Drosophila homeodomain protein Nkx6 coordinates motoneuron subtype identity and axonogenesis

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Summary

The regulatory networks acting in individual neurons to control their stereotyped differentiation, connectivity, and function are not well understood. Here, we demonstrate that homeodomain protein Nkx6 is a key member of the genetic network of transcription factors that specifies neuronal fates in *Drosophila*. Nkx6 collaborates with the homeodomain protein Hb9 to specify ventrally projecting motoneuron fate and to repress dorsally projecting motoneuron fate. While *Nkx6* acts in parallel with *hb9* to regulate motoneuron fate, we find that *Nkx6* plays a distinct role to promote axonogenesis, as axon growth of Nkx6-positive motoneurons is severely compromised in *Nkx6* mutant embryos. Furthermore, *Nkx6* is necessary for the expression of the neural adhesion molecule Fasciclin III in Nkx6-positive motoneurons. Thus, this work demonstrates that *Nkx6* acts in a specific neuronal population to link neuronal subtype identity to neuronal morphology and connectivity.

Key words: *Drosophila melanogaster*, Neuronal fate specification, Motoneurons, Interneurons, Axon outgrowth, *Nkx6*, *hb9* (exex), *lim3*, *islet*, *eve*, *vnd*

Introduction

The development of neuromuscular circuits depends critically on the specification of distinct motoneuron (MN) subtypes during development. Conserved transcriptional regulators help establish MN subtype identity (see Jurata et al., 2000; Thor and Thomas, 2002). The expression of unique combinations of transcription factors in distinct MN subtypes probably regulates the differential expression of cell-surface receptors that translate guidance cues to downstream effectors of cytoskeletal changes (e.g. Kania and Jessell, 2003). Such cytoskeletal rearrangements enable motor axons of different MN subtypes to make strikingly distinct guidance choices in a common environment of guidance cues. However, the manner in which distinct transcription factor profiles are translated into unique patterns of motor axon projections remains an outstanding question.

In many model systems, MNs that extend axons along common trajectories express similar sets of transcriptional regulators – which in turn regulate key aspects of the differentiation of these MN subtypes (for a review, see Thor and Thomas, 2002). *Drosophila* MNs are classified by the location of the body wall muscles they innervate. MNs that innervate dorsal body wall muscles in *Drosophila* express the homeodomain (HD) transcription factor Even-skipped (Eve) (Doe et al., 1988). Furthermore, genetic analyses indicate that Eve is a key determinant of the fate of dorsally projecting MNs (Landgraf et al., 1999). Eve engages in a cross-repressive interaction with the HD protein Hb9, a determinant of ventrally projecting MNs (Broihier and Skeath, 2002). Ventrally projecting MNs also express the HD transcription factors Lim3 and Islet. Functional analyses have demonstrated that these three HD factors are required for proper axon guidance of ventrally projecting MNs (Broihier and Skeath, 2002; Odden et al., 2002; Thor and Thomas, 1997; Thor et al., 1999). The genetic hierarchy governing the fate of ventrally projecting neurons has, however, remained elusive as Lim3, Islet, and Hb9 are expressed independently of each other.

Lim3, Islet, and Hb9 are conserved regulators of MN cell fate whose vertebrate homologs – Lhx3/4, Islet 1/2, and Hb9 – play key roles in vertebrate MN specification (Arber et al., 1999; Sharma et al., 1998; Thaler et al., 1999; Tsuchida et al., 1994). In vertebrates, the genetic hierarchy linking the three transcription factors appears more linear than in *Drosophila*, as Hb9 regulates Lhx3/4 and Isl1/2 expression (Arber et al., 1999; Thaler et al., 1999). As in *Drosophila*, the vertebrate Eve homolog, Evx1, is expressed in a distinct population of neurons – in this case, a subset of vertebrate interneurons (Moran-Rivard et al., 2001).

In *Drosophila* and vertebrates, Hb9, Islet1/2, and Lhx3/4 are expressed almost exclusively by postmitotic neurons. In vertebrates, the expression of these factors in MNs depends on proper establishment of the MN progenitor domain by the coordinated action of upstream HD transcription factors (Briscoe et al., 2000). For example, the pair of Nkx-class HD proteins, Nkx6.1 and Nkx6.2 (Nkx6 proteins), have complementary expression patterns in MN and interneuron progenitors (Briscoe et al., 2000; Cai et al., 1999). Nkx6.1/Nkx6.2 compound mutants exhibit a near complete loss of somatic MNs, demonstrating that Nkx6 proteins are

essential for MN generation (Sander et al., 2000; Vallstedt et al., 2001). Expression of Nkx6 proteins persists in postmitotic MNs, where they regulate proper nuclear migration and axon guidance in visceral MNs in the hindbrain (Müller et al., 2003; Pattyn et al., 2003).

To explore further the genetic networks behind neuronal diversification in *Drosophila*, we investigated the role of the *Drosophila Nkx6* homolog in regulating distinct MN fates. We characterized genetic interactions between *Nkx6* and factors essential for neuronal fate acquisition. We present evidence that *Nkx6* collaborates with *hb9* (*exex* – FlyBase) to regulate the fate of distinct neuronal populations. Our analysis of *hb9 Nkx6* double mutant embryos indicates that ventrally projecting MNs fail to develop properly in these embryos, while expression of *eve*, a key determinant of dorsally projecting MN identity, expands. In addition, we demonstrate that *Nkx6* promotes axonogenesis of Nkx6-positive neurons. Consistent with a direct regulatory role in this process, *Nkx6* activates the expression of the neural adhesion molecule Fasciclin III in ventrally projecting motoneurons. These data suggest that *Nkx6* is a primary transcriptional regulator of molecules essential for axon growth and guidance in a specific neuronal population.

Materials and methods

Fly stocks

The following fly stocks were used: $hb9^{KK30}$, $hb9^{JJ154}$, $hb9^{GAL4}$, UAS-hb9 (Broihier and Skeath, 2002), UAS-vnd (D. Mellerick), UAS-GAPGFP (A. Chiba), elavGAL4 (A. DiAntonio), UAS-eve (A. Brand), lim3-τmyc (S. Thor). $Nkx6^{P[JG]}$ was generated in an enhancer trap screen (A.K. and W.O., unpublished data). We generated a $Nkx6^{GAL4}$ enhancer trap from the $Nkx6^{P[JG(LacZ)]}$ enhancer trap via targeted transposition essentially as described by Sepp and Auld (Sepp and Auld, 1999). We verified that this line expressed GAL4 in a Nkx6-dependent manner by expressing GAP-GFP with this driver and double labeling the embryos with anti-Nkx6 and anti-GFP (data not shown). Early in embryogenesis, GFP is expressed in an identical pattern to Nkx6, though more neurons express GFP than Nkx6 at later stages (data not shown). We attribute this difference to the dynamic nature of the Nkx6 expression pattern and perdurance of GAL4 in Nkx6-negative neurons. $Nkx6^{D25}$ was generated by imprecise excision of $Nkx6^{P[JG]}$ via standard methods. All other stocks were obtained from the Bloomington Stock Center.

Nkx6 cDNA and *UAS-Nkx6*

We isolated a full-length *Nkx6* cDNA via RT-PCR from RNA prepared from a 0- to 20-hour collection of Oregon R embryos. *polyA* RNA was prepared using the RNeasy midi kit and oligotex beads (Qiagen) and converted into cDNA using superscript II reverse transcriptase (Gibco BRL). *Nkx6* cDNA was generated using primers that amplify from the predicted start to the predicted stop codon. We cloned and sequenced the 1539 bp product, which matches *Nkx6* cloned from an embryonic library (Uhler et al., 2002) at the amino acid level. To create *UAS-Nkx6*, we inserted the *Nkx6* cDNA into the *Not*1 site of pUAST (Brand and Perrimon, 1993) and created germline transformants following standard methods.

