Development 129, 853-862 (2002)
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DEV7912

The *C. elegans even-skipped* homologue, *vab-7*, specifies DB motoneurone identity and axon trajectory

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Accepted 4 November 2001

SUMMARY

Locomotory activity is defined by the specification of motoneurone subtypes. In the nematode, *C. elegans*, DA and DB motoneurones innervate dorsal muscles and function to induce movement in the backwards or forwards direction, respectively. These two neurone classes express separate sets of genes and extend axons with oppositely directed trajectories; anterior (DA) versus posterior (DB). The DA-specific homeoprotein UNC-4 interacts with UNC-37/Groucho to repress the DB gene, *acr-5* (nicotinic acetylcholine receptor subunit). We show that the *C. elegans even-skipped*-like homoedomain protein, VAB-7, coordinately regulates different aspects of the DB motoneurone fate, in part by repressing *unc-4*. Wild-type DB motoneurones express VAB-7, have posteriorly directed axons, express ACR-5 and lack expression of the homeodomain protein UNC-4. In a *vab-7* mutant, ectopic UNC-4 represses *acr-5* and induces an anteriorly directed DB axon trajectory. Thus, *vab-7* indirectly promotes DB-specific gene expression and posteriorly directed axon outgrowth by preventing UNC-4 repression of DB differentiation. Ectopic expression of VAB-7 also induces DB traits in an *unc-4*-independent manner, suggesting that VAB-7 can act through a parallel pathway. This work supports a model in which a complementary pair of homeodomain transcription factors (VAB-7 and UNC-4) specifies differences between DA and DB neurones through inhibition of the alternative fates. The recent findings that Even-skipped transcriptional repressor activity specifies neurone identity and axon guidance in the mouse and *Drosophila* motoneurone circuit points to an ancient origin for homeoprotein-dependent mechanisms of neuronal differentiation in the metazoan nerve cord.

Key words: *C. elegans*, *vab-7*, Motoneurone identity, Axons

INTRODUCTION

The nerve cords of organisms as diverse as nematodes, flies and humans embrace a common architecture with axial arrays of motoneurones distributed along midline bundles of neuronal processes (Hedgecock and Hall, 1990; Eisen, 1998; Jurata et al., 2000). In each case, locomotion depends on the coordinated activities of distinct classes of motoneurones, the interneurones that regulate them and the muscles that they innervate. The proper function of each neuronal subtype is defined by the adoption of appropriate axonal trajectory, neurotransmitter expression and synaptic connectivity. What are the mechanisms that regulate these specific traits? Recent evidence indicates that homeodomain (HD) proteins exercise key roles in the specification of cell type identity in the motoneurone circuit. Hierarchical cascades of interacting HD proteins segregate the vertebrate spinal cord into distinct progenitor domains (Briscoe et al., 2000; Muhr et al., 2001). Subsequently expressed HD proteins may induce the differentiation of specific interneurone and motoneurone subclasses within each of these regions (Tanabe et al., 1998; Jessell, 2000). The roles of many of these transcription factors appear to have been evolutionarily conserved. For example, the LIM class of HD proteins has been shown to govern axonal trajectory and targeting in both vertebrate and invertebrate motoneurone networks (Hobert and Westphal, 2000).

Three motoneurone subtypes, DA, DB and DD, are incorporated into the *C. elegans* ventral nerve cord (VNC) during embryonic development (Sulston, 1983; Sulston et al., 1983). Five additional subclasses of postembryonic motoneurones (VA, VB, VC, AS, VD) are added in the first larval stage (Sulston and Horvitz, 1977). These motoneurones are grouped on the basis of common morphological characteristics, neurotransmitter expression and presynaptic specificity (White et al., 1986). HD transcription factors that regulate subsets of these traits have been identified. The UNC-30 HD protein functions in DD and VD motoneurones (D-class) where it promotes expression of GABA pathway components and is required for normal neuronal morphogenesis (Jin et al., 1994). The LIM-HD protein, LIN-11, is expressed in postmitotic VC motoneurones and mediates VC motor axon fasiculation in the ventral nerve cord (VNC) (Hobert et al., 1998). The UNC-4 HD protein is expressed in A-class motoneurones (DA, VA) to prevent the adoption of B-

