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 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 mediolateral 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; 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).

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-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 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, SNc, 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 $hb9^{kk30}/+$ 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 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.

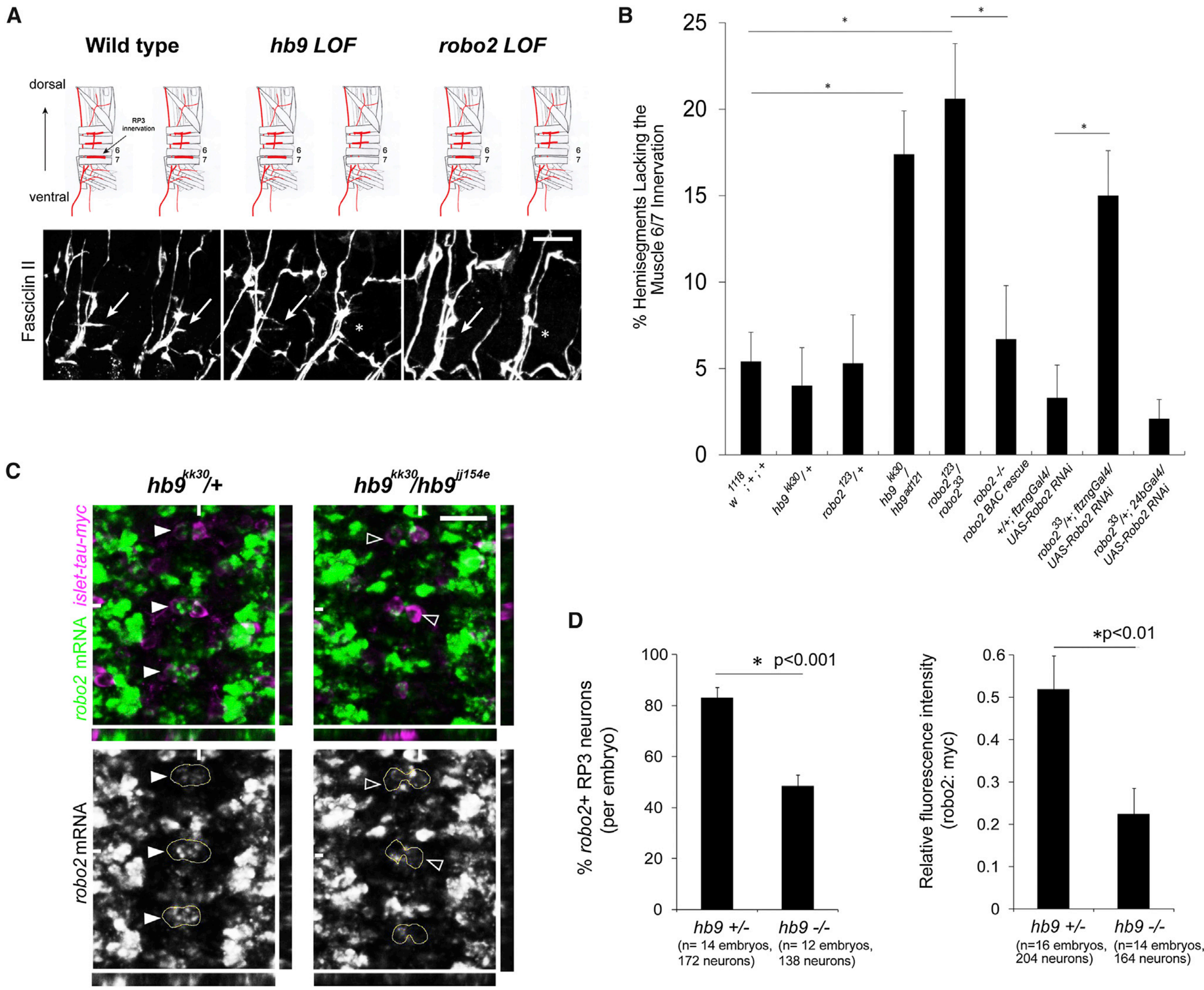

Figure 1. *Robo2* and *hb9* Mutants Have Similar Motor Axon Guidance Defects, and *hb9* Is Required for *robo2* Expression in the RP Motor Neurons

(A) Stage 17 embryos stained for 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, SEM.