Antibody production, immunofluorescent, and immunohistochemical studies

Amino acids 34-386 of Nkx6 were cloned into pET29a (Novagen) for protein expression and purification. This antigen was used to immunize rats at Pocono Rabbit Farm. Confirming antibody specificity, we failed to detect Nkx6 protein in embryos homozygous for $Nkx6^{D25}$ or Df(3L)fz-D21 (data not shown). The following primary antibodies were used: rat anti-Islet, guinea pig anti-Lim3, rabbit anti-Hb9 (Broihier and Skeath, 2002); rabbit anti-Odd (E. Ward); rabbit anti-Vnd (D. Mellerick); rabbit anti-GFP (P. Silver); rabbit anti-Eve (M. Frasch); mouse anti-Eve (N. Patel); mouse anti-Myc (Sigma); rabbit anti-βgal (ICN; mouse anti-βgal (Promega); and mouse monoclonal 1D4/Fas2 and 7G10/Fas3 were generated by C. Goodman's Laboratory and obtained from the Developmental Studies Hybridoma Bank. The anti-Lim3 antibody was affinity purified using the Ultralink Immobilization Kit (Pierce). We used the Vector ABC kit for immunohistochemistry and Alexa-488 and Alexa-594 with appropriate species specificity for immunofluorescence (Molecular Probes).

Results

Nkx6 expression and functional analysis

To identify additional regulators of neuronal fate determination in *Drosophila*, we conducted a computational search of the *Drosophila* genome for uncharacterized homologs of vertebrate genes with essential functions in neuronal fate specification (H.T.B. and J.B.S., unpublished). A detailed analysis of other regulators will be described elsewhere; here we describe our identification and characterization of *Drosophila* homeodomain protein Nkx6. We identified a single *Drosophila Nkx6* homolog (Fig. 1A) (Uhler et al., 2002) located in the 70C-D region of chromosome 3L. RNA in situ hybridization analysis indicated that *Nkx6* is expressed in the embryonic central nervous system (CNS) (data not shown).

To initiate a functional analysis of *Nkx6*, we identified a P element insertion, $P^{\{JG[LacZ]\}}$ inserted 4 KB downstream of the *Nkx6* locus. $P^{\{JG[LacZ]\}}$ is an enhancer trap and a mutant allele of *Nkx6* as Nkx6 protein levels are greatly reduced in $P^{\{JG[LacZ]\}}$ homozygotes (Fig. 1A; data not shown). Via imprecise excision of $P^{\{JG[LacZ]\}}$, we generated a 25 kB deletion that removes the 3′ end of the *Nkx6* locus ($Nkx6^{D25}$; see Materials and methods; Fig. 1A). $Nkx6^{D25}$ homozygous embryos do not express *Nkx6* RNA or protein (data not shown), indicating that $Nkx6^{D25}$ is a null allele of the *Nkx6* locus. The deletion also removes *CG13479*, a predicted gene with a single 83 amino acid ORF situated 14 KB downstream of *Nkx6*. We attribute the CNS phenotypes observed in $Nkx6^{D25}$ mutant embryos to the *Nkx6* locus for four reasons. First, CG13479 is unlikely to be an embryonically-expressed transcript since we fail to detect CG13479 expression in wild-type embryos via RNA *in situ* hybridization (data not shown) and the Berkeley Drosophila Genome Project has not identified any embryonic ESTs for CG13479. Second, the axonal phenotypes in *Nkx6* mutant embryos are largely rescued by *Nkx6* expression in the CNS (see Fig. 4). Third, we were able to phenocopy the Eve phenotype that we observe in $hb9^{KK30}$ $Nkx6^{D25}$ double mutant embryos by injection of ds *Nkx6* RNA into *hb9* mutant embryos (see below; see Fig. S1 in the supplementary material). Fourth, the cell fate and axonal outgrowth phenotypes we observe in $Nkx6^{D25}$ embryos are reciprocal to those observed when *Nkx6* is misexpressed via the GAL4/UAS system (see Fig. 5) (Brand and Perrimon, 1993).

To identify the neuronal cell types most likely to be affected by loss of *Nkx6* function, we generated Nkx6-specific antibodies. We verified antibody specificity on embryos bearing deletions of the *Nkx6* locus (Materials and methods). We find that Nkx6 exhibits a highly dynamic expression pattern

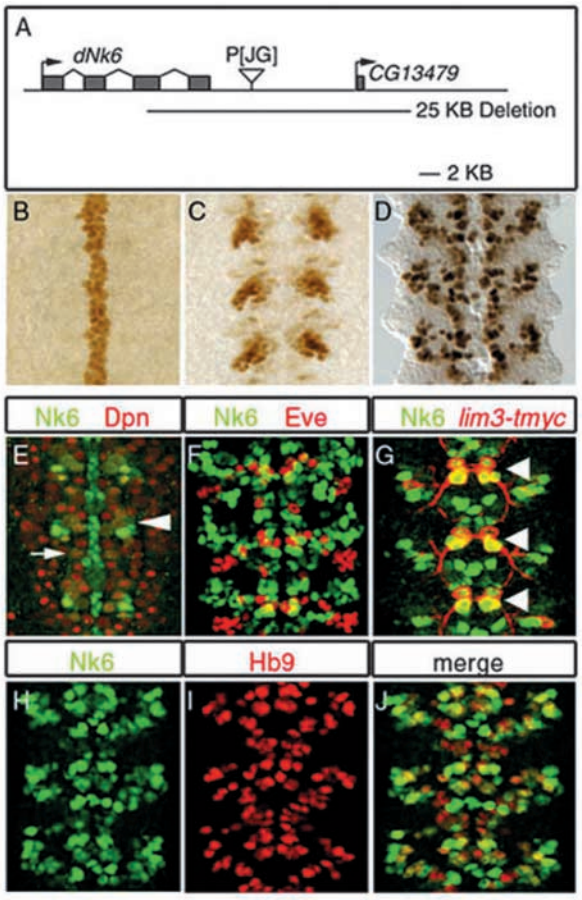

Fig. 1. *Nkx6* allele generation and expression analysis. (A) Diagram of the *Nkx6* locus, with the location of the P[JG] insertion and the extent of the D25 deletion indicated. (B) Stage 9, (C) stage 11, and (D) stage 14 wild-type embryos stained for Nkx6. (B) Nkx6 is expressed in midline precursors at stage 9. (C) At stage 11, Nkx6 is expressed in compact clusters of neural precursor cells and neurons flanking the midline. (D) Nkx6 is expressed in 30 to 40 postmitotic neurons at stage 14. Note, Nkx6 expression levels vary widely between neurons. (E-J) Wild-type embryos labeled with indicated antibodies. (E) Nkx6 and Dpn are co-expressed in medial neuroblasts in stage 11 embryos. Arrowhead points to a cluster of NBs including NBs 1-2, 3-1, 3-2, and 4-2. Arrow points to NB5-2. (F) Confocal projection of stage 14 CNS with Nkx6 and Eve expressed in complementary subsets of postmitotic neurons. (G) Nkx6 and a lim3-taumyc reporter are co-expressed in the medial 1 and 3-5 RP MNs (arrowheads) at stage 14. (H-J) Confocal projection of stage 15 embryo with Nkx6 and Hb9 expressed in largely overlapping populations of postmitotic neurons. Three segments of dissected nerve cords are shown in B-J with anterior oriented up.

