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The homeodomain transcription factor Hb9 controls axon guidance in *Drosophila* through the regulation of Robo receptors

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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 central nervous system, 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. Together, these data identify Robo2 and Robo3 as key effectors of Hb9 in regulating nervous system development.

Keywords

Axon Guidance; Lateral Position; Robo receptors; Hb9; Transcription factors

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., 2013). In most cases the

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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; Marcos-Mondéjar et al., 2012; Nóbrega-Pereira et al., 2008; Wilson et al., 2008).

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 (Fujioka et al., 2003; Labrador et al., 2005; Landgraf et al., 1999). *Eve* exhibits cross-repressive interactions with *hb9* and *nkx6*, 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).

Robo receptors regulate midline crossing and lateral position within the developing central nervous systems 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., 2000a). 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., 2000b). Robo3 is expressed in a subset of CNS neurons, and also regulates lateral position (Rajagopalan et al., 2000b; Simpson et al., 2000b).

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., 2000b). 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). A phenotypic analysis of these gene-swap 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 medial-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, while 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 defe cts. 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 and data not shown). *Robo2* heterozygotes and *robo2/+; hb9/+* double heterozygotes do not have significant defects (Figure 1 and data not shown). *Robo2*

mutants have no defects in axons forming the ISN, SNa, SNc, TN, or ISNd nerves. Importantly, restoring one copy of an 83.9 kb 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 RP motor neurons (Figure S1 and Figure 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 $hb9^{kk30}$/+ heterozygous embryos, but only 49% of RP3 neurons in $hb9^{kk30}/hb9^{jj154e}$ 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, 3 and 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 if Robo2's activity in motor axon guidance is a unique property of Robo2, we examined knock-in alleles in which the coding sequences of Robo1, Robo2, or Robo3 are knocked into the *robo2* locus, hereafter referred to as $robo2^{X}$, where X represents the inserted coding sequence (Spitzweck et al., 2010). Embryos homozygous for the $robo2^{robo2}$ allele have no significant defects in motor axon pathfinding, whereas embryos homozygous for either $robo2^{robo1}$ or $robo2^{robo3}$ 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 knock-in 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 *robo2*$^{robo2\text{-}1}$ embryos have as strong a motor axon phenotype as *robo2* mutants, while *robo2*$^{robo1\text{-}2}$ 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 over-expressing *robo2* in *hb9* mutants rescues their muscle 6/7 innervation defects. However, over-expressing 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 over-expressed. As our results with the knock-in alleles indicate a requirement for Robo2's cytodomain in motor axon guidance (Figure 2B), we tested whether over-expression 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 over-expression 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), as 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 and data not shown). Over-expressing *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 over-expressed 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 co-repressor (Broihier and Skeath, 2002; Smith and Jaynes, 1996), and a domain similar to sequences that interact with the C-terminal binding protein (CtBP)

co-repressor (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 (Hb9ΔEh) does not rescue motor axon pathfinding, and only weakly rescues *robo2* expression (Figure 3). Conversely, the CtBP-binding domain deletion (Hb9ΔCtBP) fully rescues both guidance and *robo2* expression (Figure 3). The double deletion (Hb9ΔEhΔCtBP) is not significantly different from Hb9ΔEh 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., 2004). 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) (Figure 4A and B). 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, as many cells are not labeled by *hb9gal4* or *islet-tau-myc*. Therefore, we looked for an alternative background to address whether *nkx6* regulates *robo2* in parallel with *hb9*. Removing one copy of *nkx6* in *hb9* mutants strongly enhances the motor axon phenotype (from 21.6% of hemisegments with 6/7 innervation defects in *hb9/hb9* embryos to 45% in *hb9, nkx6/hb9,+* embryos, $p<0.001$, Student's t-test) without producing the changes in markers observed in *hb9, nkx6* double mutants (Figure 4C and D). In this background *robo2* expression is significantly decreased relative to *hb9* mutants (from 41% *robo2*+ RP3 neurons in *hb9/hb9* embryos to 19% in *hb9, nkx6/hb9,+* embryos, $p<0.001$, Student's t-test) suggesting that *nkx6* promotes *robo2* expression independently of *hb9* (Figure 4B). *Nkx6* single mutants have a severe ISNb phenotype in which most ventrally-projecting motor axons fail to exit the nerve cord (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.

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). As *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 and Skeath, 2002), labeling three parallel axon tracts on

either side of the midline (Figure 5A). These align with, but are distinct from, Fasciclin II (FasII)-expressing axons, which form three bundles at specific medio-lateral positions (Figure 5A). *Hb9* mutants do not have defects in the organization of FasII axons (Figure 5A and data not shown). However, in *hb9* mutants, the two outer *hb9gal4+* bundles are often disrupted and the inner pathway appears thicker (Figure 5A). The lateral-most *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 *nkx6* activity. *Nkx6* mutants have no significant defects in the lateral position of *hb9gal4+* axons (Figure S3). However, *hb9* mutants heterozygous for *nkx6* have a significantly stronger disruption of the outer-most *hb9gal4+* pathway relative to *hb9* mutants (Figure S3), suggesting that *nkx6* 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., 2000b; 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, while *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., 2000b). 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* regulate 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, while 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 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 co-express *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, as 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 and 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). Over-expressing 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., 2000b). We found that over-expressing 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* over-expression on *robo2* and *robo3* mRNA levels. Over-expression 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 over-expression in ap neurons is due to the induction of *robo2*, we over-expressed Hb9 in *robo2* mutants. Strikingly, removing both copies of *robo2* results in a full suppression of Hb9's gain of function phenotype, and ap axons 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 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., 2000b). 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., 2000a). 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 (Figure 7A and B). 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 medial shift phenotype ($p<0.0001$, Fisher's exact test) (Figure 7B). MP1 neurons also express *nkx6*; however, we detected no significant change in *robo3* expression or in the MP1 axonal projection in *nkx6* mutants (Figure S3).

To determine if restoring Robo3 rescues the lateral position of MP1 axons in *hb9* mutants, we used *C544-Gal4* to over-express a *UAS-HARobo3* transgene. *Robo*3 over-expression 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 over-expressing 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 while variants lacking the Eh domain are slightly weaker than Hb9 FL in this assay, these differences are not statistically significant (Figure S5). While we cannot rule out that Hb9 acts as a repressor to regulate *robo3*, the observation that its Engrailed homology 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 over-expressed 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 Fasciclin III, which is normally expressed in the RP motor neurons and appears reduced in *nkx6* 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.

We have identified a new activity for *Drosophila* Robo2 in regulating motor axon guidance. While 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 anti-repulsive activity at the midline and its ability to shift axons laterally when over-expressed 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. While Hb9 can act instructively to direct lateral position when over-expressed, 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 *hb9* 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 *hb9* 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 while it is ectopically expressed in two neurons per hemisegment in *hb9* 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 Engrailed homology 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 *hb9gal4+* neurons that project to intermediate zones of the CNS. The finding that Hb9ΔEh 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 co-factors or intermediate targets. In support of this hypothesis, *hb9* over-expression in the apterous 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 have 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 10xUAS and an attB site for ΦC31-mediated targeted insertion. Hb9ΔEh (lacking amino acids 219-229) and Hb9ΔCtbp (lacking amino acids 336–340) were generated by serial overlap extension PCR. Transgenes were inserted at cytological site 51C by Best Gene (Chino Hills, CA, USA). The *22K18-robo2* BAC was obtained from BACPAC Resources (Children's Hospital, Oakland) and inserted at 51C by Rainbow Transgenics (Carmarillo, CA, USA).