(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, SEM. Right panel shows that the average relative fluorescence intensity of *robo2* mRNA is significantly lower in *hb9* mutants than in *hb9* heterozygotes (Student's t test, $p < 0.01$). The mean gray value of the *robo2* mRNA signal in RP3 neurons was normalized to the mean gray value of the myc signal. Error bars, SEM. 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.

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 *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

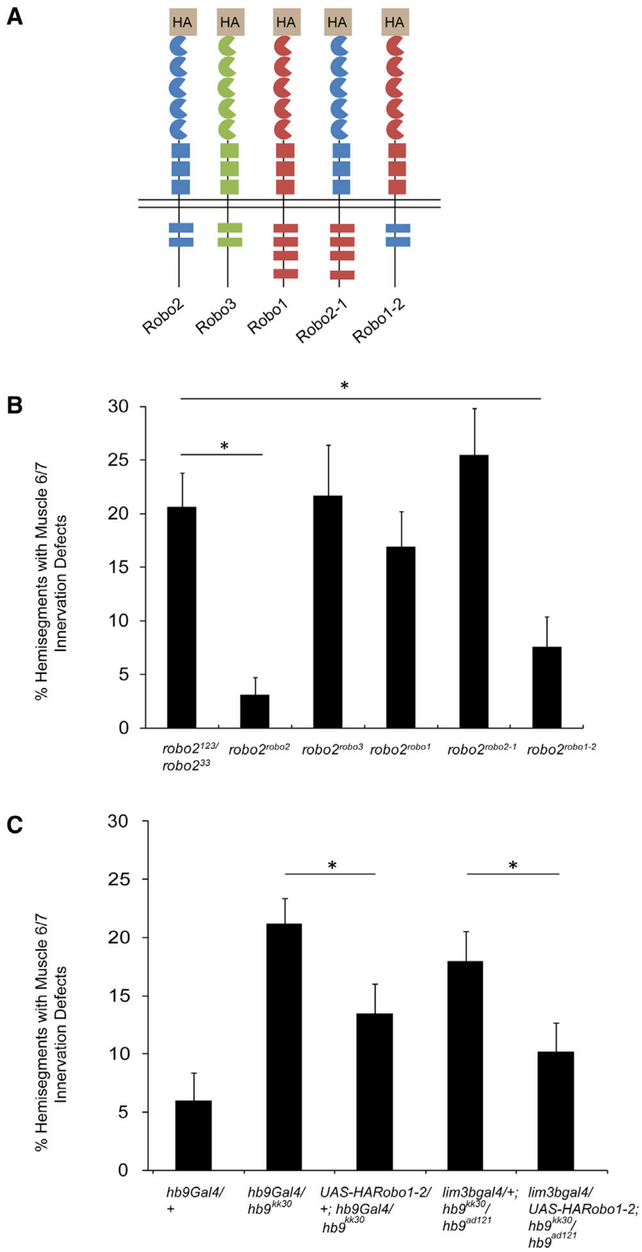

Figure 2. Restoring Robo2 Activity in *hb9* Mutants Rescues Motor Axon Guidance Defects

(A) Schematic of the Robo receptors analyzed for their ability to replace endogenous Robo2.

(B) Embryos homozygous for knockin 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, SEM.

(C) *Hb9* mutant embryos overexpressing *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, SEM.

See also Figure S2.