within the embryonic CNS (Fig. 1B-D). Nkx6 is first expressed in CNS midline precursors at embryonic stage 9 (Fig. 1B); this expression is transient and is extinguished by stage 10. At stage 10, we observe relatively weak Nkx6 expression in neural precursors or neuroblasts (NBs). To identify the Nkx6-positive NBs, we assayed Nkx6 expression relative to Svp-LacZ and Deadpan – characterized markers of NB identity. These experiments illustrate that NBs 1-1, 1-2, 2-2, 3-1, 3-2, 4-2 and 5-2 express Nkx6 at low to moderate levels (Fig. 1E; data not shown). By stage 11, Nkx6 is expressed in medial clusters of approximately 15 cells (Fig. 1C). Based on their position and size, these cells appear to be a mixture of intermediate precursor cells called ganglion mother cells (GMCs) and postmitotic neurons. Beginning at stage 12, neurons in the intermediate and lateral regions of the CNS activate dNkx6 expression. By stage 14, Nkx6 is expressed in a complex pattern of 30-40 neurons in each hemisegment. Notably, Nkx6 expression levels vary dramatically and reproducibly between neurons in late-stage embryos (Fig. 1D,F,H). The dynamic pattern of Nkx6 expression in the CNS suggests *Nkx6* may function in the development of specific CNS cell types.

To establish the identity of Nkx6-positive neurons, we compared Nkx6 expression to markers of defined neuronal subsets. We first investigated whether Nkx6 is expressed in MN and interneuron populations in *Drosophila*. We compared Nkx6 expression to that of Odd-skipped (Odd), and find that Nkx6 and Odd are co-expressed in the MP1 and dMP2 interneurons (data not shown). We then asked whether Nkx6 is present in distinct MN groups. To this end, we compared Nkx6 expression to that of Hb9 and Eve. Hb9 is expressed in ventrally and laterally projecting MNs while Eve is expressed in dorsally projecting MNs (Broihier and Skeath, 2002; Landgraf et al., 1999; Odden et al., 2002). Like Hb9 and Eve, Nkx6 and Eve are also expressed in complementary patterns (Fig. 1F). On the other hand, the majority of Nkx6-expressing cells express Hb9, although Nkx6 is expressed in slightly more neurons than Hb9 (Fig. 1H-J). The extensive co-expression of Nkx6 and Hb9 suggested that Nkx6 is also expressed in ventrally projecting MNs. Confirming this, we find that Nkx6 is co-expressed with a Lim3-τmyc transgene, a marker of RP 1,3,4,5 (RP MNs) – a group of well-characterized ventrally projecting MNs (arrowheads in Fig. 1G) (Thor et al., 1999). This analysis established that Nkx6 is expressed in both interneurons and ventrally projecting MNs.

The co-expression of *Nkx6* and *hb9* in ventrally projecting MNs raised the possibility that they act in a linear genetic pathway to control the development of these MNs. In addition, vertebrate *Nkx6.1* is expressed in MN progenitors and is necessary for the activation of *Hb9* in postmitotic MNs (Sander et al., 2000; Vallstedt et al., 2001). However, we find that Nkx6 and Hb9 are expressed independently of each other in the *Drosophila* CNS (data not shown). Thus, if *Nkx6* regulates neuronal fate, it does so independently of regulating *hb9* transcription. Instead, the independent regulation of *Nkx6* and *hb9* combined with their similar expression profiles suggests they may act in parallel to regulate neuronal fate.

Nkx6 and *hb9* act in parallel to repress *vnd*

During early CNS development, Nkx6 is co-expressed with Ventral nervous system defective (Vnd) in a subset of medial column NBs (see Fig. S2 in the supplementary material), prompting us to investigate the genetic relationship between *vnd* and *Nkx6*. Vnd expression marks medial column CNS NBs and is required for the development of these cells (Chu et al., 1998; Jimenez and Campos-Ortega, 1990; Skeath et al., 1994). We first compared Nkx6 and Vnd expression in wild-type embryos. Surprisingly, while Nkx6 and Vnd are co-expressed in a subset of medial column NBs, their expression patterns are otherwise complementary (Fig. 2A-C). At stage 9, Nkx6 is expressed in CNS midline precursors, while Vnd is expressed

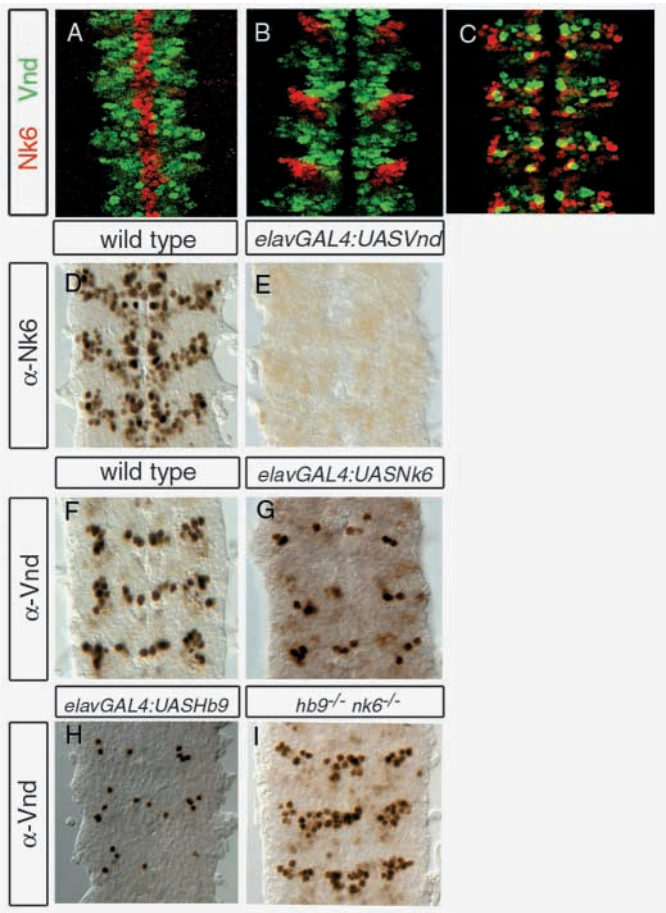

Fig. 2. *Nkx6* and *hb9* antagonize *vnd*. (A) Stage 9, (B) stage 11, (C) stage 14 wild-type embryos stained for Nkx6 and Vnd. (A) At stage 9 Nkx6 is expressed in midline precursor cells and Vnd is expressed in flanking ventral neuroectoderm. (B) At stage 11 Nkx6 is expressed in clusters of GMCs and neurons situated within stripes of Vnd-positive GMCs and neurons. (C) At stage 14 Nkx6 and Vnd are expressed in mutually exclusive populations of postmitotic neurons. (D) In stage 15 wild-type embryos approximately 30 neurons express Nkx6 while (E) *elavGAL4:UAS-vnd* embryos are devoid of Nkx6-positive neurons. (F) In stage 15 wild-type embryos approximately ten neurons express Vnd while (G) *elavGAL4:UAS-Nkx6* mutants and (H) *elavGAL4:UAS-hb9* mutants exhibit fewer Vnd-positive neurons. (I) Stage 15 $hb9^{KK30}$ $Nkx6^{D25}$ double mutant embryo displays ectopic Vnd-positive neurons. Three segments of dissected nerve cords with anterior up are shown in all panels.

in ventral neuroectoderm flanking the midline (Fig. 2A). During stage 10, low-level Nkx6 expression initiates in five Vnd-positive NBs per hemisegment. At stage 11, Vnd and Nkx6 are expressed in non-overlapping groups of GMCs and postmitotic neurons. Notably, at this stage clusters of Nkx6-expressing cells are nestled within stripes of Vnd-expressing cells (Fig. 2B). The complementary patterns of Nkx6 and Vnd in GMCs and neurons are maintained throughout embryogenesis (Fig. 2C). These data raised the possibility that opposing activities of *Nkx6* and *vnd* help establish and maintain their respective expression patterns.

