). To quantify the fluorescent robo2 mRNA signal in RP3 neurons, we measured pixel intensity and normalized the mRNA signal to the myc signal from islet-tau-myc. The average relative fluorescence intensity of robo2 mRNA in hb9 heterozygotes is more than twice the average value measured in hb9 mutants (p < 0.01, Student’s t test) (Figure 1D). We conclude that hb9 is an essential regulator of robo2 in the RP motor neurons.
Robo2’s Activity in Motor Axon Guidance Depends on Unique Features of Its Cytodomain

Robo2 has multiple activities in the embryonic CNS, some of which cannot be substituted for by the other Robo receptors (Evans and Bashaw, 2010; Spitzweck et al., 2010). To determine whether Robo2’s activity in motor axon guidance is a unique property of Robo2, we examined knockin alleles in which the coding sequences of Robo1, Robo2, or Robo3 are knocked into the Robo2 locus, hereafter referred to as robo2X, where X represents the inserted coding sequence (Spitzweck et al., 2010). Embryos homozygous for the robo2robo2 allele have no significant defects in motor axon pathfinding, whereas embryos homozygous for either robo2robo1 or robo2robo3 have as many RP3 innervation defects as Robo2 mutants (Figure 2B). To define
the protein domains required for Robo2’s activity in motor axon guidance, we examined knockin alleles encoding either of two chimeric receptors: Robo2-1 (Robo2’s ectodomain and Robo1’s cytodomain); or Robo1-2 (Robo1’s ectodomain and Robo2’s cytodomain) (Spitzweck et al., 2010) (Figure 2A). We found that robo2robo2-1 embryos have as strong a motor axon phenotype as robo2 mutants, whereas robo2robo1-2 embryos are phenotypically normal (Figure 2B). Together, these results suggest that neither Robo1 nor Robo3 can substitute for Robo2 in motor axon guidance and that this Robo2-specific activity maps to its cytodomain.

Restoring Robo2 Activity in hb9 Mutants Rescues Motor Axon Guidance Defects

To determine if Robo2 acts as an effector of Hb9 during motor axon guidance, we tested whether overexpressing robo2 in hb9 mutants rescues their muscle 6/7 innervation defects. However, overexpressing a UAS-Robo2 transgene using hb9GAL4 in otherwise wild-type embryos produces severe motor axon defects, affecting RP3 innervation in more than 50% of hemisegments (Figure S2). We therefore sought to identify a variant of the Robo2 receptor that retains its endogenous activity in ISNb pathfinding but does not generate defects when overexpressed. Because our results with the knockin alleles indicate a requirement for Robo2’s cytodomain in motor axon guidance (Figure 2B), we tested whether overexpression of a chimeric receptor that contains the ectodomain of Robo1 and the cytodomain of Robo2 (Robo1-2) results in motor axon guidance defects. We found that overexpression of UAS-Robo1-2 with hb9GAL4 does not result in 6/7 innervation defects, whereas expressing the reciprocal chimera (Robo2-1) produces significant errors in motor axon pathfinding (Figure S2).

We could now test if expressing a receptor that is functional in robo2’s endogenous context (Robo1-2) rescues motor axon guidance in hb9 mutants. We used the hb9GAL4 enhancer trap to perform this experiment (Broihier and Skeath, 2002) because we have found that when placed over a null hb9 allele, this allelic combination results in nearly undetectable levels of hb9 protein and has as strong a motor axon phenotype as the null itself (Figure 2C; data not shown). Overexpressing UAS-Robo1-2 in hb9 mutants using hb9GAL4 significantly rescues RP3 innervation defects (22% of hemisegments to 13%; p = 0.03, Student’s t test) (Figure 2C). A similar result is observed using the lim3bGAL4 driver (Certel and Thor, 2004) and a different hb9 allelic combination (18% to 10%; p = 0.04, Student’s t test) (Figure 2C). The incomplete rescue may be a consequence of the timing or expression levels caused by GAL4-driven expression. Alternatively, robo2 may be one of multiple downstream targets of hb9, and restoring Robo2 activity might not be sufficient to fully rescue hb9 mutants. Nevertheless, together with the loss-of-function phenotypes and the requirement for hb9 in promoting robo2 expression, these results strongly suggest that Robo2 acts as a downstream effector of Hb9 during motor axon guidance.