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 (Yang et al., 2009); see the supplemental experimental procedures.

Immunostaining and imaging

Embryo fixation and staining were performed as described (Kidd et al., 1998). Images were acquired with Volocity using a spinning disk confocal (Perkin Elmer) using a Nikon 40x objective with a Hamamatsu C10600-10B CCD camera and Yokogawa CSU-10 scanner head. Images were processed using ImageJ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Allan DW, Park D, St Pierre SE, Taghert PH, Thor S. Regulators acting in combinatorial codes also act independently in single differentiating neurons. Neuron. 2005; 45:689–700. [PubMed: 15748845]

Arber S, Han B, Mendelsohn M, Smith M, Jessell TM, Sockanathan S. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron. 1999; 23:659–674. [PubMed: 10482234]

Bashaw GJ, Kidd T, Murray D, Pawson T, Goodman CS. Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. Cell. 2000; 101:703–715. [PubMed: 10892742]

Baumgardt M, Miguel-Aliaga I, Karlsson D, Ekman H, Thor S. Specification of neuronal identities by feedforward combinatorial coding. PLoS Biol. 2007; 5:e37. [PubMed: 17298176]

Van den Berghe V, Stappers E, Vandesande B, Dimidschstein J, Kroes R, Francis A, Conidi A, Lesage F, Dries R, Cazzola S, et al. Directed migration of cortical interneurons depends on the cell-autonomous action of Sip1. Neuron. 2013; 77:70–82. [PubMed: 23312517]

Bravo-Ambrosio A, Mastick G, Kaprielian Z. Motor axon exit from the mammalian spinal cord is controlled by the homeodomain protein Nkx2.9 via Robo-Slit signaling. Development. 2012; 139:1435–1446. [PubMed: 22399681]

Broihier HT, Skeath JB. Drosophila homeodomain protein dHb9 directs neuronal fate via crossrepressive and cell-nonautonomous mechanisms. Neuron. 2002; 35:39–50. [PubMed: 12123607]

Broihier HT, Kuzin A, Zhu Y, Odenwald W, Skeath JB. Drosophila homeodomain protein Nkx6 coordinates motoneuron subtype identity and axonogenesis. Development. 2004; 131:5233–5242. [PubMed: 15456721]

Certel SJ, Thor S. Specification of Drosophila motoneuron identity by the combinatorial action of POU and LIM-HD factors. Development. 2004; 131:5429–5439. [PubMed: 15469973]

Crowner D, Madden K, Goeke S, Giniger E. Lola regulates midline crossing of CNS axons in Drosophila. Development. 2002; 129:1317–1325. [PubMed: 11880341]

Dasen, JS. Transcriptional networks in the early development of sensory-motor circuits. Elsevier Inc; 2009.

Evans TA, Bashaw GJ. Functional diversity of Robo receptor immunoglobulin domains promotes distinct axon guidance decisions. Curr Biol. 2010; 20:567–572. [PubMed: 20206526]

Fambrough D, Goodman CS. The Drosophila beaten path gene encodes a novel secreted protein that regulates defasciculation at motor axon choice points. Cell. 1996; 87:1049–1058. [PubMed: 8978609]

Fan X, Labrador JP, Hing H, Bashaw GJ. Slit stimulation recruits Dock and Pak to the roundabout receptor and increases Rac activity to regulate axon repulsion at the CNS midline. Neuron. 2003; 40:113–127. [PubMed: 14527437]

Fujioka M, Lear BC, Landgraf M, Yusibova GL, Zhou J, Riley KM, Patel NH, Jaynes JB. Even-skipped, acting as a repressor, regulates axonal projections in Drosophila. Development. 2003; 130:5385–5400. [PubMed: 13129849]

Furrer MP, Kim S, Wolf B, Chiba A. Robo and Frazzled/DCC mediate dendritic guidance at the CNS midline. Nature. 2003; 6:223–230.

Hidalgo A, Brand AH. Targeted neuronal ablation : the role of pioneer neurons in guidance and fasciculation in the CNS of Drosophila. Development. 1997; 124:3253–3262. [PubMed: 9310320]

Hobert O. Regulation of terminal differentiation programs in the nervous system. Annu Rev Cell Dev Biol. 2011; 27:681–696. [PubMed: 21985672]

Huang Z, Yazdani U, Thompson-peer KL, Kolodkin AL, Terman JR. Crk-associated substrate (Cas) signaling protein functions with integrins to specify axon guidance during development. Development. 2007; 134:2337–2347. [PubMed: 17537798]

Jaworski A, Tessier-Lavigne M. Autocrine/juxtaparacrine regulation of axon fasciculation by Slit-Robo signaling. Nat Neurosci. 2012; 15:367–369. [PubMed: 22306607]

Jaworski A, Long H, Tessier-Lavigne M. Collaborative and specialized functions of Robo1 and Robo2 in spinal commissural axon guidance. J Neurosci. 2010; 30:9445–9453. [PubMed: 20631173]

Jinushi-Nakao S, Arvind R, Amikura R, Kinameri E, Liu AW, Moore AW. Knot/Collier and Cut control different aspects of dendrite cytoskeleton and synergize to define final arbor shape. Neuron. 2007; 56:963–978. [PubMed: 18093520]

Kania A, Jessell TM. Topographic motor projections in the limb imposed by LIM homeodomain protein regulation of ephrin-A:EphA interactions. Neuron. 2003; 38:581–596. [PubMed: 12765610]

Kania A, Johnson RL, Jessell TM. Coordinate roles for LIM homeobox genes in directing the dorsoventral trajectory of motor axons in the vertebrate limb. Cell. 2000; 102:161–173. [PubMed: 10943837]

Kastenhuber E, Kern U, Bonkowsky JL, Chien CB, Driever W, Schweitzer J. Netrin-DCC, Robo-Slit, and heparan sulfate proteoglycans coordinate lateral positioning of longitudinal dopaminergic diencephalospinal axons. J Neurosci. 2009; 29:8914–8926. [PubMed: 19605629]

Kidd T, Brose K, Mitchell KJ, Fetter RD, Tessier-lavigne M, Goodman CS, Tear G. Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell. 1998; 92:205–215. [PubMed: 9458045]

Kidd T, Bland KS, Goodman CS. Slit is the midline repellent for the robo receptor in Drosophila. Cell. 1999; 96:785–794. [PubMed: 10102267]

Kramer SG, Kidd T, Simpson JH, Goodman CS. Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration. Science. 2001; 292:737–740. [PubMed: 11326102]

Labrador JP, O'keefe D, Yoshikawa S, McKinnon RD, Thomas JB, Bashaw GJ. The homeobox transcription factor even-skipped regulates netrin-receptor expression to control dorsal motor-axon projections in Drosophila. Curr Biol. 2005; 15:1413–1419. [PubMed: 16085495]

Landgraf M, Bossing T, Technau GM, Bate M. The origin, location, and projections of the embryonic abdominal motorneurons of Drosophila. J Neurosci. 1997; 17:9642–9655. [PubMed: 9391019]

Landgraf M, Roy S, Prokop A, VijayRaghavan K, Bate M. even-skipped determines the dorsal growth of motor axons in Drosophila. Neuron. 1999; 22:43–52. [PubMed: 10027288]