To investigate whether the complementary expression patterns of Nkx6 and Vnd arise due to their opposing activities, we asked if *vnd* misexpression represses *Nkx6*. These analyses focus on the genetic relationship between *Nkx6* and *vnd* in postmitotic neurons since these genes exhibit mutually exclusive patterns in these cells. We used the elav-GAL4 driver to express *vnd* in postmitotic neurons and found this abolishes CNS expression of *Nkx6* (Fig. 2D,E). We were unable to obtain meaningful loss-of-function data for *vnd* because nearly all medial column NBs and their progeny, many of which are Nkx6-positive, fail to develop in *vnd* mutant embryos. The requirement of *vnd* to promote medial column NB formation inhibited our ability to assay the effect of removing *vnd* function on Nkx6. Nevertheless, the ability of *vnd* misexpression to abolish *Nkx6* expression supports the model that *vnd* represses *Nkx6* to help establish the complementary expression patterns of Nkx6 and Vnd.

In the reciprocal experiment, we found that postmitotic misexpression of *Nkx6* dramatically reduces the number of Vnd-positive neurons (compare Fig. 2F,G). Normally, 10.0±1.3 neurons express Vnd per hemisegment (n=30) whereas only 4.2±1.8 neurons express Vnd per hemisegment (n=53) in *Nkx6* misexpression embryos. However, Vnd expression is wild type in *Nkx6* mutant embryos (data not shown). Thus, *Nkx6* is sufficient but not necessary to repress *vnd* expression.

These data suggest that while high levels of Nkx6 and Vnd are cross-repressive in postmitotic neurons, these factors function in concert with other regulators during normal development to limit each other's expression. Given the similar expression profiles of Nkx6 and Hb9 and their independent regulation we asked whether *Nkx6* and *hb9* act in parallel to repress *vnd* expression. As observed for *Nkx6*, *hb9* misexpression in postmitotic neurons significantly reduces the number of Vnd-positive CNS neurons (2.8±1.7 neurons per hemisegment; n=48; Fig. 2H) while *hb9* mutants exhibit wild-type Vnd expression (data not shown). However, removal of both *hb9* and *Nkx6* leads to an overproduction of Vnd-positive neurons as 13.6±2.1 Vnd-positive neurons (n=41) develop in double mutant embryos relative to ten in wild type (compare Fig. 2F and 2I). These results show that *hb9* and *Nkx6* act in parallel to repress *vnd*, and support the model that the complementary patterns of *Nkx6* and *vnd* arise at least in part due to their opposing activities.

Nkx6 and *hb9* collaborate to regulate MN fate

We next explored the regulatory relationship between *Nkx6* and *hb9* – both of which are expressed in ventrally projecting motoneurons – and the dorsal motoneuron determinant *eve*. *eve* and *hb9* engage in a cross-repressive relationship to maintain their expression in distinct neuronal populations (Broihier and Skeath, 2002). Since Nkx6 and Eve are also expressed in non-overlapping populations of neurons (Fig. 1E), we asked whether they repress each other. We first asked whether *eve* is sufficient to repress *Nkx6* by misexpressing *eve* in all postmitotic neurons. Eve misexpression results in a near complete suppression of Nkx6 expression by embryonic stage 16 (compare Fig. 3A and 3B), demonstrating that *eve* is sufficient to repress *Nkx6*.

In a reciprocal manner, misexpression of *Nkx6* in all postmitotic neurons severely reduces Eve expression in the U MNs and EL neurons, though Eve expression in RP2 and aCC/pCC appears grossly normal (compare Fig. 3D,E).

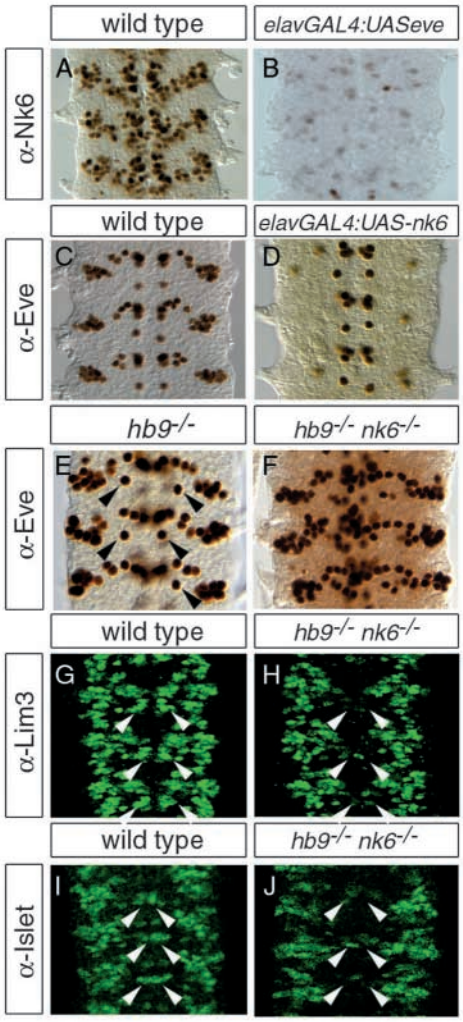

Fig. 3. *Nkx6* and *hb9* collaborate to regulate neuronal fate. (A) Stage 15 wild-type and (B) stage 15 *elavGAL4:UAS-eve* embryos stained for Nkx6. Ectopic *eve* expression largely abolishes *Nkx6* expression. Stage 15 (C) wild-type, (D) *elavGAL4:UAS-Nkx6* (E) $hb9^{KK30}$, and (F) $hb9^{KK30}$ $Nkx6^{D25}$ embryos labeled with α-Eve.
(D) *elavGAL4:UAS-Nkx6* embryos exhibit a specific reduction of Eve expression in the U MNs and EL interneurons, but not in RP2 or a/pCC. (E) *hb9* mutant displays two ectopic Eve-positive neurons per hemisegment (arrowheads). (F) *hb9 Nkx6* mutant displays roughly six ectopic Eve neurons per hemisegment. (G-J) Confocal projections of stage 15 (G,I) wild-type and (H, J) *hb9 Nkx6* mutant embryos stained for Lim3 (G,H) or Islet (I,J). The 1 and 3-5 RP MNs express Lim3 (G) and Islet (I) while in *hb9 Nkx6* double mutants, Lim3 (H) and Islet (J) are no longer expressed in RP MNs (arrowheads). Three segments of dissected nerve cords with anterior up are shown in all panels.

However, as observed for *vnd*, Eve expression is normal in *Nkx6* mutant embryos (data not shown). Thus, *Nkx6* is sufficient but not necessary to repress *eve*.