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 *robo2*$^{robo2\text{-}1}$ embryos have as strong a motor axon phenotype as *robo2* mutants, whereas *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 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

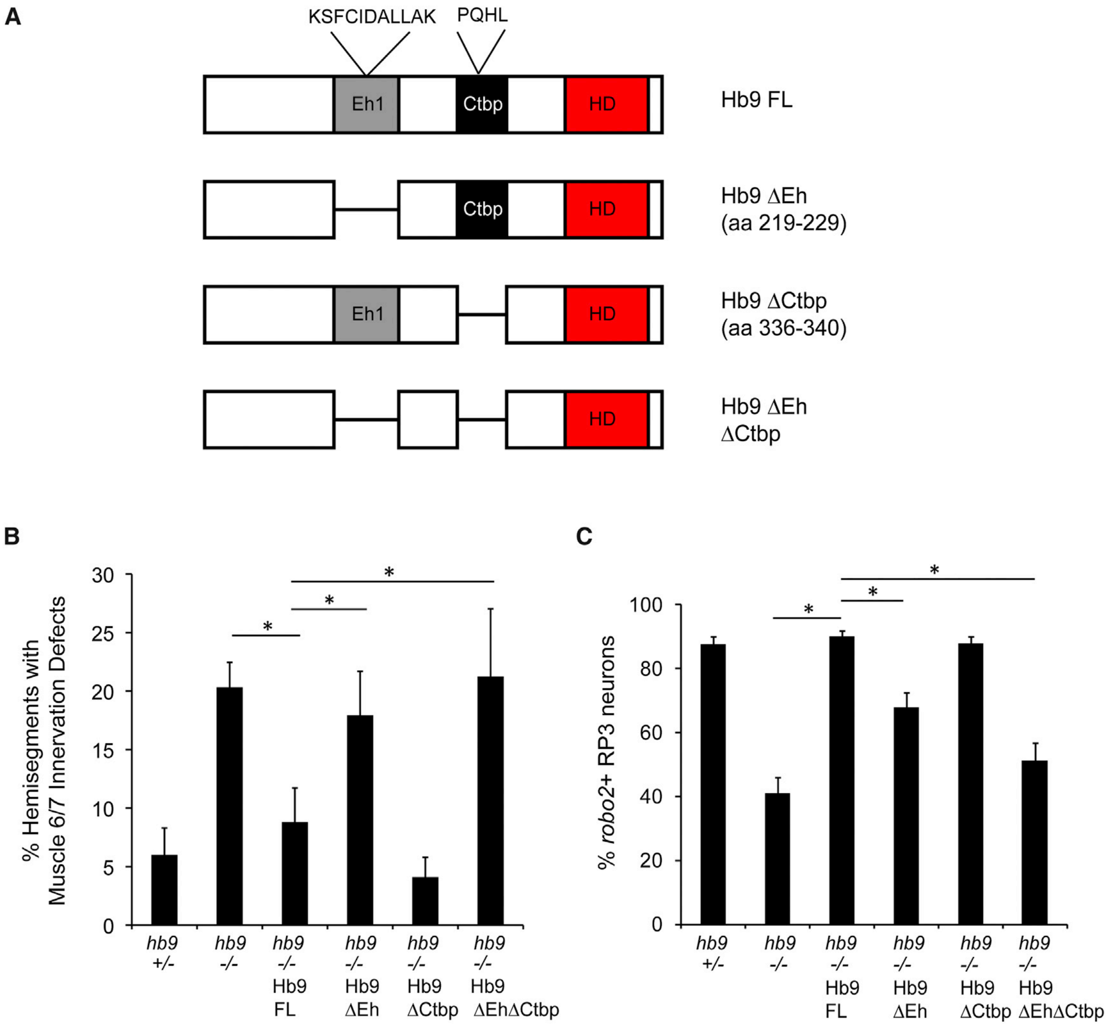

Figure 3. Hb9's Eh Domain Is Required for Its Activity in Motor Axon Guidance and for *robo2* Regulation

(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 the rescue of *robo2* expression.