Hb9 Requires Its Conserved Repressor Domain and Functions in Parallel with Nkx6 to Regulate robo2

Vertebrate Hb9 acts as a repressor to regulate gene expression when overexpressed in the spinal cord, but the requirement for
Hb9’s repressor activity for axon guidance has not been studied (Lee et al., 2008; William et al., 2003). Two conserved putative repressor domains are found in *Drosophila* Hb9: an Engrailed homology (Eh) domain similar to sequences that interact with the Groucho corepressor (Broihier and Skeath, 2002; Smith and Jaynes, 1996); and a domain similar to sequences that interact with the C-terminal binding protein (CtBP) corepressor (William et al., 2003). To test the contribution of these domains to Hb9 function, we generated Hb9 transgenes in which either or both domains were deleted and compared their ability to rescue *hb9* mutants relative to full-length Hb9 (Figure 3). All transgenes are inserted in the same genomic location and are expressed at similar levels (data not shown). We found that whereas a full-length Hb9 transgene (Hb9 FL) fully rescues both muscle 6/7 innervation defects and *robo2* expression in *hb9* mutants, the Eh domain deletion (Hb9 ΔEh) does not rescue motor axon guidance defects in *hb9* mutants. Conversely, the CtBP-interacting domain deletion (Hb9 ΔCtBP) fully rescues both guidance and *robo2* expression (Figure 3). The double deletion (Hb9 ΔEh ΔCtBP) is not significantly different from Hb9 ΔEh in either assay (Figure 3). These results suggest that Hb9 indirectly activates *robo2*, perhaps by repressing a direct regulator of *robo2*, likely through a Groucho-dependent mechanism.

The embryonic expression patterns of *hb9* and the homeodomain transcription factor *nkx6* largely overlap, and genetic analyses suggest that Hb9 and Nkx6 act in parallel to regulate motor axon guidance and multiple transcription factors (Broihier et al.,...
We hypothesized that robo2 might be a shared downstream target of hb9 and nkx6. Indeed, nkx6 mutants have a significant decrease in robo2 expression in the RP motor neurons (81% robo2+ RP3 neurons in nkx6 heterozygotes versus 51.4% robo2+ RP3 neurons in nkx6 mutants; p < 0.001, Student’s t test) (Figures 4A and 4B). To determine if hb9 and nkx6 function in parallel to regulate robo2, we examined robo2 expression in hb9, nkx6 double mutants and observed a decrease relative to either single mutant (data not shown). However, we were not able to quantify robo2 expression in the double mutants because many cells are not labeled by the lim3a-tau-myc transgene (magenta). Filled arrowheads point to robo2+ RP3 neurons; empty arrowheads indicate robo2− neurons.

We removed one copy of nkx6 in the RP motor neurons and that they act in parallel to regulate ISNb guidance and achieve normal levels of robo2 expression, thus demonstrating how a combination of transcription factors regulates axon guidance by impinging on a common downstream target.

**Hb9 Regulates Lateral Position in a Subset of Neurons**

Robo2 regulates midline crossing and lateral position within the embryonic CNS (Rajagopalan et al., 2000a, 2000b; Simpson et al., 2000a, 2000b). Because hb9 is expressed in many neurons other than the RP motor neurons, we asked if it acts through robo2 to regulate axon guidance in other contexts. The enhancer trap hb9GAL4 is expressed in all neurons that endogenously express hb9 (Broihier et al., 2004), implying that Nkx6 regulates downstream targets other than robo2. Nevertheless, our data argue that Hb9 and Nkx6 are essential regulators of robo2 in the RP motor neurons and that they act in parallel to regulate ISNb guidance and achieve normal levels of robo2 expression, thus demonstrating how a combination of transcription factors regulates axon guidance by impinging on a common downstream target.
and the inner pathway appears thicker (Figure 5A). The lateralmost hb9GAL4+ pathway is missing or discontinuous in approximately 30% of hemisegments, and the intermediate pathway is missing in close to 50% of hemisegments (Figure 5B). These defects are fully rescued by expression of a UAS-Hb9 transgene (Figure 5). No changes in the number of hb9GAL4+ neurons are observed (data not shown). To determine if nxk6 also regulates the trajectory of hb9GAL4+ axons, we examined the organization of these pathways in embryos with reduced nxk6 activity. Nxk6 mutants have no significant defects in the lateral position of hb9GAL4+ axons (Figure S3). However, hb9 mutants heterozygous for nxk6 have a significantly stronger disruption of
the outermost hb9GAL4+ pathway relative to hb9 mutants (Figure S3), suggesting that nkk6 also regulates lateral position, although its requirement is only revealed in the absence of hb9. Robo2 and robo3 are major regulators of lateral position in the developing CNS (Evans and Bashaw, 2010; Rajagopalan et al., 2000b; Simpson et al., 2000a; Spitzweck et al., 2010). Their expression patterns mirror their requirements: robo2 is expressed on axons that select a lateral trajectory and is required for the formation of lateral pathways, whereas robo3 is expressed in both lateral and intermediate zones and is required for the formation of intermediate pathways (Rajagopalan et al., 2000b; Simpson et al., 2000a). Gene-swap experiments underscored the importance of the transcriptional regulation of robo2 and robo3 for their function in lateral position (Spitzweck et al., 2010), but upstream regulators within the CNS remain unknown.