Lee S, Lee B, Joshi K, Pfaff SL, Lee JW, Lee SK. A regulatory network to segregate the identity of neuronal subtypes. Dev Cell. 2008; 14:877–889. [PubMed: 18539116]

Liu QX, Hiramoto M, Ueda H, Gojobori T, Hiromi Y, Hirose S. Midline governs axon pathfinding by coordinating expression of two major guidance systems. Genes Dev. 2009; 23:1165–1170. [PubMed: 19451216]

Long H, Sabatier C, Ma L, Plump A, Yuan W, Ornitz DM, Tamada A, Murakami F, Goodman CS, Tessier-Lavigne M. Conserved roles for Slit and Robo proteins in midline commissural axon guidance. Neuron. 2004; 42:213–223. [PubMed: 15091338]

Luria V, Krawchuk D, Jessell TM, Laufer E, Kania A. Specification of Motor Axon Trajectory by Ephrin-B: EphB Signaling: Symmetrical Control of Axonal Patterning in the Developing Limb. Neuron. 2008; 60:1039–1053. [PubMed: 19109910]

De Marco Garcia NV, Jessell TM. Early motor neuron pool identity and muscle nerve trajectory defined by postmitotic restrictions in Nkx6.1 activity. Neuron. 2008; 57:217–231. [PubMed: 18215620]

Marcos-Mondéjar P, Peregrín S, Li JY, Carlsson L, Tole S, López-Bendito G. The lhx2 transcription factor controls thalamocortical axonal guidance by specific regulation of robo1 and robo2 receptors. J Neurosci. 2012; 32:4372–4385. [PubMed: 22457488]

Muhr J, Andersson E, Persson M, Jessell TM, Ericson J. Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. Cell. 2001; 104:861–873. [PubMed: 11290324]

Nóbrega-Pereira S, Kessaris N, Du T, Kimura S, Anderson S, Marín O. Postmitotic Nkx2-1 controls the migration of telencephalic interneurons by direct repression of guidance receptors. Neuron. 2008; 59:733–745. [PubMed: 18786357]

Odden JP, Holbrook S, Doe CQ. Drosophila HB9 is expressed in a subset of motoneurons and interneurons, where it regulates gene expression and axon pathfinding. J Neurosci. 2002; 22:9143–9149. [PubMed: 12417636]

Polleux F, Ince-Dunn G, Ghosh A. Transcriptional regulation of vertebrate axon guidance and synapse formation. Nat Rev Neurosci. 2007; 8:331–340. [PubMed: 17453014]

Rajagopalan S, Nicolas E, Vivancos V, Berger J, Dickson BJ. Crossing the midline: roles and regulation of Robo receptors. Neuron. 2000a; 28:767–777. [PubMed: 11163265]

Rajagopalan S, Vivancos V, Nicolas E, Dickson BJ. Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the Drosophila CNS. Cell. 2000b; 103:1033–1045. [PubMed: 11163180]

Sabatier C, Plump AS, Le M, Brose K, Tamada A, Murakami F, Lee EYHP, Tessier-Lavigne M. The divergent Robo family protein Rig-1/Robo3 is a negative regulator of slit responsiveness required for midline crossing by commissural axons. Cell. 2004; 117:157–169. [PubMed: 15084255]

Sakai N, Insolera R, Sillitoe RV, Shi SH, Kaprielian Z. Axon sorting within the spinal cord marginal zone via Robo-mediated inhibition of N-cadherin controls spinocerebellar tract formation. J Neurosci. 2012; 32:15377–15387. [PubMed: 23115176]

Sander M, Paydar S, Ericson J, Briscoe J, Berber E, German M, Jessell TM, Rubenstein JLR. Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. Genes Dev. 2000; 14:2134–2139. [PubMed: 10970877]

Shirasaki R, Pfaff SL. Transcriptional codes and the control of neuronal identity. Annu Rev Neurosci. 2002; 25:251–281. [PubMed: 12052910]

Simpson JH, Kidd T, Bland KS, Goodman CS. Short-range and long-range guidance by slit and its Robo receptors. Robo and Robo2 play distinct roles in midline guidance. Neuron. 2000a; 28:753–766. [PubMed: 11163264]

Simpson JH, Bland KS, Fetter RD, Goodman CS. Short-range and long-range guidance by Slit and its Robo receptors: a combinatorial code of Robo receptors controls lateral position. Cell. 2000b; 103:1019–1032. [PubMed: 11163179]

Smith ST, Jaynes JB. A conserved region of engrailed, shared among all en-, gsc-, Nk1-, Nk2-and msh-class homeoproteins, mediates active transcriptional repression in vivo. Development. 1996; 122:3141–3150. [PubMed: 8898227]

Spitzweck B, Brankatschk M, Dickson BJ. Distinct protein domains and expression patterns confer divergent axon guidance functions for Drosophila Robo receptors. Cell. 2010; 140:409–420. [PubMed: 20144763]

Thaler J, Harrison K, Sharma K, Lettieri K, Kehrl J, Pfaff SL. Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. Neuron. 1999; 23:675–687. [PubMed: 10482235]

Thor S, Thomas JB. The Drosophila islet gene governs axon pathfinding and neurotransmitter identity. Neuron. 1997; 18:397–409. [PubMed: 9115734]

Thor S, Andersson SGE, Tomlinson A, Thomas JB. A LIM-homeodomain combinatorial code for motor- neuron pathway selection. Nature. 1999; 397:76–80. [PubMed: 9892357]

Vallstedt A, Muhr J, Pattyn A, Pierani A, Mendelsohn M, Sander M, Jessell TM, Ericson J. Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. Neuron. 2001; 31:743–755. [PubMed: 11567614]

Wheeler SR, Kearney JB, Guardiola AR, Crews ST. Single-cell mapping of neural and glial gene expression in the developing Drosophila CNS midline cells. Dev Biol. 2006; 294:509–524. [PubMed: 16631157]

William CM, Tanabe Y, Jessell TM. Regulation of motor neuron subtype identity by repressor activity of Mnx class homeodomain proteins. Development. 2003; 130:1523–1536. [PubMed: 12620979]

Wilson JM, Hartley R, Maxwell DJ, Todd AJ, Lieberam I, Kaltschmidt Ja, Yoshida Y, Jessell TM, Brownstone RM. Conditional rhythmicity of ventral spinal interneurons defined by expression of the Hb9 homeodomain protein. J Neurosci. 2005; 25:5710–5719. [PubMed: 15958737]

Wilson SI, Shafer B, Lee KJ, Dodd J. A molecular program for contralateral trajectory: Rig-1 control by LIM homeodomain transcription factors. Neuron. 2008; 59:413–424. [PubMed: 18701067]

Winberg ML, Noordermeer JN, Tamagnone L, Comoglio PM, Spriggs MK, Tessier-Lavigne M, Goodman CS. Plexin A is a neuronal semaphorin receptor that controls axon guidance. Cell. 1998; 95:903–916. [PubMed: 9875845]

Yang L, Bashaw GJ. Son of sevenless directly links the Robo receptor to rac activation to control axon repulsion at the midline. Neuron. 2006; 52:595–607. [PubMed: 17114045]

Yang L, Garbe DS, Bashaw GJ. A frazzled/DCC-dependent transcriptional switch regulates midline axon guidance. Science. 2009; 324:944–947. [PubMed: 19325078]

Zarin AA, Asadzadeh J, Labrador JP. Transcriptional regulation of guidance at the midline and in motor circuits. Cell Mol Life Sci. 2013