Error bars, SEM. *Hb9* +/− denotes $hb9^{GAL4}/TM3$. *Hb9* −/− denotes $hb9^{GAL4}/hb9^{kk30}$. *Hb9* −/− Hb9 (variant) denotes *UAS-Hb9 (variant)/+;* $hb9^{GAL4}/hb9^{kk30}$.

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 (Hb9ΔEh) does not rescue motor axon pathfinding and only weakly rescues *robo2* expression (Figure 3). Conversely, the CtBP-interacting 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.,

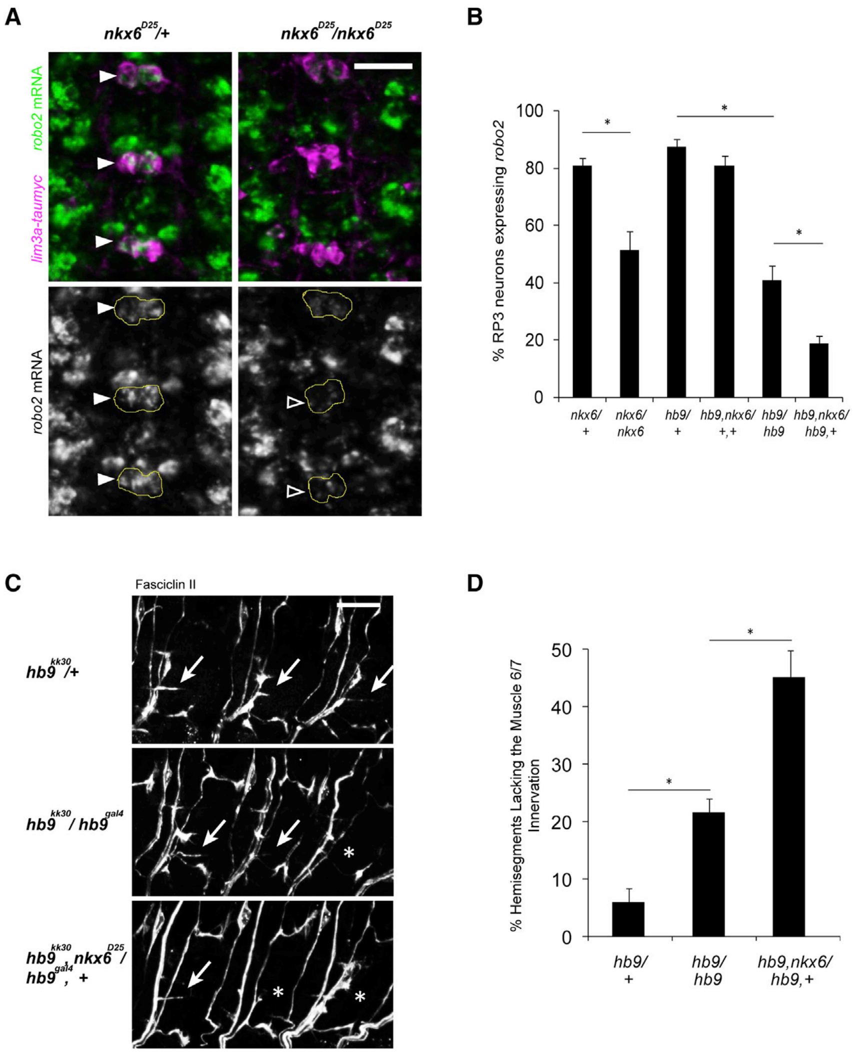

Figure 4. Hb9 and Nkx6 Function in Parallel to Regulate Motor Axon Guidance and *robo2*

(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, SEM.

(C) Stage 17 embryos stained for FasII. Anterior is left. The arrows point to the muscle 6/7 innervation, whereas 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, SEM.

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.