We find, however, that *Nkx6* and *hb9* also act in parallel to repress *eve*. Stage 15 *hb9* mutant embryos contain 19.4±2.0 Eve-positive neurons per hemisegment (n=58; Fig. 3E). This number represents an increase of two Eve-positive neurons relative to wild type (Broihier and Skeath, 2002). Significantly, stage 15 $hb9^{KK30}$ $Nkx6^{D25}$ double mutant embryos display 24.0±3.7 Eve-positive neurons per hemisegment (n=50; Fig. 3F), representing an increase of six Eve-positive neurons relative to wild type. The ectopic Eve-positive neurons arise at multiple positions within the CNS, suggesting they develop from multiple NB lineages; however, a number are situated close to the midline (see Discussion). To confirm this phenotype is caused by loss of *Nkx6* activity from an *hb9* mutant background, we injected double-stranded *Nkx6* RNA into $hb9^{KK30}$ mutant embryos. We find an average of 24.8±5.9 Eve-positive neurons per hemisegment in these embryos (n=39; see Fig. S1 in the supplementary material), demonstrating that injection of *Nkx6* RNA into *hb9* mutants phenocopies the Eve phenotype observed in $hb9^{KK30}$ $Nkx6^{D25}$ mutants. The further increase of Eve-positive neurons in *hb9 Nkx6* mutant embryos relative to *hb9* mutant embryos demonstrates that *Nkx6* and *hb9* collaborate to repress Eve.

hb9 and *Nkx6* thus act together to limit the expression of *eve*, a key determinant of dorsally projecting MN identity. We next investigated whether *hb9* and *Nkx6* coordinate the specification of ventrally projecting MN identity. RP1,3,4,5 MNs are large Nkx6-positive cells that lie close to the midline and project their axons contralaterally to ventral muscles within ISNb (Fig. 1F, Fig. 6A) (Sink and Whitington, 1991a; Sink and Whitington, 1991b; Schmid et al., 1999). Since both Nkx6 and Hb9 are expressed in RP1,3,4,5 (Fig. 1G) (Broihier and Skeath, 2002), we asked whether these neurons develop properly in *hb9 Nkx6* double mutant embryos. Islet and Lim3 are markers of RP1,3,4,5 identity (Thor and Thomas, 1997; Thor et al., 1999) and are expressed in the these MNs in embryos singly mutant for *Nkx6* or *hb9* (data not shown) (Broihier and Skeath, 2002). However, expression of Islet and Lim3 in the RP1,3,4,5 MNs is strongly reduced in *hb9 Nkx6* double mutant embryos (arrowheads in Fig. 3G-J). Interestingly, the requirement of *Nkx6* and *hb9* to promote Islet and Lim3 expression is relatively specific to these RP MNs, since Islet and Lim3 expression is otherwise grossly normal in these embryos. The absence of these early determinants of RP1,3,4,5 MN identity strongly suggests that RP MNs are specified incorrectly in the absence of *Nkx6* and *hb9* activity. Hence, *Nkx6* and *hb9* act in parallel to control the fate of distinct MN subsets. They collaborate to restrict the expression of Eve, a key determinant of dorsally projecting MN identity, and to promote the expression of Islet and Lim3 in a well-defined subset of ventrally projecting MNs. While these functions of *Nkx6* and *hb9* may be distinct, we favor the model that *Nkx6* and *hb9* promote ventrally projecting MN identity by repressing *eve* expression in RP MNs (see Discussion).

Nkx6 is necessary for axon outgrowth

Nkx6 is co-expressed with Hb9, Lim3, and Islet in populations of ventrally projecting MNs. Since *hb9*, *lim3*, and *islet* are known to be required for proper axon guidance of ventrally projecting axons (Broihier and Skeath, 2002; Odden et al., 2002; Thor and Thomas, 1997; Thor et al., 1999), we asked whether *Nkx6* was also necessary for the axonal development of this MN population. Using Fas2 to label motor axon pathways (mAB 1D4) (Van Vactor et al., 1993), we find that two of the four major nerve branches that innervate ventral and lateral muscles exhibit highly penetrant phenotypes in *Nkx6*

mutant embryos. Specifically, both secondary branches of the ISN, ISNb and ISNd, are absent in a significant proportion of *Nkx6* mutant hemisegments (arrowheads in Fig. 4A-C). Notably, the Nkx6-positive RP1,3,4,5 MNs project within ISNb. We quantified the ISNb phenotype in four allelic combinations of *Nkx6* (Fig. 4E), including embryos transheterozygous for $Nkx6^{D25}$ and an unrelated deficiency of the region. We scored the ISNb as completely absent if we failed to detect axon extension into the ventral muscle field (Fig. 4B). Likewise, we scored ISNb as reduced if any axons grew into the ventral muscle field, even if they initially bypassed their normal choicepoint (Fig. 4C). In all allelic combinations, we found defects in ISNb outgrowth in at least half of all hemisegments. In fact, for $dNkx6^{D25}$ homozygotes or $Nkx6^{D25}/Df(3L)fz\text{-}D21$ transheterozygotes, the penetrance is greater than 90% (Fig. 4E). The penetrance of the ISNd phenotype is roughly equivalent to that of ISNb (Fig. 4A-C; data not shown). Hence, *Nkx6* activity promotes proper axonal development of ISNb and ISNd.

To ensure that loss of *Nkx6* activity is responsible for the observed axonal phenotypes, we assayed whether *Nkx6* misexpression in a *Nkx6* mutant background rescues the ISNb outgrowth phenotype. We used targeted transposition to engineer an $Nkx6^{GAL4}$ enhancer trap from the $Nkx6^{P[JG(LacZ)]}$ enhancer trap (Materials and methods) (Sepp and Auld, 1999). We then used the $Nkx6^{GAL4}$ driver to express *Nkx6* in $Nkx6^{GAL4}/Nkx6^{D25}$ mutant embryos (Fig. 4E). We find that *Nkx6* expression is sufficient to rescue ISNb outgrowth in 72% of hemisegments (n=154), compared to 27% in the absence of *Nkx6* misexpression (n=166). The ability of *Nkx6* expression to largely rescue the observed motor axon phenotypes provides strong evidence that loss of *Nkx6* is responsible for the axonal phenotypes in $Nkx6^{D25}$ mutant embryos.

Since *Nkx6* and *hb9* act in parallel to regulate neuronal fate (see above), we wondered whether they also act in parallel to regulate axon growth. However, the motor axon phenotypes in *hb9 Nkx6* mutant embryos are nearly identical to those in *Nkx6* mutants (Fig. 4E). Therefore, while *Nkx6* and *hb9* collaborate to regulate multiple neuronal fates, *Nkx6* plays a specific non-redundant role to promote axonogenesis.

To examine the role of Nkx6 during axonogenesis in more detail, we focused on axon projections of Hb9-positive neurons. Since Hb9 and Nkx6 are normally expressed in largely overlapping neuronal subsets, this enriched for Nkx6-positive axons relative to Fas2, which labels all motor axons. In wild-type embryos, Hb9-positive axons project in ISNb and synapse with their appropriate targets (Fig. 4F). However, we fail to detect Hb9-positive axons in ISNb in $Nkx6^{D25}$ homozygous mutant embryos (Fig. 4G). In fact, few Hb9-positive axons are observed in the periphery of *Nkx6* mutant embryos, suggesting these motor axons may remain in the nerve cords of *Nkx6* mutants. To test this, we followed Hb9-positive axons in the nerve cords of *Nkx6* mutant embryos. In wild type, Hb9-positive interneurons extend axons in multiple longitudinal fascicles in the CNS (Fig. 4H). In contrast, in *Nkx6* mutant embryos, we observe very few Hb9-positive axons projecting along longitudinal fascicles (Fig. 4I). Since all Hb9-positive neurons appear to be specified in *Nkx6* mutants, the motor axon phenotypes observed in *Nkx6* mutants do not represent motoneuron to interneuron transformations. Rather, these data argue that *Nkx6* potentiates axon growth of Nkx6-expressing neurons.