class (DB, VB) traits (Miller and Niemeyer, 1995). In *unc-4* mutants, A-class motoneurones are morphologically normal but express a B-type nicotinic acetylcholine receptor (nAChR) subunit, *acr-5* (Winnier et al., 1999). In addition, mutations that disrupt *unc-4* function result in the miswiring of VA motoneurones with presynaptic inputs normally reserved for B-class motoneurones (White et al., 1992). The dependence of these UNC-4 activities on physical interaction with the Groucho-like transcriptional co-repressor protein, UNC-37, indicates that UNC-4 is likely to function as a negative regulator of B-class genes (Pflugrad et al., 1997; Winnier et al., 1999).

We show that the *C. elegans Even-skipped* homologue, VAB-7, is expressed in DB class motoneurones where it functions as a negative regulator of A-class traits. In *vab-7* mutants, ectopic UNC-4 in DB motoneurones results in the adoption of DA type axonal trajectory and repression of the B-class *acr-5* gene. Ectopic expression experiments indicate that *vab-7* may also promote expression of B-class genes through a parallel pathway that does not depend on *unc-4* function. These findings indicate that the proper differentiation of DA and DB motoneurones depends on HD transcription factors that reciprocally repress DB and DA traits, respectively. A related scheme appears to have been used on a grand scale in the vertebrate spinal cord where a similar but more elaborate set of mutually repressive HD proteins specify separate neuronal progenitor domains (Briscoe et al., 2000; Muhr et al., 2001). Involvement of Eve HD proteins in cross-repression of neuronal fates might be conserved, as Eve in *Drosophila* and mouse blocks expression of an alternative program of neuronal differentiation that would otherwise disrupt the function of the motoneurone circuit (Moran-Rivard et al., 2001; Pierani et al., 2001). This work points to the utility of exploiting an organism with a simple, well-defined nervous system and powerful genetics to uncover evolutionarily conserved mechanisms of neuronal differentiation in the metazoan nerve cord.

MATERIALS AND METHODS

Strains

Strains were grown and maintained as described by (Brenner, 1974). The following strains were used in this study: *vab-7(e1562), unc-4(e120), unc-37(e262)*; NC120, *dpy-20(e1282); wdIs1[unc-4::lacZ dpy-20(+)]*; JA1234, *vab-7(e1562); evIs82 [unc-129::gfp dpy-20(+)]*; JA1236, *unc-4(e120); vab-7(e1562)*; *evIs82*; JA1261, *unc-37(e262); vab-7(e1562); evIs82*; JA1262, *unc-37(e262); evIs82*; JA1276, *unc-4(e120); evIs82*; JA1278, *evIs82;weEx43[unc-3::vab-7 rol-6 (d)]*; JA1299, *dpy-20 (e1282); weIs10[unc-3::vab-7 unc-17::gfp dpy-20(+)]*; JA1301, *unc-129(ev554);weIs10*; RM1872, *pha-1(e2123); mdEx72[unc-17::gfp pha-1(+)]*; JA1303, *vab-7(e1562); mdEx72*; JA1304, *unc-4(e120); mdEx72*; JA1305, *weIs10; wdIs1*; JA1313, *weEx52[acr-5::gfplacZ rol-6(d)]*; JA1314, *weIs10;weEx52*; JA1316, *vab-7(e1562); weEx54[vab-7::gfpvab-7 rol-6 (d)]*; NW1099, *dpy-20 (e1282); evIs82*; NC257, *vab-7(e1562);dpy-20(e1282);wdIs4[unc-4::gfp, dpy-20(+)]*; NC244, *vab-7(e1562);dpy-20(e1282);wdEx60[acr-5::gfp dpy-20(+)]*; NC241, *unc-4(e120);vab-7(e1562);dpy-20(e1282);wdEx60*; NC237, *unc-37(e262);vab-7(e1562);dpy-20(e1282);wdEx60.*