Zlatic M, Landgraf M, Bate M. Genetic specification of axonal arbors: atonal regulates robo3 to position terminal branches in the Drosophila nervous system. Neuron. 2003; 37:41–51. [PubMed: 12526771]

Highlights

- Hb9 acts through Robo2 to regulate motor axon pathfinding
- Robo2's novel activity in motor axon guidance maps to its cytoplasmic domain
- Hb9 regulates medio-lateral position in longitudinal axons through *robo2* and *robo3*
- Hb9 requires its conserved repressor domain to regulate *robo2*, but not *robo3*

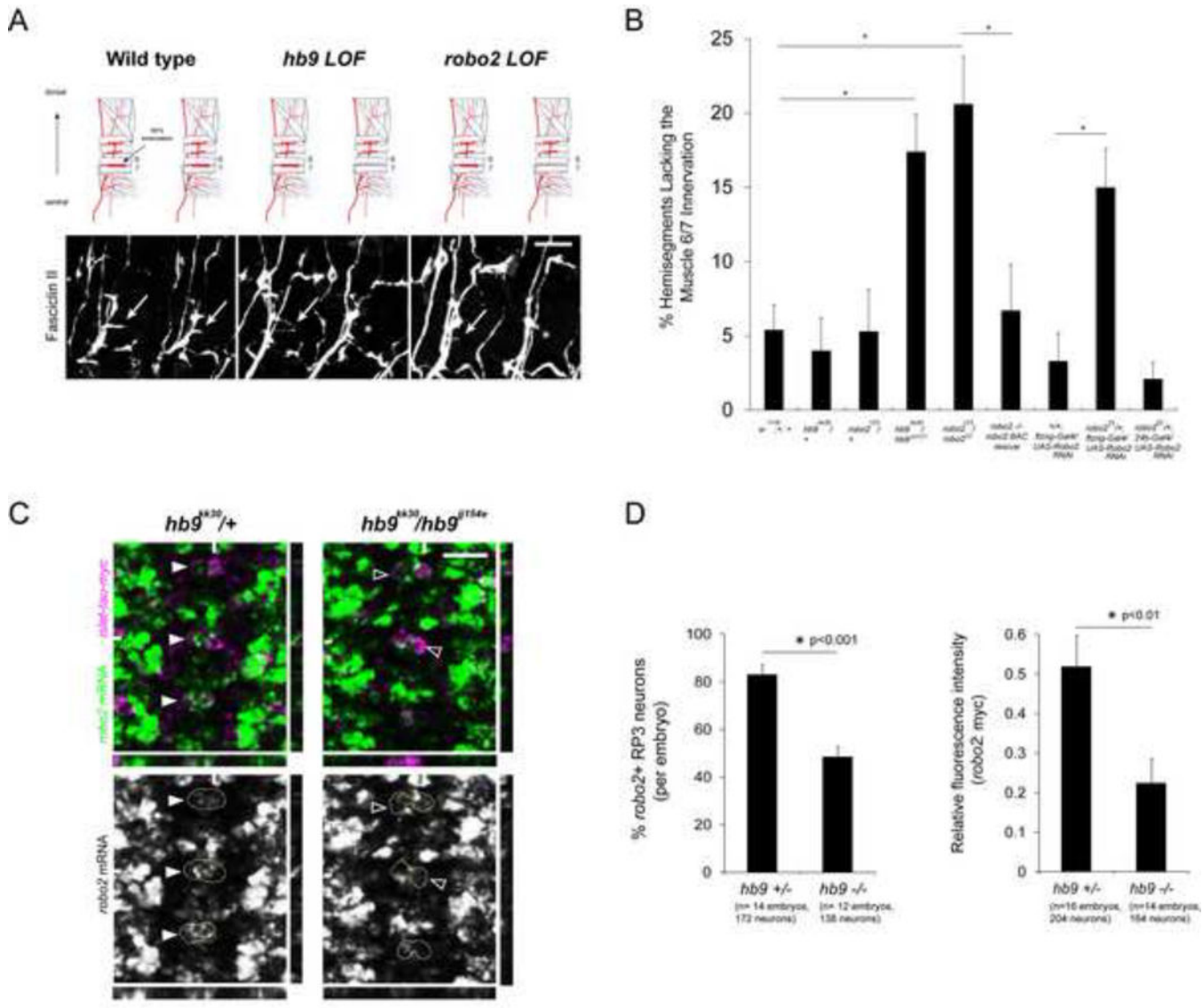

Figure 1. *Robo2* and *hb9* mutants have similar motor axon guidance defects, and *hb9* is required for *robo2* expression in the RP motor neurons

A: Stage 17 embryos stained for Fasciclin II (FasII). Anterior is left. Arrows point to the muscle 6/7 innervation, which is often absent in *hb9* or *robo2* mutants (asterisks). B: The percentage of hemisegments lacking the 6/7 innervation is shown; asterisks indicate a significant difference (Student's t-test, $p<0.01$). Error bars = s.e.m. C: Fluorescent *in situ* for *robo2* mRNA in Stage 16 embryos. Anterior is up. The RP3 motor neurons are labeled by the *islet-tau-myc* transgene, and circled in the single-channel images. Most RP3 neurons express *robo2* in *hb9* heterozygotes (filled arrowheads), whereas many RP3 neurons do not express *robo2* in *hb9* mutants (empty arrowheads). YZ and XZ cross-sections are shown; hash marks indicate the planes of the sections. D, Left: RP3 neurons were scored as positive or negative for *robo2*. *Hb9* mutants have significantly fewer *robo2*+ RP3 neurons than heterozygous siblings (Student's t-test, $p<0.001$). Error bars = s.e.m. D, Right: The mean gray value of the *robo2* mRNA signal in RP3 neurons was normalized to the mean gray

value of the myc signal. The average relative fluorescence intensity of *robo2* mRNA is significantly lower in *hb9* mutants than in *hb9* heterozygotes (Student's t-test, $p<0.01$). Error bars = s.e.m. Numbers of embryos and neurons analyzed are shown in parentheses. Scale bars represent 10 μm. *Robo2 −/− robo2 BAC rescue* denotes $robo2^{123}$, *22K18robo2BAC/* $robo2^{33}$. *Hb9 +/−* denotes $hb9^{kk30}$, *isl-taumyc/TM3. Hb9 −/−* denotes $hb9^{kk30}$, *isl-taumyc/*$hb9^{jj154e}$. See also Figure S1.