To determine if hb9 regulates medio-lateral position through robo2 or robo3, we first asked whether robo2 or robo3 regulates the position of axons labeled by hb9GAL4. In robo2 mutants, the outer hb9GAL4+ pathway is missing in approximately 30% of hemisegments (Figure 5B). The intermediate pathway is mildly affected, whereas the medial pathway appears intact (Figure 5). In robo3 mutants, the intermediate hb9GAL4+ pathway is absent or strongly shifted in close to 50% of hemisegments, the outer pathway is not disrupted, and the medial pathway is intact (Figure 5). Robo2, robo3 double mutants have a stronger phenotype in which the outer two hb9GAL4+ pathways are disrupted in a majority of hemisegments (Figure 5). However, the dramatic decrease in the width of the nerve cord in robo2, robo3 double mutants made it difficult to quantify the presence of lateral pathways. We conclude that a loss of robo2 and robo3 reproduces the lateral position defects observed in hb9 mutants.

Hb9 Can Regulate Lateral Position by Inducing robo2

To test whether hb9 regulates lateral position through robo2 or robo3, we searched for hb9-expressing neurons that also express robo2 or robo3 and project to intermediate or lateral zones. Several hb9+ cells coexpress robo2, including a cluster of neurons found immediately anterior and slightly dorsal to dMP2 (Figure S4). We scored robo2 expression in these cells and observed a decrease in the percentage expressing robo2 mRNA in hb9 mutants compared to heterozygotes (52% to 24%; p < 0.0001, Student’s t test; Figure S4). However, we were not able to achieve the resolution necessary to determine whether these neurons contribute to lateral pathways. It is likely that most of these cells are interneurons because few motor neuron cell bodies reside in this area of the nerve cord (Landgraf et al., 1997). Together with the similarity in the lateral position defects of hb9 and robo2 mutants, as well as the observation that Robo2 is an effector of hb9 in motor neurons, these data suggest that hb9 may endogenously regulate the medio-lateral position of a subset of interneurons via its effect on robo2.

To study the consequences of manipulating hb9 levels on lateral position in a defined group of neurons, we used the apterous-GAL4 driver, which labels ipsilateral interneurons that normally do not express hb9, and express little robo2 and robo3 (Figure 6; data not shown). In wild-type embryos, the apterous (ap) axons form a fascicle that projects along the medial FasII bundle on either side of the midline (Figure 6B). Overexpressing Robo2 or Robo3 in the ap neurons causes their axons to shift laterally away from the midline (Evans and Bashaw, 2010; Rajagopalan et al., 2000b; Simpson et al., 2000a). We found that overexpressing Hb9 produces a very similar phenotype, in which ap axons are shifted in more than 75% of hemisegments, now aligning with the intermediate or lateral FasII tracts (Figure 7B). To determine if this phenotype is due to the induction of robo2 or robo3, we examined the effect of hb9 overexpression on robo2 and robo3 mRNA levels. Overexpression of Hb9 in ap neurons does not result in robo3 induction (data not shown). In contrast, we observed significant upregulation of robo2 (Figure 6A). In control embryos, robo2 mRNA is detected in less than 20% of ventral ap cells, whereas more than 60% of ventral ap neurons express robo2 when Hb9 is present (p < 0.001, Student’s t test) (Figure 6A). Interestingly, we do not observe robo2 induction in the dorsal ap neurons (data not shown), which express a different transcription factor profile than their ventral counterparts (Allan et al., 2005; Baumgardt et al., 2007).