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) (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 *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 (Figures 4C and 4D). 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). 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 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; data not shown). However, in *hb9* mutants, the two outer *hb9GAL4*+ bundles are often disrupted,

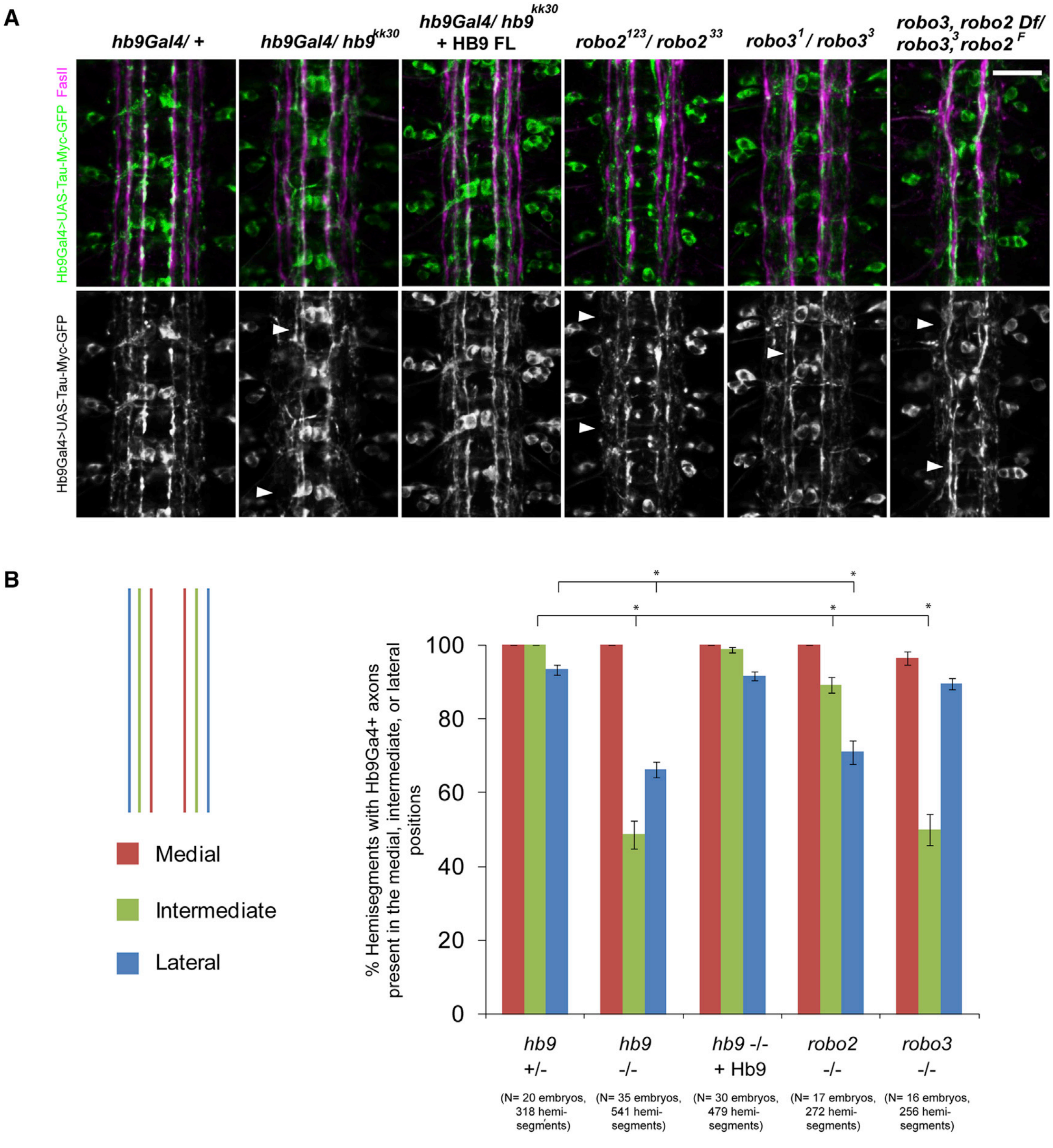

Figure 5. The Lateral Position of *hb9GAL4*-Expressing Axons Is Disrupted in the Absence of *hb9*, *robo2*, or *robo3*

(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, SEM. Numbers of embryos and hemisegments scored are shown in parentheses. Scale bars, 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.