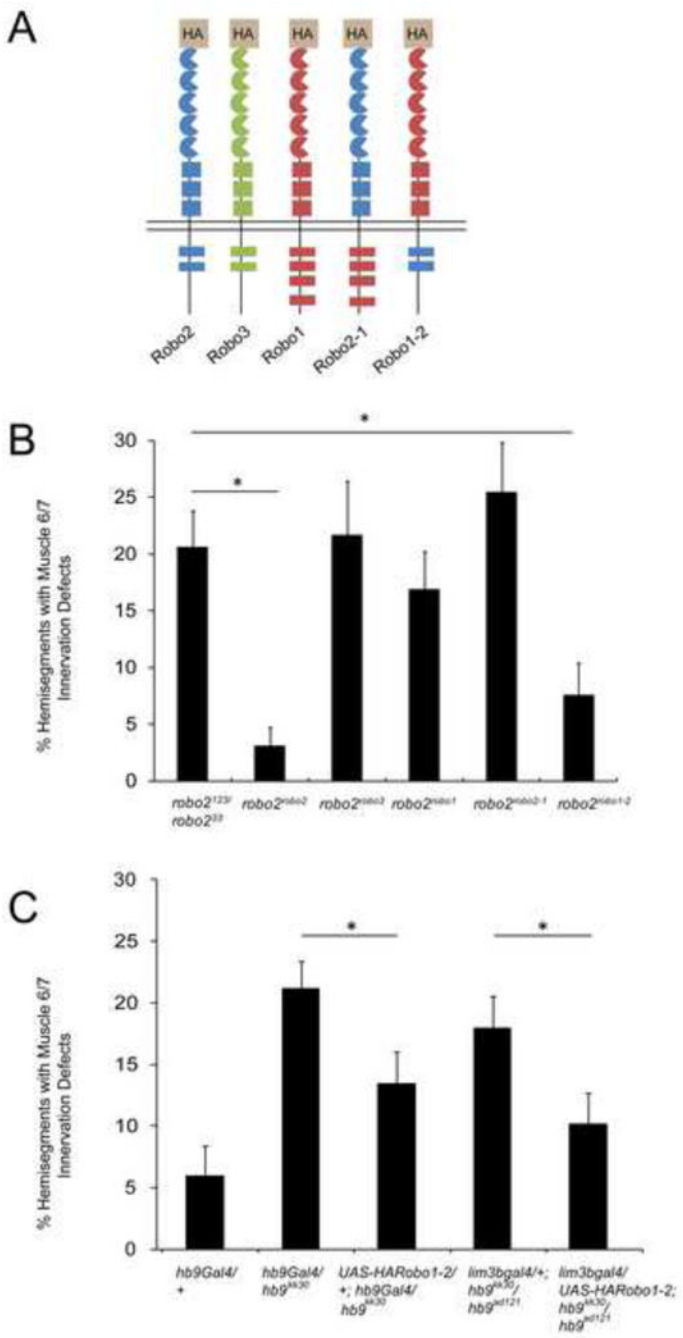

Figure 2. Restoring Robo2 activity in *hb9* mutants rescues motor axon guidance defects

A: Schematic of the Robo receptors analyzed for their ability to replace endogenous Robo2. B: Embryos homozygous for knock-in alleles in which the coding sequences of Robo2, Robo3, Robo1, Robo2-1, or Robo1-2 are inserted in the *robo2* locus were analyzed for motor axon guidance defects. Only Robo2 and Robo1-2 can restore muscle 6/7 innervation. Asterisks indicate a significant difference (Student's t-test, $p<0.01$). Error bars = s.e.m. B: *Hb9* mutant embryos over-expressing *UAS-HARobo1-2* have fewer defects than mutants

lacking the transgene (Student's t-test, $p<0.05$). All *hb9* mutants were scored blind to genotype. Error bars = s.e.m. See also Figure S2.

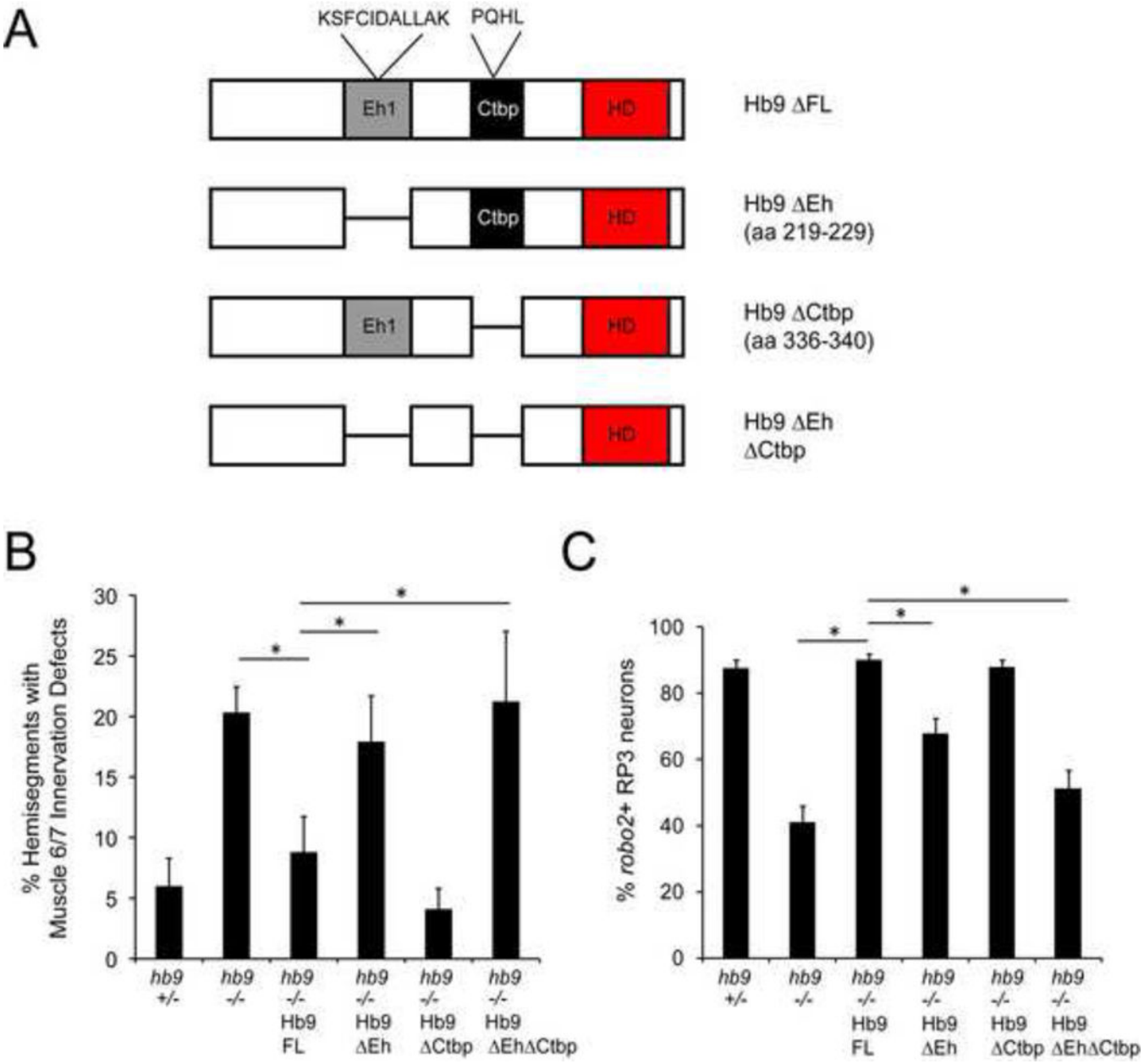

Figure 3. Hb9's Eh domain is required for its activity in motor axon guidance and for *robo2* regulation

A: Schematic of the Hb9 variants analyzed for their ability to rescue *hb9* mutants. B: Muscle 6/7 innervation was quantified in late Stage 17 embryos; asterisks indicate a significant difference (Student's t-test, $p<0.01$). Hb9 transgenes lacking the Eh domain failed to rescue motor axon guidance defects in *hb9* mutants. C: The percentage of *robo2*+ RP3 neurons per embryo is shown; asterisks indicate a significant difference (Student's t-test, $p<0.01$). Hb9's Eh domain is required for rescue of *robo2* expression. Error bars = s.e.m. *Hb9 +/−* denotes $hb9^{gal4}/TM3$. *Hb9 −/−* denotes $hb9^{gal4}/hb9^{kk30}$. *Hb9 −/−* Hb9 (variant) denotes *UAS-Hb9 (variant)/+;* $hb9^{gal4}/hb9^{kk30}$.