To determine if the lateral shift phenotype caused by Hb9 overexpression in ap neurons is due to the induction of robo2, we overexpressed Hb9 in robo2 mutants. Strikingly, removing both copies of robo2 results in a full suppression of Hb9’s gain-of-function phenotype, and ap neurons appear wild-type (Figure 6B). Together, these data indicate that ectopic expression of Hb9 is sufficient to induce robo2 and that Hb9-driven changes in robo2 expression can dramatically affect the medio-lateral position of axons.

Hb9 Endogenously Regulates Lateral Position through robo3

The requirement for hb9 in regulating the position of intermediate hb9GAL4+ axons suggests that it may also regulate robo3, which is expressed on axons that project to intermediate regions of the nerve cord and is essential for the formation of intermediate axonal pathways (Rajagopalan et al., 2000b; Simpson et al., 2000a). The peptidergic midline neuron MP1 expresses both hb9 and robo3 and is one of the pioneers for the intermediate FasII pathway (Broihier and Skeath, 2002; Hidalgo and Brand, 1997; Simpson et al., 2000b). We used the C544-GAL4 driver (Wheeler et al., 2006) to identify MP1 neurons and score robo3 expression and the position of the MP1 axon. The mosaic expression of C544-GAL4 allowed us to score the axonal trajectory of individual cells. Whereas almost all MP1 neurons express high levels of robo3 mRNA and project along the intermediate FasII bundle in hb9 heterozygous embryos, in hb9 mutants, 56% of MP1 neurons do not express robo3, and 47% of MP1 axons project along the medial FasII tract (Figures 7A and 7B). A strong correlation between robo3 expression and the lateral position of a cell’s axon is detected in both hb9 heterozygotes and mutants, suggesting that the loss of robo3 is responsible for the medio shift phenotype (p < 0.0001, Fisher’s exact test) (Figure 7B). MP1 neurons also express nkk6; however, we detected no significant change in robo3 expression or in the MP1 axonal projection in nkk6 mutants (Figure S3).

To determine if restoring Robo3 rescues the lateral position of MP1 axons in hb9 mutants, we used C544-GAL4 to overexpress a UAS-HARobo3 transgene. Robo3 overexpression produces no effect on the lateral position of MP1 axons in hb9 heterozygous embryos (data not shown) but results in a robust rescue
of the lateral position defects of hb9 mutants: 50.4% of MP1 axons shifted medially in hb9 mutants versus 19% in hb9 mutants overexpressing Robo3 (p < 0.0001, Fisher’s exact test) (Figure 7C). We conclude that in at least one defined group of neurons, hb9 acts through robo3 to direct the selection of an intermediate pathway.

Interestingly, all of the Hb9 deletion variants fully rescue the lateral position defects of the intermediate hb9GAL4+ axons in hb9 mutants (Figure S5). Moreover, they all rescue robo3 expression in MP1 neurons, and whereas variants lacking the Eh domain are slightly weaker than Hb9 FL in this assay, these differences are not statistically significant (Figure S5). Although we cannot rule out that Hb9 acts as a repressor to regulate robo3, the observation that its Eh domain is not required for robo3 regulation suggests the intriguing possibility that Hb9 may regulate robo2 and robo3 via distinct mechanisms.

**DISCUSSION**

We have demonstrated a functional relationship between Hb9 and the Robo2 and Robo3 receptors in multiple contexts in the Drosophila embryo. In the RP motor neurons, hb9 is required for robo2 expression, and genetic rescue experiments indicate that robo2 acts downstream of hb9. Hb9 requires its conserved repressor domain and acts in parallel with Nkx6 to regulate robo2 and motor axon guidance. Moreover, hb9 contributes to the endogenous expression patterns of robo2 and robo3 and the lateral position of a subset of axons in the CNS, and can redirect axons laterally when overexpressed via upregulation of robo2. Finally, restoring Robo3 rescues the medial shift of MP1 axons in hb9 mutants, indicating that hb9 acts through robo3 to regulate medio-lateral position in a defined subset of neurons.