Plasmid constructs

To construct *unc-3::vab-7*, the *vab-7* promoter was removed from the pJA17 *vab-7*-rescuing plasmid (Ahringer, 1996) by *Sac*II and *Sph*I digestion. A 4.14kb DNA fragment containing putative *unc-3* promoter to the beginning of exon 2 was amplified by PCR from the *pBP6-1* plasmid (Prasad et al., 1998) using UNC-3PP4 (5′-AAACTGCAGCCGCGGATGCCTGCAGGTCGAC-3′) and UNC-3PP2 (5′-TTTCTGCAGCATGCCAGACCTGAGTAAGGTATTC-3′) primers, cut with *Sac*II and *Sph*I and cloned into pJA17/*Sac*II/*Sph*Ito create plasmid pJA55. The UNC-3::VAB-7 protein product will contain the first 30 amino acids of UNC-3 upstream of VAB-7, and an extra glycine residue at the fusion site that was created to allow in-frame cloning. This *unc-3::vab-7* construct rescues the forward movement defect of *vab-7* mutants (not shown). The *acr-5::gfplacZ* plasmid pJA63 was constructed by subcloning a 4.2kb *Sph*I fragment containing the *acr-5* promoter (from pJR7) (Winnier et al., 1999) into the *gfp::lacZ* expression plasmid pPD96.62.

Generation of transgenic lines

DNA microinjection experiments were performed as previously described (Mello and Fire, 1995). *weEx43* was generated by injection of wild-type hermaphrodites with 100 μg/ml of pRF4 [*rol-6(d)*] (Mello et al., 1991) and 30 μg/ml of pJA55 (*unc-3::vab-7)*. The *weIs10* transgene was made by integration of an extrachromosomal array generated by injection of 30 μg/ml pJA55 (*unc-3::vab-7)* and 100 μg/ml pMH86 (*dpy-20(+)*; (Clark et al., 1995) into a *dpy-20(e1282)* background. The *weIs10* line was integrated as described by Mello and Fire (Mello and Fire, 1995) and was out crossed three times before analysis. *weEx52* (*acr-5::gfplacZ + rol-6(d)*) was generated by injection of 30 μg/ml pJA63 and 100 μg/ml pRF4. β-Galactosidase staining was performed as previously described (Fire et al., 1990).

Generation of anti-VAB-7

A fragment containing the entire *vab-7*-coding region was subcloned into the His tag vector pRSETB (Clontech) to create plasmid pJA18. Protein was purified on a nickel column after denaturation. A mouse was injected seven times over a period of 11 months with 15 μg of His-tagged VAB-7 in Freunds complete adjuvant, followed by intravenous injection of 10 μg protein in phosphate-buffered saline (PBS). The animal was sacrificed 4 days later and the spleen frozen. After fusion of spleen cells, one monoclonal line (2C4) that gave bright staining with no background was obtained.

Immunostaining of embryos, larvae and adults

Immunostaining experiments were performed as follows: embryos isolated by hypochlorite treatment were placed on a poly-lysine coated slide, squashed under a coverslip and frozen on dry ice for 10 minutes. After freezing, the coverslip was flicked off, and 100 μl of 5% formaldehyde in PBS placed on the sample for 20 minutes in a humid chamber. After incubation, the slides were immediately placed in 100% methanol for 4 minutes, in PBS with 0.2% Tween (PBST) for 4 minutes, blocked in 1% non-fat milk in PBST for 10 minutes, then placed in PBST for 10 minutes. Monoclonal anti-VAB-7 primary antibody (from cell culture supernatant) or anti-β-galactosidase antibody (Cappel) was incubated overnight at 4°C; secondary antibodies (FITC anti-mouse or Texas Red Amersham) were incubated for 1-2 hours at room temperature. Samples were mounted using mowiol. For staining of larvae and adults, worms were washed four times in 15 ml distilled water in 15 ml centrifuge tubes before they were placed on poly-lysine-coated slides for adhesion, and then treated as above.