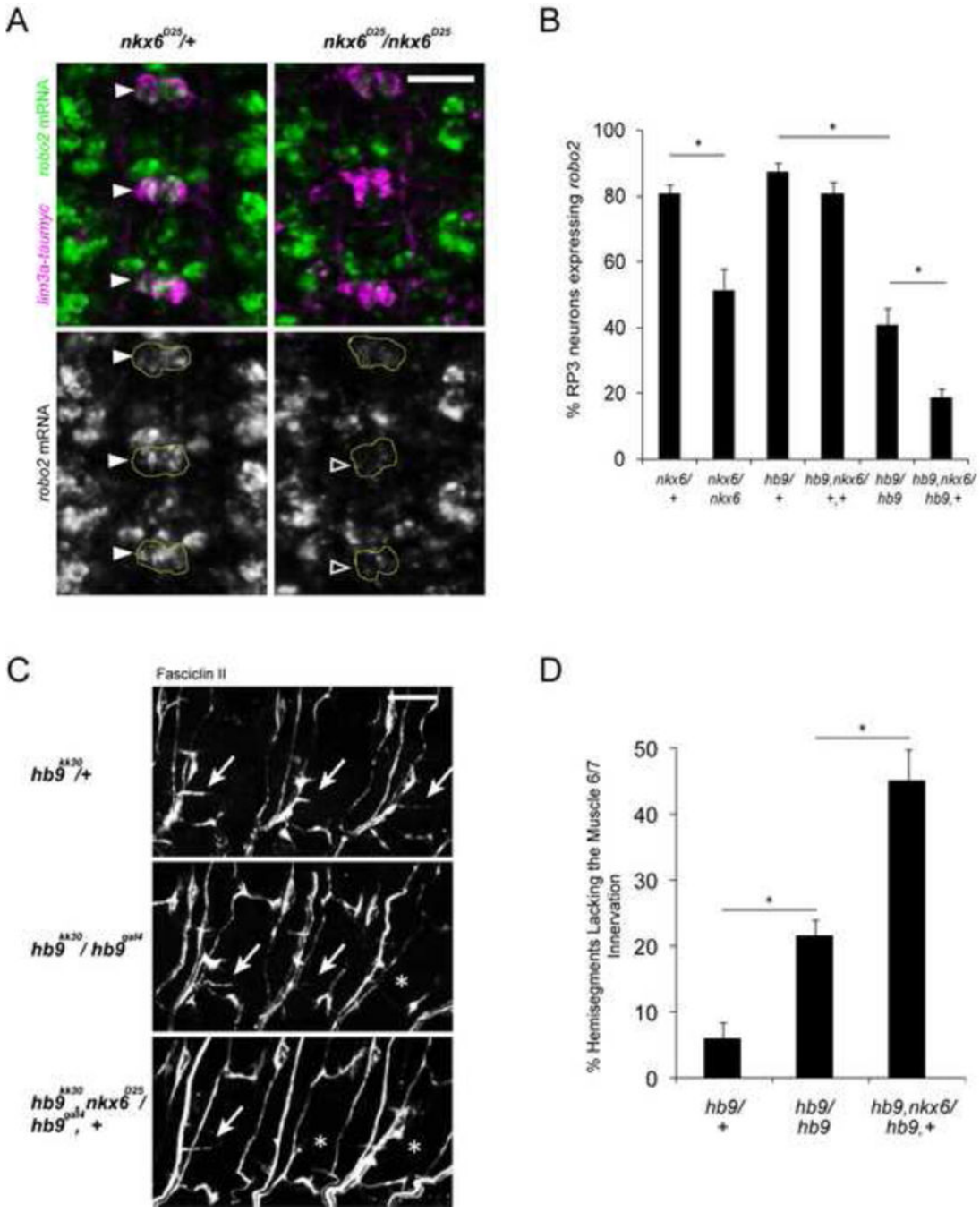

Figure 4. Hb9 and Nkx6 function in parallel to regulate motor axon guidance and *robo2*

A: Fluorescent *in situ* for *robo2* mRNA (green) in Stage 16 embryos. Anterior is up. The RP motor neurons are labeled by the *lim3a-taumyc* transgene (magenta). Filled arrowheads point to *robo2*+ RP3 neurons; empty arrowheads indicate *robo2*- neurons. B: *Nkx6* mutants have fewer *robo2*+ RP3 neurons than *nkx6* heterozygotes ($p<0.001$, Student's t-test). Removing one copy of *nkx6* enhances the loss of *robo2* in *hb9* mutants ($p<0.001$, Student's t-test). Error bars = s.e.m. C: Stage 17 embryos stained for FasII. Anterior is left. The arrows point to the muscle 6/7 innervation, while asterisks indicate its absence. D: The percentage

of hemisegments lacking the 6/7 innervation was quantified; asterisks indicate a significant difference ($p<0.001$, Student's t-test). Loss of *nkx6* dominantly enhances the 6/7 innervation defects of *hb9* mutants. Error bars = s.e.m. Scale bars represent 10 μm. *Nxk6/+* denotes $nkx6^{D25}/TM6B$. *Nkx6/nkx6* denotes $nkx6^{D25}/nkx6^{D25}$. *Hb9/+* denotes $hb9^{kk30}/TM3$. *Hb9, nxk6/+,+* denotes $hb9^{gal4}$, $nkx6^{D25}/TM3$. *Hb9/hb9* denotes $hb9^{gal4}/hb9^{kk30}$. *Hb9, nkx6/hb9,+* denotes $hb9^{gal4}$, $nkx6^{D25}/hb9^{kk30}$. See also Figure S3.