**Robo2 Is a Downstream Effector of Hb9 during Motor Axon Guidance**

Hb9 and nkx6 are required for the expression of robo2 in motor neurons, and rescue experiments suggest that the loss of robo2 contributes to the phenotype of hb9 mutants. However, nkx6 mutants and hb9 mutants heterozygous for nkx6 have a stronger ISNb phenotype than robo2 mutants, implying the existence of additional downstream targets. One candidate is the cell adhesion molecule FasIII, which is normally expressed in...
the RP motor neurons and appears reduced in nkh6 mutant embryos (Broihier et al., 2004). Identifying the constellation of effectors that function downstream of Hb9 and Nkx6 will be key to understanding how transcription factors expressed in specific neurons work together to drive the expression of the cell surface receptors that regulate axon guidance and target selection.

Robo2’s activity in motor axon guidance appears distinct from the previously described activities of the Drosophila Robo receptors. Although Robo1 can replace Robo2’s repulsive activity at the midline (Spitzweck et al., 2010), Robo2’s function in motor axon guidance is not shared by either Robo1 or Robo3. Moreover, Robo2’s antirepulsive activity at the midline and its ability to shift axons laterally when overexpressed both map to Robo2’s ectodomain, whereas we have found that Robo2’s activity in motor axon guidance maps to its cytodomain (Evans and Bashaw, 2010; Spitzweck et al., 2010). The signaling outputs of Robo2’s cytodomain remain unknown, as it lacks the conserved motifs within Robo1 that engage downstream signaling partners (Bashaw et al., 2000; Fan et al., 2003; Yang and Bashaw, 2006).

How does Robo2 function during motor axon guidance? In mice, Robo receptors are expressed in spinal motor neurons and prevent the defasciculation of a subset of motor axons (Jaworski and Tessier-Lavigne, 2012). Does Drosophila Robo2 regulate motor axon fasciculation? The levels of adhesion between ISNb axons and other nerves must be precisely controlled during the different stages of motor axon growth and target selection, and several regulators of adhesion are required for ISNb guidance (Fambrough and Goodman, 1996; Huang et al., 2007; Winberg et al., 1998). Furthermore, whereas Slit can be detected on ventral muscles, it is not visibly enriched in a pattern that suggests directionality in guiding motor axons (Kramer et al., 2001), making it difficult to envision how Robo2-mediated repulsive or attractive signaling might contribute to ISNb pathfinding. Future work will determine how Robo2’s cytodomain mediates motor axon guidance, whether this activity is Slit dependent, and whether Robo2 signals attraction, repulsion, or modulates adhesion in Drosophila motor axons.
**Hb9 Regulates Lateral Position through robo2 and robo3**

Elegant gene-swap experiments revealed the importance of transcriptional regulation in establishing the different expression patterns and functions of the Drosophila Robo receptors (Spitzweck et al., 2010). By analyzing a previously uncharacterized subset of axon pathways, we have uncovered a requirement for Hb9 in regulating lateral position in the CNS. Although Hb9 can act instructively to direct lateral position when overexpressed, its endogenous expression in a subset of medially projecting neurons suggests that its ability to shift axons laterally is context dependent. A complex picture emerges in which multiple factors act in different groups of neurons to regulate robo2 and robo3. In a subset of interneurons, Hb9 is endogenously required for lateral position through the upregulation of robo3 and likely robo2. In other neurons, such as those that form the outer FasII tracts, the expression patterns of robo2 and robo3 rely on additional upstream factors. What might be the significance of a regulatory network in which multiple sets of transcription factors direct lateral position in different groups of neurons? One possibility is that Hb9-expressing neurons may share specific functional properties, such as the expression of particular neurotransmitters or ion channels. Alternatively, Hb9 may regulate other aspects of connectivity. Indeed, Robo receptors mediate dendritic targeting in the Drosophila CNS (Furrer et al., 2003), raising the exciting possibility that Hb9 regulates both axonal and dendritic guidance through its effects on guidance receptor expression.