Identification of neurones in the VNC

Identities of neurones in the ventral cord were assigned based on the position of their nuclei in the VNC, their commissures and their axonal processes in the DNC (Sulston, 1983; Sulston et al., 1983; White et al., 1986). Identification of postembryonic neurones expressing VAB-7 was aided by the *unc-129::gfp* marker, which is expressed in DA and DB motoneurones (Colavita et al., 1998). Non-

DB motoneurones were identified by comparing DAPI and *unc-129::gfp* staining data with that of White et al. (White et al., 1986). For identification of neurones with ectopic ACR-5::GFPLACZ in *weIs10;weEx52 (acr-5::gfplacZ)* animals, the *unc-17::gfp* neuronal marker which is expressed in DA, DB, AS, VA, VB and VC motoneurones in the VNC was used (Lickteig et al., 2001) (Rand et al., 2000).

RESULTS

VAB-7 is expressed in DB motoneurones

vab-7 mutants exhibit defects in forward locomotion, as well as in the patterning of posterior muscle and epidermal cells (Ahringer, 1996). *vab-7* mutant L1 larvae are virtually immobile. Beginning with the second larval stage, after post-embryonically derived motoneurones have been added to the ventral cord circuit, *vab-7* mutants show normal backwards movement but curl ventrally when induced to move forwards. These findings indicate that *vab-7* may be important for embryonic motoneurone development. However, previous work using a *vab-7::lacZ* reporter gene did not detect expression in the nervous system; only posterior muscle and epidermal expression was seen (Ahringer, 1996).

To discover whether VAB-7 is expressed in the nervous system, a mouse monoclonal antibody was generated against VAB-7 recombinant protein. Staining embryos with this anti-VAB-7 antibody confirmed that VAB-7 is expressed in posterior muscle and epidermal cells (Fig. 1A,B). However, we also discovered a second phase of VAB-7 expression in embryonic ventral nerve cord (VNC) neurones, beginning at the 1.5-fold stage (Fig. 1 C). At the threefold stage VAB-7 is expressed in nine neuronal nuclei (seven in the VNC and two nuclei in the head), and in the five most posterior epidermal nuclei, which form the hyp8 to hyp11 cells (Fig. 1C-E). The *vab-7(e1562)* allele [which introduces an early stop codon (Ahringer, 1996)] appears to be null, as VAB-7 is not detectable in these embryos (data not shown).

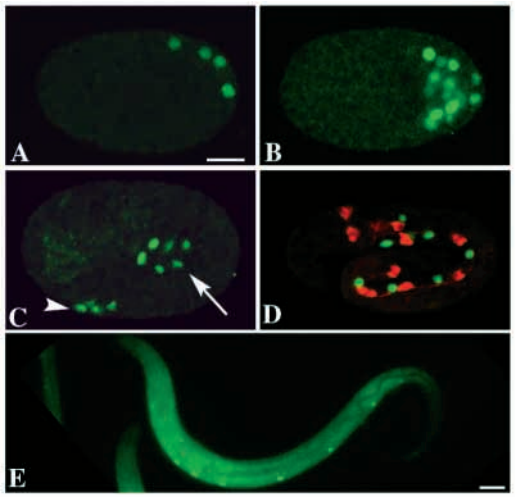


Fig. 1. VAB-7 is expressed in DB motoneurones, as well as in posterior mesodermal and epidermal precursors. Immunostaining of VAB-7 in (A) 100 cell stage embryo and (B) bean stage embryo is in mesodermal and epidermal prescursors. (C) Two-fold embryo. VAB-7 is found in five posterior epidermal cells (hyp8-hyp11; arrowhead), seven DB motoneurones in the ventral nerve cord (arrow) and two unidentified neurones in the head. One DB and the two neurones in the head are not visible in this image. (D) A threefold embryo showing VAB-7 (green) and *unc-25::lacZ*, a DD motoneurone marker (red). (E) An L1 larva showing expression of VAB-7 in all seven DB motoneurones. Anterior is towards the left. Scale bar: 10 μm.