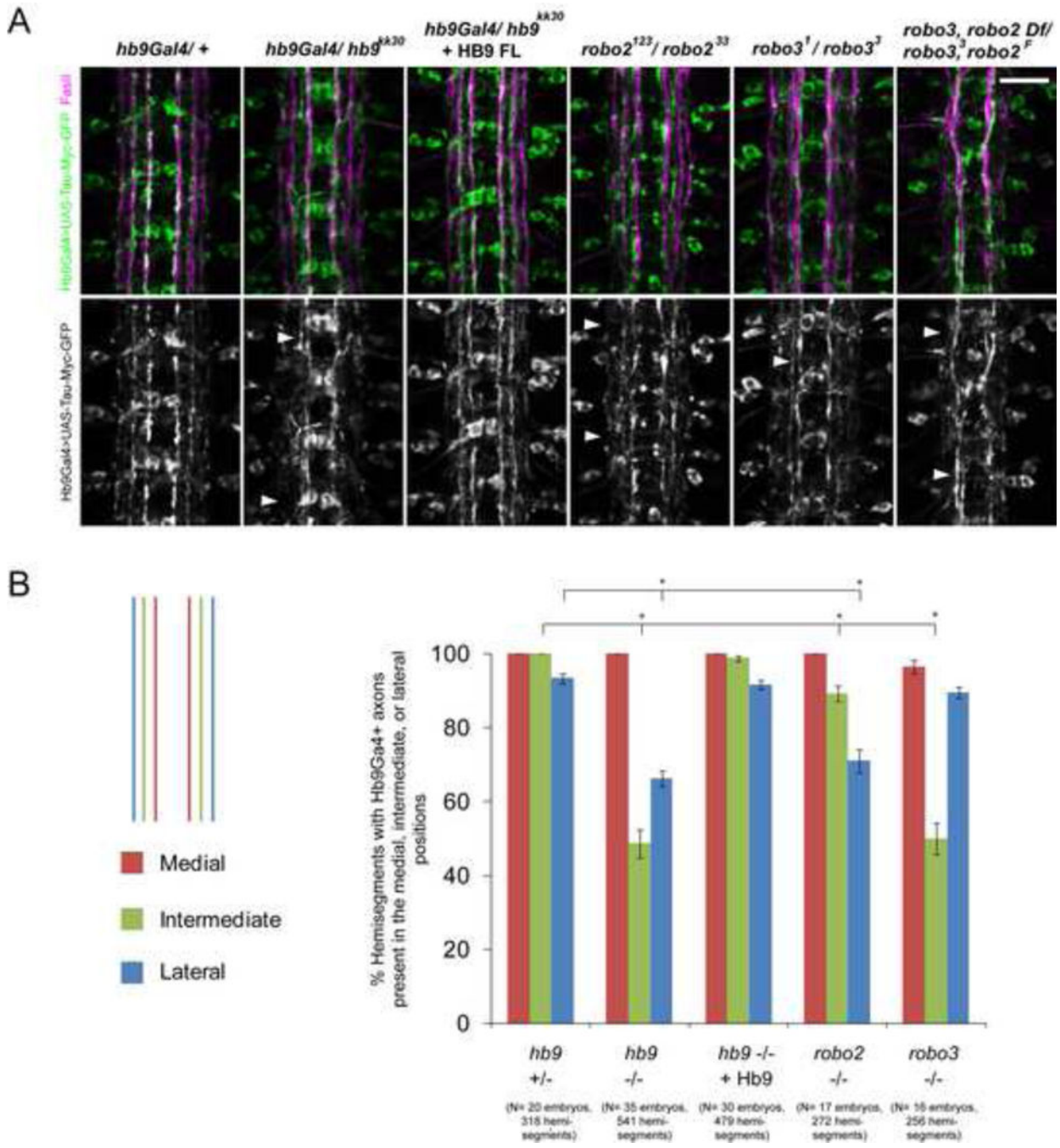

Figure 5. The lateral position of *hb9Gal4-expressing* axons is disrupted in the absence of *hb9, robo2*, or *robo3*

A: Stage 17 embryos, anterior is up. FasII staining is shown in magenta. *Hb9Gal4> UAS-TauMycGFP* (green) labels axons that form three bundles on each side of the midline in *hb9* heterozygotes. In *hb9* mutants, the outer *hb9Gal4+* pathways are disrupted or shifted medially (arrowheads). *Robo2* and *robo3* mutants partially phenocopy these defects (arrowheads). B: The percentage of hemisegments containing *hb9Gal4+* axons in the medial, intermediate, or lateral positions is shown. Asterisks indicate a significant difference

(Student's t-test, $p<0.001$). Error bars = s.e.m. Numbers of embryos and hemisegments scored are shown in parentheses. Scale bars represent 10 μm. *Hb9 +/−* denotes $hb9^{gal4}$/ *TM6B*. *Hb9 −/−* denotes $hb9^{gal4}/hb9^{kk30}$. *Hb9 −/− + HB9* denotes *UAS-Hb9/+;* $hb9^{gal4}$/ $hb9^{kk30}$. *Robo2 −/−* denotes $robo2^{123}/robo2^{33}$; $hb9^{gal4}$/+. *Robo3 −/−* denotes $robo3^{1}$ / $robo3^{3}$; $hb9^{gal4}$/+. *Robo3, robo2 Df/* $robo3^{3}$, $robo2^{F}$ denotes *Df(2L)ED108/* $robo2^{F}$, $robo3^{3}$; $hb9^{gal4}$/+. See also Figure S4.

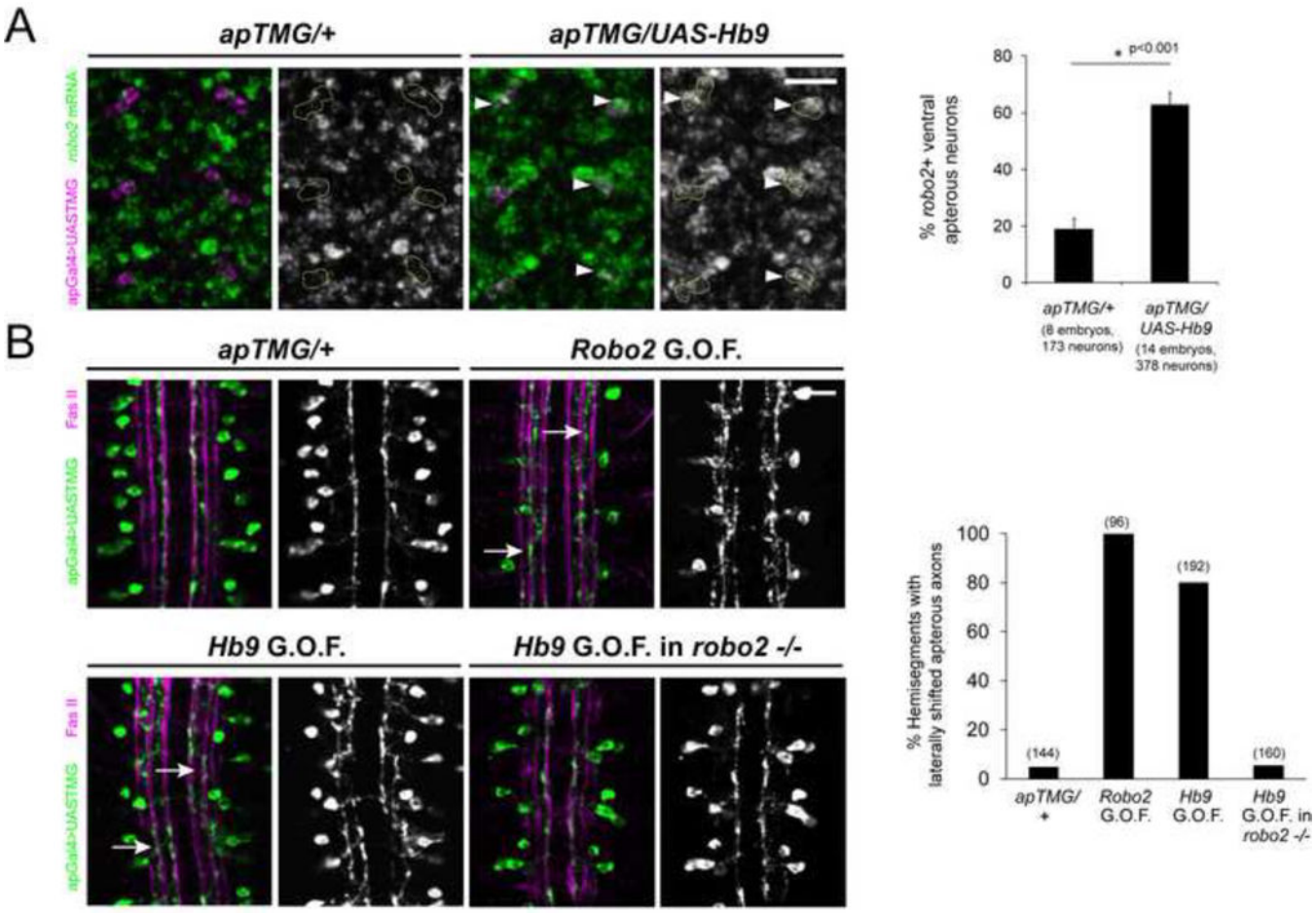

Figure 6. *Hb9* gain of function in ap neurons induces *robo2* expression and a *robo2*-dependent lateral shift