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 outermost *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., 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 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 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 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 overexpress a *UAS-HARobo3* transgene. *Robo*3 overexpression produces no effect on the lateral position of MP1 axons in *hb9* heterozygous embryos (data not shown) but results in a robust rescue

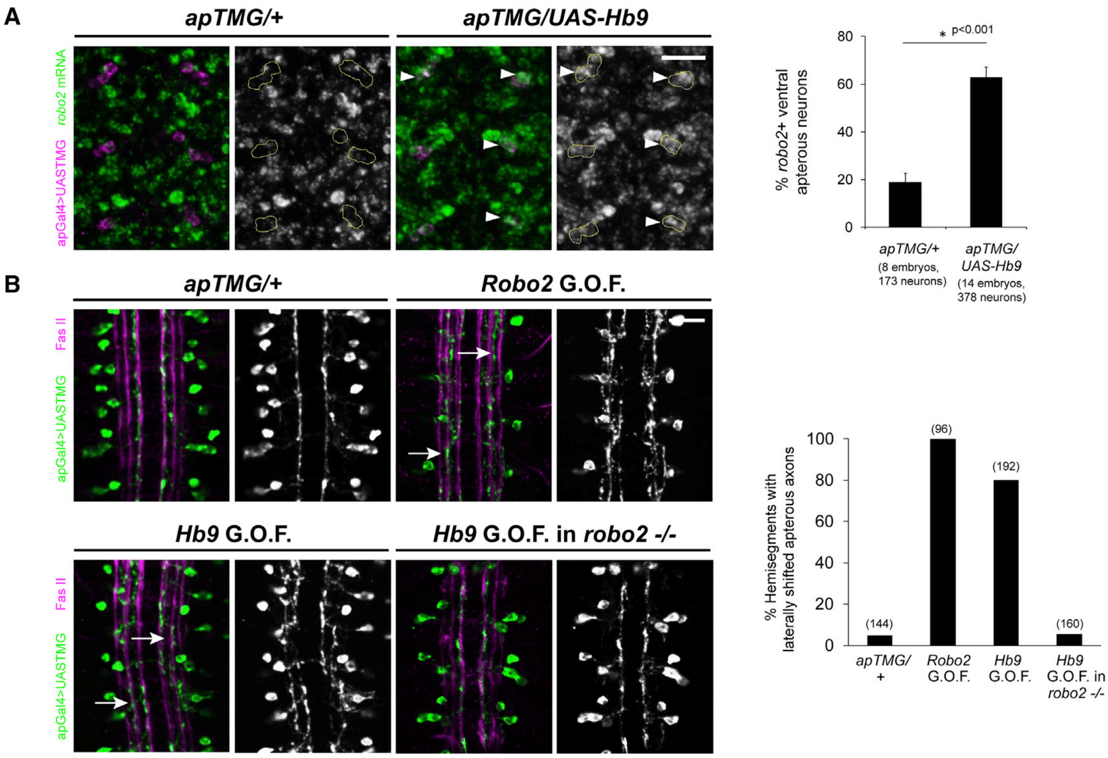

Figure 6. *Hb9* Gain of Function in ap Neurons Induces *robo2* Expression and a *robo2*-Dependent Lateral Shift

(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). Right panel shows the percentage of ventral ap neurons expressing *robo2*. *Hb9* gain of function results in a significant increase compared to controls ($p < 0.001$, Student's t test). Error bars, SEM.