Three classes of motoneurones (DA, DB and DD) are present in the embryonic VNC; DAs and DBs are excitatory cholinergic motoneurones that stimulate dorsal muscles (Fig. 2A). The DDs are GABAergic neurones (McIntire et al., 1993) that are believed to coordinate body bending by inhibiting bodywall muscles on the side opposite to a region of cholinergic motoneurone excitation (i.e. dorsal versus ventral). All three of these motoneurone classes have cell bodies and processes in the VNC, commissures that travel around the body to the dorsal side, and processes in the dorsal nerve cord (DNC) (White et al., 1986). To identify the VAB-7-expressing neurones in the VNC, we compared VAB-7 antibody staining with that of two neuronal markers, *unc-25::lacZ* and *unc-4::lacZ*, that are expressed in DD and DA motoneurones, respectively (Miller and Niemeyer, 1995; Jin et al., 1999). These experiments showed that VAB-7 is expressed in the seven DB class motoneurones (Fig. 1D and data not shown); VAB-7 staining is also seen in two additional head neurones that have not been identified. DB motoneurones are required for normal forward movement (Chalfie et al., 1985); therefore, the lack of *vab-7* expression in the DBs could explain the forward locomotory defect of *vab-7* mutants.

DB neurones express VAB-7 throughout development (Fig. 1 and data not shown). In addition, we found that VAB-7 is expressed continuously in the hypodermal syncitium hyp10, and from the L1 stage in four unidentified neurones in the tail. Finally, at the adult stage, VAB-7 is detected in three VC neurones: VC1, VC2 and VC6 (data not shown).

DB motoneurones exhibit reversed axonal polarity in *vab-7* mutants

To determine if the lack of *vab-7* expression perturbs the generation of DB motoneurones, we viewed DB neurones using *unc-129::gfp*, a promoter fusion that drives GFP expression in DA and DB cell bodies and processes (Colavita et al., 1998). We found that all DB neurones are generated in *vab-7* mutants (compare Fig. 2B with 2C), indicating that *vab-7* is not required for the production of these cells. In addition, two other DB characteristics are normal in *vab-7* mutants. First, each DB commissure (White et al., 1986) travels around the body on the correct side (i.e. right or left) to reach the dorsal nerve cord (data not shown). Second, as in wild-type, DB neurones express *unc-17/cha-1::gfp* (see Fig. 6B,D and data not shown), a marker for acetylcholine production (Lickteig et al., 2001) (Rand et al., 2000).

The above findings showed that DB neurones are present in *vab-7* mutants and that they retain at least some of the key characteristics of excitatory motoneurones. We next examined their axonal polarity, a DB-specific fate. In wild-type animals, DB neurones extend posteriorly directed processes in the VNC and in the DNC; DA neurones adopt a similar structure, but send out axons (DNC) and dendrites (VNC) with the opposite or anteriorly directed trajectory (Fig. 2A,D; Table 1) (White et al., 1986). Strikingly, in *vab-7* mutants, the axons of DB neurones, like the DAs, turn anterior rather than posterior when