A, Left: Fluorescent *in situ* for *robo2* mRNA (green) in Stage 15 embryos. Anterior is up. The ventral ap neurons are labeled in magenta and circled in the single channel images. Wild-type embryos express little *robo2* in the ap neurons, whereas many ventral ap neurons express *robo2* when Hb9 is present (arrowheads). A, Right: The percentage of ventral ap neurons expressing *robo2* is shown. *Hb9* gain of function results in a significant increase compared to controls ($p<0.001$, Student's t-test). Error bars = s.e.m. B, Left: Stage 17 embryos stained for FasII (magenta) and GFP (green), which labels the ap axons. Over-expression of *robo2* or *hb9* in ap neurons shifts their axons laterally (arrows). *Hb9* over-expression in *robo2* mutants does not induce a lateral shift phenotype. B, Right: The percentage of hemisegments in which ap axons project along the intermediate or lateral FasII tracts is shown. Numbers of hemisegments scored are indicated in parentheses. Scale bars represent 10 μm. *apTMG/+* denotes *apGal4,UAS-TauMycGFP/CyO*. *Robo2* G.O.F. denotes *UAS-HARobo2.T1/apGal4, UAS-TauMycGFP*. *Hb9* G.O.F denotes *UAS-Hb9/ apGal4,UAS-TauMycGFP*. *Hb9* G.O.F. in *robo2* −/− denotes $robo2^{123}$,*UAS-Hb9*/$robo2^{33}$, *apGal4; UAS-TauMycGFP/+*.

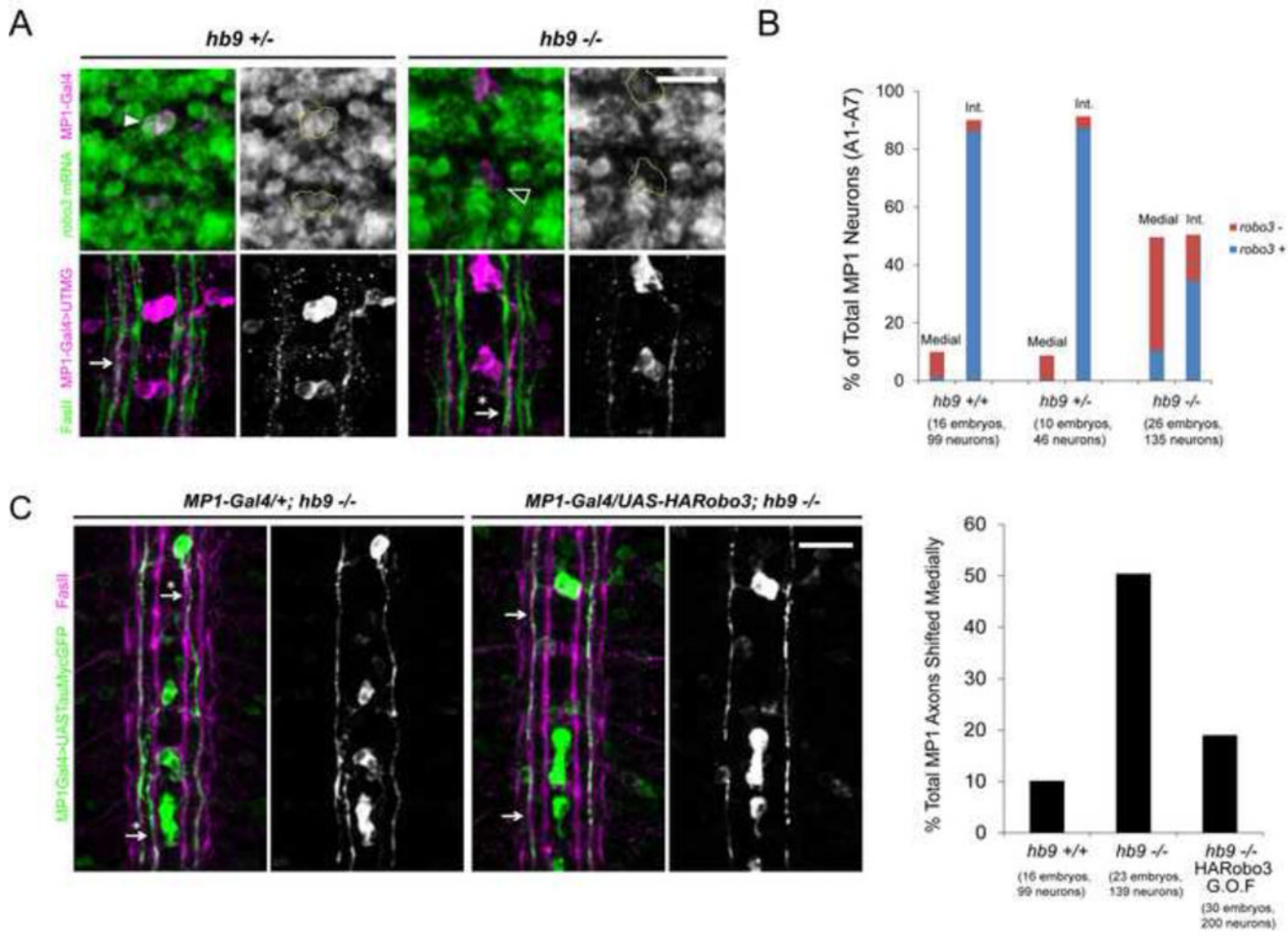

Figure 7. Robo3 acts downstream of Hb9 to direct the lateral position of MP1 axons

A, Top: Fluorescent *in situ* for *robo3* mRNA (green) in Stage 16 embryos. Anterior is up. MP1 neurons are labeled by *C544-Gal4* in magenta and circled in the single-channel images. Many MP1 neurons do not express *robo3* in *hb9* mutants (empty arrowhead). A, Bottom: MP1 axons project along the intermediate FasII bundle in *hb9* heterozygotes (arrow), but are often shifted to the medial pathway in *hb9* mutants (arrow with an asterisk). B: MP1 neurons were scored as *robo3+* or *robo3−* and as projecting along the medial or intermediate (Int.) FasII tract. A significant correlation was detected between *robo3* expression and lateral position in both *hb9 +/− and hb9−/−* embryos (Fisher's exact test, $p<0.001$). C: Over-expressing *robo3* rescues the medial shift phenotype of MP1 axons in *hb9* mutants ($p<0.001$, Fisher's Exact Test). Arrows point to MP1 axons in the correct position; arrows with asterisks point to medially shifted axons. All mutants were scored blind to genotype. Scale bars represent 10 μm. *Hb9 +/+* denotes *C544-Gal4/+; UAS-TauMycGFP/+*. *Hb9 +/−* denotes *C544-Gal4/+; hb9^{ad121}, UAS-TauMycGFP/TM3. Hb9 −/−* denotes *C544-Gal4/+; hb9^{ad121}, UAS-TauMycGFP/hb9^{kk30}. Hb9 −/−* HARobo3 G.O.F. denotes *C544-Gal4/UAS-HARobo3.T15; hb9^{ad121}, UAS-TauMycGFP/hb9^{kk30}*. See also Figure S5.