(B) Left: stage 17 embryos stained for FasII (magenta) and GFP (green), which labels the ap axons. Overexpression of *robo2* or *hb9* in ap neurons shifts their axons laterally (arrows). *Hb9* overexpression in *robo2* mutants does not induce a lateral shift phenotype. Right panel shows the percentage of hemisegments in which ap axons project along the intermediate or lateral FasII tracts. Numbers of hemisegments scored are indicated in parentheses. Scale bars, 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/+*. G.O.F., gain of function.

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

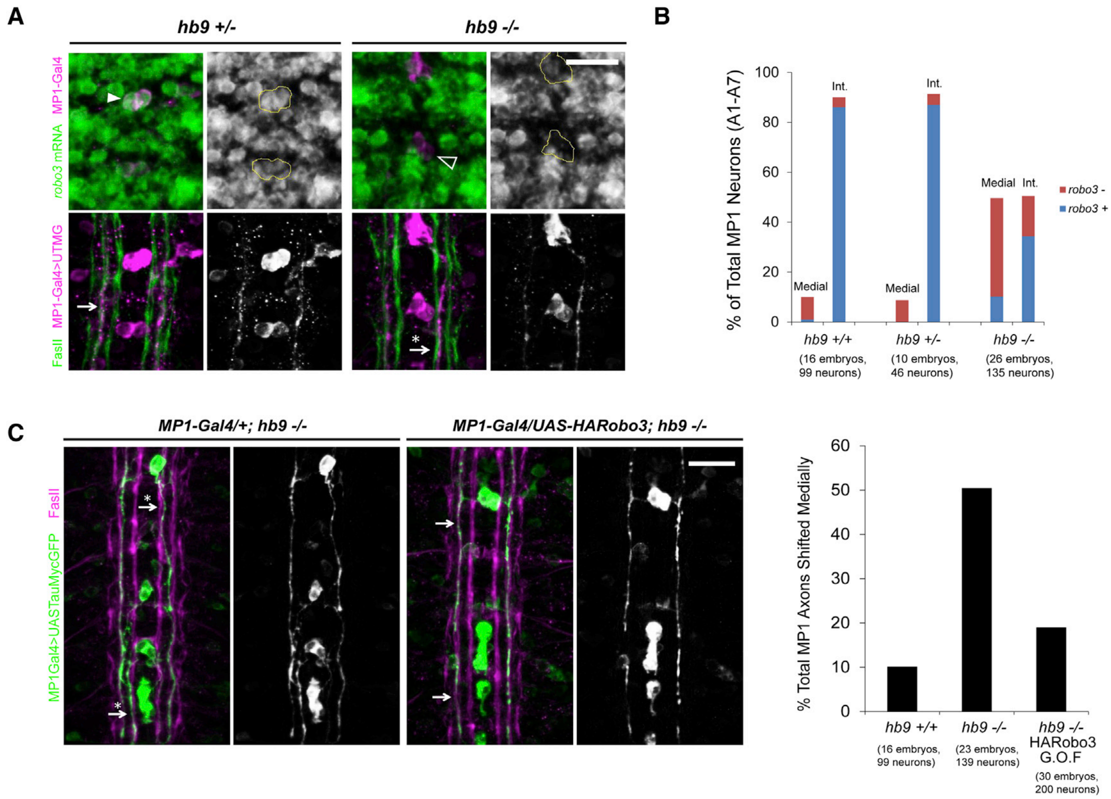

Figure 7. Robo3 Acts Downstream of Hb9 to Direct the Lateral Position of MP1 Axons

(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). Bottom panel shows that 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) Overexpressing *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, 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.

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.

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 *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 whereas 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 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 *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 cofactors or intermediate targets. In support of this hypothesis, *hb9* 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 10× UAS 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. 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 Volocity using a spinning disk confocal microscope (PerkinElmer) using a Nikon 40× objective with a Hamamatsu C10600-10B CCD 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.

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