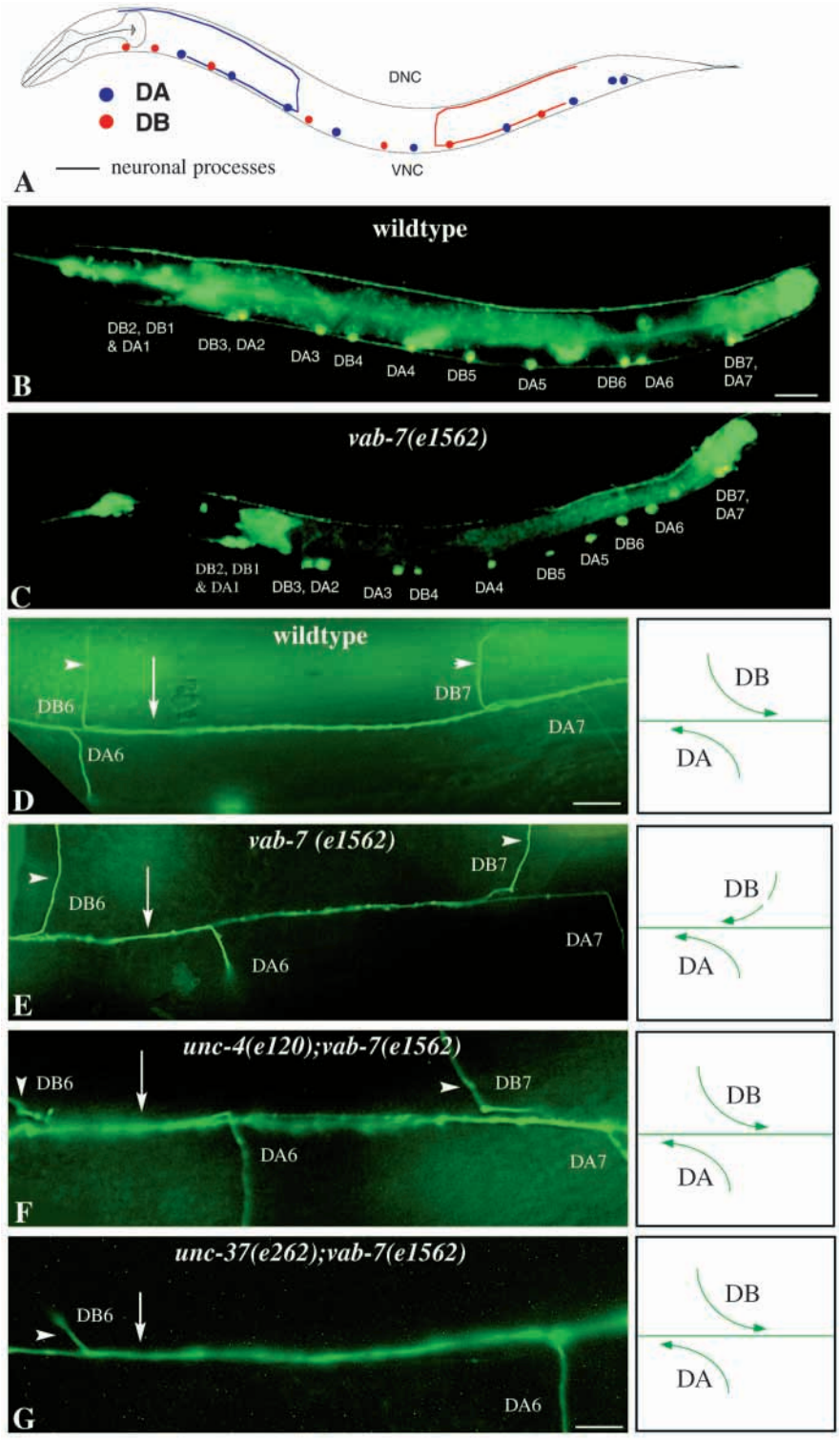


Fig. 2. *vab-7* specifies DB posterior axonal polarity. (A) Morphologies of DA and DB motoneurones. DA (nine) and DB (seven) cell bodies are located in the ventral nerve cord (VNC). Representative DA (blue) and DB (red) neurones are composed of dendritic processes in the VNC, circumferential commissures, and axons extending along the dorsal nerve cord (DNC). Note dendritic and axonal projections from DA (anterior) and DB (posterior) adopt opposite trajectories. (B,C) The cell bodies of DA and DB motoneurones visualized with *unc-129*::GFP. (B) Wild type. (C) A *vab-7(e1562)* mutant; both DA and DB are present. (High background in A,B is due to *unc-129*::GFP expression in intestinal cells.) (D-G) Dorsal views of adult animals. Anterior is towards left. Arrows point to the DNC and arrowheads identify DB commissures. (D) DB motor axons project posteriorly in the DNC of a wild-type animal. (E) *vab-7* is required for posterior DB axonal outgrowth. In *vab-7(e1562)*, DB motor axons adopt a DA-like anterior trajectory. Mutations in *unc-4* (F) and *unc-37* (G) restore posterior DB axonal polarity. Scale bars: in B, 50 μm in B,C; in D, 10 μm in D-F; in G, 4 μm.

they enter the DNC (Fig. 2E; Table 1). DA motor axon trajectories are not perturbed. Therefore, *vab-7* is required for proper (posterior) DB axonal polarity.