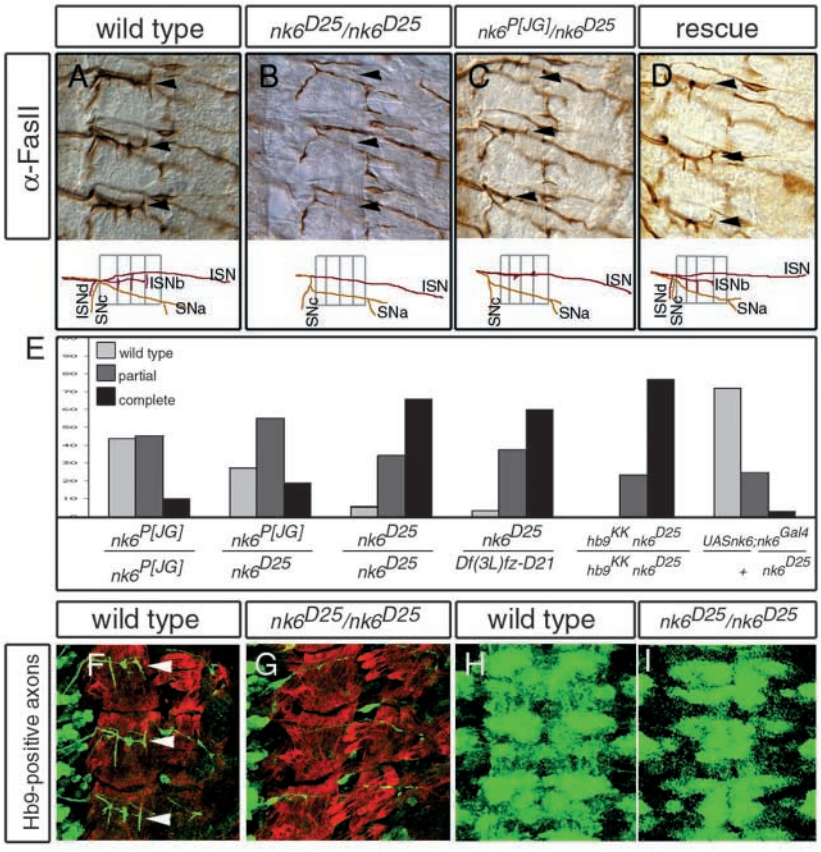

Fig. 4. *Nkx6* is necessary for axon growth. Three hemisegments of Stage 16 dissected (A) wild-type (B) $Nkx6^{D25}/Nkx6^{D25}$ (C) $Nkx6^{P[JG]}/Nkx6^{D25}$, and (D) *UAS-Nkx6/+;* $Nkx6^{Gal4}/Nkx6^{D25}$ embryos labeled with α-Fas2 to mark motor axon projections. Schematics summarizing the observed phenotype are shown below each panel. (A) In wild type, ISNb (arrowheads) and ISNd innervate the ventral muscle field. (B) In $Nkx6^{D25}$ homozygous embryos, ISNb/d do not innervate their muscle targets. Arrowheads indicate ventral muscle field. (C) In $Nkx6^{P[JG]}/Nkx6^{D25}$ mutants the ventral muscle field is not innervated, though presumptive ISNb axons extend along ISN (arrowheads). (D) In *Nkx6* rescue embryos ISNb axons usually contact their appropriate target muscles. (E) Penetrance of mutant phenotypes for different *Nkx6* allelic combinations. See text for details. (F) Three hemisegments of wild-type and (G) *UAS-GAPGFP/+;* $hb9^{GAL4}$ $Nkx6^{D25}/Nkx6^{D25}$ mutants labeled with α-GFP (green) to mark Hb9-positive axons and α-MHC (red) to mark muscles. Hb9-positive axons extend along ISNb to contact ventral muscles in wild-type (F), but not mutant (G) embryos. (H) Three segments of wild-type and (I) *UAS-GAPGFP/+;* $hb9^{GAL4}$ $Nkx6^{D25}/Nkx6^{D25}$ mutant nerve cords labeled with α-GFP to mark Hb9-positive axons. In wild type (H), numerous Hb9-positive longitudinal fascicles are visible, while in I very few Hb9-positive axons are present. Anterior is up in all panels.

Nkx6 overexpression leads to ISNb overgrowth

The impaired axon extension observed in *Nkx6* mutant embryos argues that *Nkx6* is a positive mediator of axon growth. To test this model, we analyzed axon growth in embryos that over-expressed *Nkx6* in all postmitotic neurons. To ensure high levels of Nkx6 expression in neurons, we used embryos carrying *elavGAL4* and two copies of *UAS-Nkx6* and again focused on ISNb. The overall pattern and thickness of ventral motor axon projections (including ISNb) is normal in these embryos suggesting that postmitotic overexpression of *Nkx6* does not result in widespread transformations of neurons to the ventrally projecting MN fate (Fig. 5). In support of this, Hb9 expression is wild type in *elavGAL4:2XUAS-Nkx6* embryos (data not shown). However, in this background a significant proportion of ISNb axons exhibit phenotypes consistent with overgrowth (Fig. 5). For example, we observed at least one ISNb branch with a clearly expanded terminal arbor in 28% of hemisegments (*n*=176; Fig. 5B) compared to 6% in wild type (*n*=155). We also observed two phenotypes in *Nkx6* overexpression embryos that we never observed in wild type (*n*=155). Namely, we observed excessive axonal branching in ISNb in 14% of hemisegments (*n*=176; Fig. 5C). In 4% of hemisegments, ISNb axons from adjacent segments extend across the segment boundary and fuse together (*n*=176; Fig. 5D). These data support the conclusion that Nkx6 overexpression in ISNb-projecting neurons leads to axonal overgrowth, probably via the upregulation of molecules that promote axon growth and regulate guidance. Furthermore, the reciprocal effects of loss of function and overexpression of *Nkx6* on axon growth argue that *Nkx6* activates genes that promote axonogenesis.

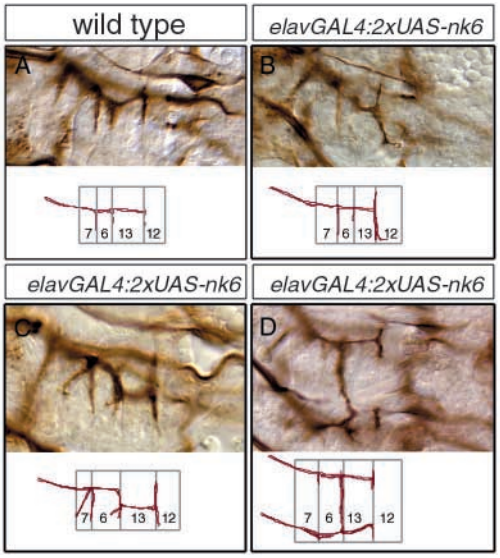

Fig. 5. *Nkx6* overexpression drives ISNb overgrowth. (A) wild-type and (B-D) *elavGAL4:2xUAS-Nkx6* embryos labeled with α-Fas2 to mark motor axons. Schematics outlining motor axon phenotypes are below each panel. Single hemisegments are shown in (A-C), in (D) adjacent hemisegments are shown. (A) In wild type, ISNb innervates ventral muscles 7, 6, 13, and 12 in a stereotyped fashion. (B-D) In *Nkx6* overexpression embryos ISNb appears to exhibit exuberant growth. In (B) the terminal arbor between muscles 13 and 12 is expanded. (C) ISNb contains supernumerary branches. (D) ISNb in adjacent hemisegments have crossed the segment boundary and fused. Anterior is up in all panels.