**How Does Hb9 Regulate robo2 and robo3?**

What is the mechanism by which Hb9 regulates the expression of robo2, robo3, and its other downstream effectors? We have found that Hb9 requires its conserved putative repressor domain and acts in parallel with Nkx6 to regulate robo2 and motor axon guidance. It has previously been shown that h9b and nkx6 function in parallel to regulate several transcription factors (Broihier and Skeath, 2002; Broihier et al., 2004). Hb9, nkx6 double mutants show decreased expression of islet and lim3 and upregulation of eve and the Nkx2 ortholog vnd (Broihier et al., 2004). Are Hb9 and Nkx6 regulating robo2 or robo3 through any of their previously identified targets? Hb9 and Nkx6 single mutants show no change in islet, lim3, or vnd expression (Broihier and Skeath, 2002; Broihier et al., 2004), arguing that h9b and nkx6 do not act solely through these factors to regulate robo2 or robo3. Eve expression is unaffected in nkx6 mutants (Broihier et al., 2004), and whereas it is ectopically expressed in two neurons per hemisegment in h9b mutants (Broihier and Skeath, 2002), these do not correspond to RP3 or MP1, the identifiable cells in which we can detect changes in robo2 and robo3 (data not shown). Therefore, our data do not support the hypothesis that Hb9 and Nkx6 regulate robo2 or robo3 primarily through their previously identified targets islet, lim3, vnd, or eve.

Gain-of-function experiments in vertebrates suggest that Hb9 and Nkx6 act as repressors to regulate gene expression in the spinal cord (Lee et al., 2008; Muhr et al., 2001; William et al., 2003). Our finding that Hb9’s Eh domain is required for motor axon pathfinding and robo2 regulation suggests that Hb9 acts as a repressor in this context as well, most likely through a previously unidentified intermediate target. On the other hand, the Eh domain is not required for Hb9’s ability to regulate robo3 or lateral position in h9bGAL4+ neurons that project to intermediate zones of the CNS. The finding that Hb9αEh retains significant activity in rescuing lateral position and robo3 expression indicates that Hb9 may regulate robo2 and robo3 via distinct mechanisms, perhaps involving different transcriptional cofactors or intermediate targets. In support of this hypothesis, h9b overexpression in the ap neurons can induce robo2, but not robo3. These data raise the intriguing possibility that Hb9’s ability to regulate robo2 and robo3 via different mechanisms contributed to the diversification of their expression patterns in the CNS.

Determining how Hb9 and Nkx6 regulate their effectors will be key to achieving a complete understanding of how these conserved transcription factors control changes in cell morphology and axon pathfinding during development. Of note, Hb9 mutant mice exhibit defects in a subset of motor nerves, including the phrenic and intercostal nerves, which are also affected in Robo mutants (Arber et al., 1999; Jaworski and Tessier-Lavigne, 2012; Thaler et al., 1999). It will be of great interest to determine if despite the vast divergence in the evolution of nervous system development between invertebrates and vertebrates, Hb9 or Nkx6 has retained a role for regulating Robo receptors across species.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**

Hb9 constructs with an N-terminal Myc tag were cloned into a pUAST vector containing 10x UAS and an attB site for flC31-mediated targeted insertion. Hb9αEh (lacking amino acids 219–229) and Hb9αCtbp (lacking amino acids 336–340) were generated by serial overlap extension PCR. Transgenes were inserted at cytological site 51C by Best Gene. The 22K18-robo2 BAC was obtained from BACPAC Resources (Children’s Hospital, Oakland) and inserted at 51C by Rainbow Transgenics.

**Fluorescent In Situ Hybridization and Quantification**

Fluorescent mRNA in situ hybridization was performed as described (Labrador et al., 2005). Fluorescence quantification was performed using ImageJ as described by Yang et al. (2009); see the Supplemental Experimental Procedures.

**Immunostaining and Imaging**

Embryo fixation and staining were performed as described by Kidd et al. (1998). Images were acquired with Velocity using a spinning disk confocal microscope (PerkinElmer) using a Nikon 40x objective with a Hamamatsu C10600-10B CDF camera and Yokogawa CSU-10 scanner head. Images were processed using ImageJ.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.02.037.

**ACKNOWLEDGMENTS**

We thank members of the G.J.B. lab for thoughtful feedback during the development of this manuscript. In particular, we thank Tim Evans, Melissa Hernandez, Alexandra Neuhaus-Follini, and Mike O’Donnell for intellectual and experimental contributions. We thank Drs. Barry Dickson, Lawrence Zipursky, Stephen Crews, and James Skeath for fly stocks and antibodies. C.S. was supported by an NSF predoctoral training grant. This work was...
supported by National Institutes of Health grants NS-046333 and NS054739 and March of Dimes grant #1-FY12-445 to G.J.B.

Received: November 12, 2013
Revised: February 6, 2014
Accepted: February 25, 2014
Published: March 27, 2014

REFERENCES