Nkx6 regulates axon development and gene expression of ventrally projecting RP MNs

The preceding analysis indicates that *Nkx6* promotes axon outgrowth of a subset of MNs. To investigate this possibility in more detail, we followed the well-characterized axon projections of the RP1,3,4,5 MNs in wild type and *Nkx6* mutant backgrounds. We utilized a *lim3-taumyc* transgene (Thor et al., 1999) to follow RP motor axon projections in wild-type and *Nkx6* mutant embryos (Fig. 6A-D). In wild type, we are able to detect RP motor axons exiting the CNS in 86% of hemisegments scored (*n*=160; arrowheads in Fig. 6A). In contrast, we could trace motor axons leaving the CNS in only 39% of hemisegments of *Nkx6* mutant embryos (*n*=210; posterior hemisegments in Fig. 6C; anterior right hemisegment in Fig. 6D). In most mutant hemisegments, the motor axons appear thinner than in wild type (posterior hemisegments in Fig. 6C). Furthermore, in 61% of *Nkx6* mutant hemisegments, RP motor axons remain within the CNS (*n*=210; all

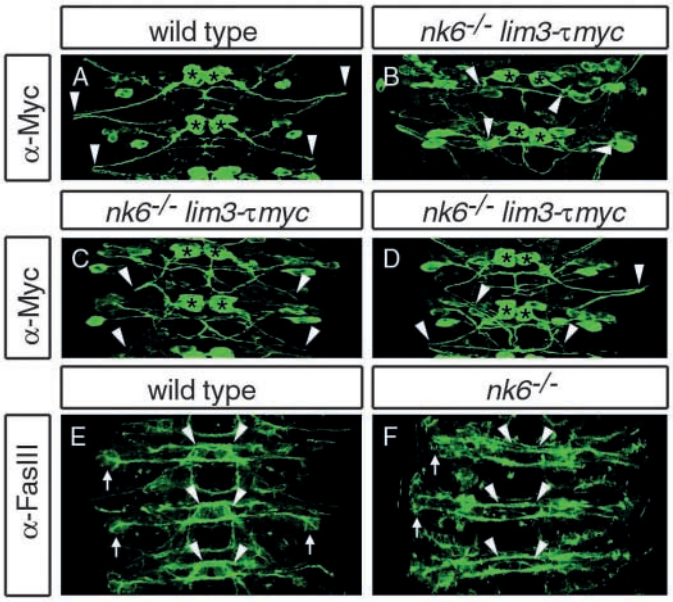

Fig. 6. *Nkx6* promotes axon growth and guidance of RP1,3,4,5 MNs. Confocal projections of four micron series from dissected stage 16 (A) wild-type and (B-D) $Nkx6^{D25}$ nerve cords carrying a *lim3-tmyc* transgene. α-Myc marks axon projections of RP1,3,4,5. (A-D) An asterisk indicates the RP MN cell bodies and arrowheads mark the extent of the RP axon projection. (A) In wild type, RP motor axons leave the CNS. $Nkx6^{D25}$ mutant embryos RP motor axons display various defects. (B) RP motor axons frequently truncate and exhibit aberrant morphology. (C) RP motor axons in the bottom left and both right hemisegments are markedly thinner than in wild-type embryos. (D) RP motor axons in the top left hemisegment diverge from their normal course and turn toward the midline. (E,F) Confocal projections of four micron series from dissected stage 16 nerve cords from (E) wild-type and (F) $Nkx6^{D25}$ mutant embryos labeled with Fas3. (E) In wild type, the Nkx6-positive RP MNs express Fas3 (arrowheads) as do Nkx6-negative lateral neurons (arrows). (F) In *Nkx6* mutant embryos, RP MNs lose Fas3 expression (arrowheads), while expression in more lateral Nkx6-negative neurons is unaffected (arrows). Anterior is up in all panels.

hemisegments in Fig. 6B), compared to 14% of wild type. The morphology of these truncated axons is often aberrant, suggesting their outgrowth has stalled. For example, we frequently observed enlarged growth cones with a club-like appearance (Fig. 6B). Finally, in 10% of mutant hemisegments, RP motor axons make dramatic guidance errors, often turning back inappropriately and extending toward the midline (n=210; anterior left hemisegment in Fig. 6D). These data demonstrate that *Nkx6* activity is critical for proper growth and guidance of the RP1,3,4,5 MNs. Furthermore, the axon phenotypes exhibited by these MNs probably reflect a general requirement of *Nkx6* in promoting axonogenesis of Nkx6-expressing neurons.

The *Nkx6* axonal phenotypes strongly suggest that *Nkx6* regulates – probably directly – molecules that control axonal outgrowth and guidance. Fasciclin III (Fas3), a cell adhesion molecule, is a possible target of *Nkx6* action in MNs as it is expressed by the RP1,3,4,5 MNs and promotes target recognition by their motor axons (Fig. 6E) (Chiba et al., 1995; Patel et al., 1987). Notably, Fas3 expression is strongly reduced in the RP MNs in *Nkx6* mutant embryos relative to wild type (arrowheads in Fig. 6E,F). Consistent with a specific role for *Nkx6* in regulating Fas3 expression, more lateral neurons that express Fas3 (arrows, Fig. 6E,F) but not Nkx6 (data not shown) exhibit wild-type Fas3 expression in *Nkx6* mutants (Fig. 6E,F). Together, these data show that *Nkx6* promotes proper RP motor axon growth, and indicate that *Nkx6* controls RP motor axon growth by regulating the transcription of adhesion and guidance molecules – one of which is Fas3.

Discussion

The nervous system must precisely coordinate neuronal fate and morphology to establish functional neural circuits during development. In fact, neuronal identity is often intimately connected to cellular morphology – for example, MNs can be classified based on common patterns of axonal growth as well as by transcription factor expression profiles. Motor axons of distinct MN subtypes make joint guidance decisions as they navigate toward their muscle targets, suggesting that MN subtypes express related sets of receptor and signaling molecules. Despite this elegant model, little is known about the transcriptional mechanisms acting in populations of neurons to produce characteristic axonal trajectories. Here we present an analysis of *Drosophila* homeodomain protein Nkx6. We find that *Nkx6* has roles in both the specification and differentiation of ventrally projecting MNs (Fig. 7). In particular, we find that *Nkx6* plays a pivotal role in promoting axon growth of these MNs and regulates the expression of a key determinant of synaptic target selection. Hence *Nkx6* activity links neuronal identity and cellular morphology.

Nkx6 and neuronal subtype identity

This work places *Nkx6* in the regulatory circuit that specifies distinct postmitotic neuron fates in the *Drosophila* CNS (Fig. 7). In the mouse, Nkx6 protein function in MN progenitors regulates Hb9 expression in postmitotic MNs (Arber et al., 1999; Sander et al., 2000; Thaler et al., 1999; Vallstedt et al., 2001). We find that *Drosophila* Nkx6 is expressed in neural precursors and postmitotic neurons while Hb9 expression is nearly exclusive to postmitotic neurons (this work) (Broihier and Skeath, 2002; Odden et al., 2002). However, in contrast to the linear relationship of *Nkx6.1/2* and *Hb9* in vertebrates, our experiments establish that *Nkx6* and *hb9* act in parallel to specify neuronal fate in *Drosophila* (Figs 2, 3, 7). *Nkx6* and *hb9* act in concert both to repress expression of the dorsal MN determinant Eve and to promote expression of Lim3 and Islet in ventrally projecting RP MNs. It will be of interest to extend this genetic analysis to other groups of ventrally projecting MNs. It will be also be important to examine the directness of these genetic interactions. Both *Nkx6* and *hb9* contain conserved TN domains that in vertebrate HD proteins have been shown to interact with the Groucho co-repressor, suggesting that *Nkx6* and *hb9* function as transcriptional repressors (Broihier and Skeath, 2002; Jiménez et al., 1997; Muhr et al., 2001; Uhler et al., 2002). This raises the possibility that *Nkx6* and *hb9* bind to sequences in the *eve* enhancer and directly repress its transcription. In addition, *Nkx6* and *hb9* activate *lim3*/*islet* gene expression within ventrally projecting MNs, raising the possibility that they do so by repressing an unidentified repressor of ventrally projecting MN identity.

eve represents an appealing candidate for the unidentified repressor in this model (Fig. 7). Ectopic Eve expression in RP MNs in *hb9 Nkx6* double mutants may repress Lim3 and Islet. Consistent with this, though we were unable to unambiguously identify the ectopic Eve neurons in *hb9 Nkx6* mutants, many of them are situated close to the midline (Fig. 3F) – suggesting they may represent mis-specified RP MNs. Furthermore, pan-neuronal *eve* expression represses Lim3 and Islet expression in the RP MNs demonstrating that Eve can repress Lim3 and Islet (data not shown) (Landgraf et al., 1999). A direct test of this model will require resolving the identity of the ectopic Eve neurons in *hb9 Nkx6* mutant embryos.

While *Nkx6* and *hb9* play conserved roles in MN specification in *Drosophila* and in vertebrates, the genetic network within which they act differs. In vertebrates, *Nkx6* is upstream of *dHb9*, while in *Drosophila*, *Nkx6* and *hb9* display a parallel requirement in MN generation. Why might the genetic relationship between *Nkx6* and *hb9* vary between *Drosophila* and vertebrates? We propose that the reason may relate to the different relationship between regional identity

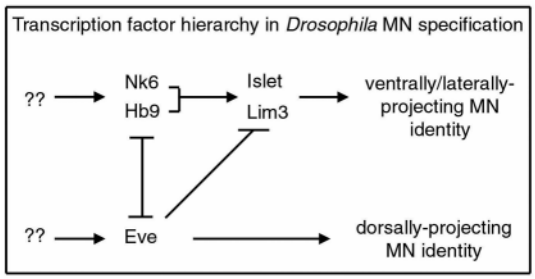

Fig. 7. Transcription factor hierarchy in *Drosophila* MN specification. The factors activating *Nkx6* and *hb9* in ventrally and laterally projecting MNs are largely unknown. In ventrally and laterally projecting MNs, *Nkx6* and *hb9* act in parallel to activate the Lim-HD proteins Lim3 and Islet. *Nkx6* and *hb9* also repress the dorsally projecting MN determinant Eve. Since Eve also represses Lim3 and Islet, it is possible that *eve* repression is responsible for loss of Islet and Lim3 expression in *Nkx6 hb9* double mutant embryos.

and neuronal subtype identity in *Drosophila* relative to vertebrates. In vertebrates, a given neuronal population is generated at a distinct dorsoventral position in the spinal cord in response to graded Sonic hedgehog levels. Thus, gene expression in neural precursors can simultaneously promote both precursor and neuronal subtype identity. In *Drosophila*, no obvious link ties regional identity to neuronal subtype. For example, *Drosophila* NBs arise within three dorsoventral columns (for a review, see Skeath, 1999). However, the dorsoventral position of neural precursors does not regulate postmitotic neuronal identity. NBs at all dorsoventral positions give rise to diverse populations of neurons, and neurons of given subtypes develop at many dorsoventral positions. For example, MNs are generated from NBs across the dorsoventral axis (Schmid et al., 1999). Therefore in *Drosophila* embryos, gene expression in NBs does not directly promote neuronal subtype identity.

A possible mechanism that might contribute to neuronal subtype identity in *Drosophila* is suggested by the temporal gene cascade in NBs (Isshiki et al., 2001; Kambadur et al., 1998). In this cascade, Hunchback (Hb) is expressed in the earliest-born one or two GMCs in a NB lineage followed by sequential expression of Kruppel, Pdm, and Castor in later-born GMCs. The majority of MNs arise from early-born GMCs (Schmid et al., 1999), consistent with the idea that *hb* promotes MN identity. Thus while regional identity promotes postmitotic identity in vertebrates, temporal identity may play a similar role in *Drosophila*. However, many early-born GMCs do not produce MNs, indicating additional layers of complexity. Regional identity may interface with the temporal gene cascade to activate the proper combination of transcription factors to promote the MN fate in a subset of early-born GMCs. In this paradigm, MN specification occurs relatively late in development, suggesting that cells may need to rapidly activate and execute the genetic pathways leading to MN identity. As a result, near simultaneous activation of factors – such a *Nkx6* and *hb9* – that act in parallel to promote MN identity, might be required.

Nkx6 and axonogenesis

While *Nkx6* and *hb9* exhibit parallel requirements in cell fate specification, *Nkx6* plays a specific non-redundant role to promote axon growth and guidance in Nkx6-positive neurons. *Nkx6* is, therefore, probably an element of the transcriptional code regulating the differential transcription of receptor and signal transduction molecules required to promote unique patterns of axon growth and guidance in distinct MN subsets. In support of this, *Nkx6* activity is necessary for Fas3 expression in ventrally projecting RP MNs. Clearly, it will be necessary to elucidate the entire cassette of genes that *Nkx6* activates to promote axonogenesis. In this regard, determining whether *Nkx6* activates the same gene battery in all Nkx6-positive neurons or if *Nkx6* regulation of such genes is cell-type-specific will be of interest.The singular requirement for *Nkx6* in axon growth combined with the redundant functions of *Nkx6* and *hb9* in neuronal specification hints at the transcriptional complexity of neuronal specification and differentiation. It will be important to further distinguish the specification and differentiation functions of Nkx6 – either by identifying additional interacting proteins, or by identifying protein domains within Nkx6 required specifically to promote either specification or differentiation. Precedence for the latter comes from the elucidation of distinct domains within the bHLH proteins Mash1 and Math1 required to promote neuronal differentiation and specification (Nakada et al., 2004).

Nkx6 is one of a number of transcription factors that have been implicated in controlling fundamental aspects of neuronal morphology. In *Drosophila*, several transcription factors have recently been shown to regulate dendritic morphogenesis (Brenman et al., 2001; Grueber et al., 2003; Moore et al., 2002). For example, different levels of the homeodomain protein Cut have been shown to regulate distinct dendritic branching patterns of peripheral nervous system (PNS) neurons, with higher Cut levels directing the development of more complex dendritic arbors (Grueber et al., 2003). Interestingly, we find that Nkx6 protein levels vary dramatically and reproducibly between CNS neurons (Fig. 1). This raises the possibility that Nkx6 directs distinct patterns of axon outgrowth as a function of expression level – potentially adding another layer of complexity to *Nkx6*-transcriptional output.

Our study also indicates that Nkx6 proteins have evolutionarily conserved functions in neuronal fate specification. In *Drosophila*, the role of Nkx6 in neuronal specification is uncovered in *hb9 Nkx6* double mutants. The phenotype we observe in *Nkx6* mutants is a dramatic block to axon growth of Nkx6-positive neurons. Is it possible that this activity of *Nkx6* in postmitotic neurons is conserved? Similar to *Nkx6*, vertebrate Nkx6.1 is expressed in postmitotic neurons, consistent with a functional role in neuronal differentiation. Interestingly, Müller et al. show that hindbrain visceral MNs in *Nkx6*.1 mutant mice display aberrant axon guidance and ectopically express the Ret and Unc5h3 receptors (Müller et al., 2003). Hence, Nkx6-class proteins appear to play conserved roles in regulating axon growth and guidance. It will be critical to determine whether the downstream factors they regulate are conserved as well.

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We dedicate this paper to the memory of Nancy C. Tarczy.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/21/5233/DC1

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