The congruent polarities of DA and DB motor axons in the dorsal nerve cord of *vab-7(e1562)* animals caused us to examine the exit trajectories of commissures emanating from DA and DB soma in the ventral nerve cord. In the wild type, DA commissures exit the soma in a posterior direction, whereas DB commissures project anteriorly from the cell body (White et al., 1986). In *vab-7* mutant animals, most of the DB commissures adopt the posterior trajectory normally reserved for DA motor axons in the ventral nerve cord (Table 2). Thus, DB neurones assume both the posterior exit trajectory of DA commissures in the ventral nerve cord as well as the anterior polarity of DA motor axons in the dorsal nerve cord.

The reversed axonal polarity of the DBs is due to ectopic UNC-4 expression

The finding that DB motoneurones adopt the axonal morphology of DA motoneurones in *vab-7* mutants indicated that DBs might also adopt other DA-specific traits. We investigated this possibility by examining the expression of *unc-4::gfp* in *vab-7(e1562)* mutants. *unc-4* encodes a homeodomain protein (Miller et al., 1992) that is expressed in the DAs embryonically (Fig. 3A), and in two other classes of ventral cord motoneurones (VA and VC) post-embryonically (Miller and Niemeyer, 1995; Lickteig et al., 2001). Examination of *vab-7(e1562)* animals showed ectopic expression of *unc-4::gfp* in the DBs (Fig. 3B; Table 3). This result indicates that *vab-7* represses *unc-4* in the DBs, either directly or indirectly. By contrast, *unc-4* does not appear to regulate *vab-7* as VAB-7 expression is normal in *unc-4* mutants (data not shown).

Is ectopic *unc-4* expression in the DBs responsible for the reversal of DB axonal polarity in *vab-7* mutants? If it is, then the removal of *unc-4* activity from a *vab-7* mutant background

Table 1. Percentage of neurones with wild-type axonal polarity

Genotype	DA (anterior)	DB (posterior)	AS (anterior)	*n*
Wild type	99% (247)	100% (207)	100% (60)	50
vab-7(e1562)	99% (200)	18% (161)	100% (59)	65
unc-4(e120)	100% (50)	100% (50)	100% (44)	22
vab-7(e1562); unc-4(e120)	100% (77)	100% (67)	nd	21
unc-37(e262)	100% (50)	100% (50)	nd	10
vab-7(e1562); unc-37(e262)	100% (150)	100% (150)	nd	30
weIs10 (unc-3::vab-7)	22% (91)	99% (80)	6% (102)	25
weIs10; vab-7(e1562)	nd	100% (30)	nd	15

Percentages refer to wild-type axonal polarities; the remainder had the opposite polarity (e.g. 82% of DB neurons of *vab-7(e1562)* animals had anterior, rather than posterior polarity).

n, the number of animals examined; the numbers of neurones scored are in parentheses; nd, not determined.

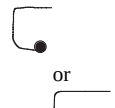
Dorsal axonal polarities were visualized with either *evIs82 (unc-129::gfp)* (DA and DB axons) or *mdEx72 (unc-17::gfp)* (DA, DB and AS axons).

weIs10 refers to the integrated *unc-3::vab-7; unc-17::gfp* strain.

should restore normal polarity. To test this model, we examined DB polarity in *unc-4; vab-7* double mutants. In this mutant background, DB motoneurones adopt the wild-type (posterior) polarity (Fig. 2F; Table 1). Therefore, ectopic expression of *unc-4* in the DBs reverses their axonal polarity from posterior to anterior in *vab-7* mutants.

UNC-4 function depends on physical interaction with UNC-37, a ubiquitously expressed Groucho-like transcriptional co-repressor (Miller et al., 1993; Pflugrad et al., 1997). Furthermore, the UNC-4/UNC-37 complex is known to function as a negative regulator of DB and VB motoneurone-specific genes (Winnier et al., 1999). We used the missense allele, *unc-37(e262)*, to determine whether the UNC-4-induced reversal of DB axonal polarity also requires wild-type UNC-37 activity. As shown Fig. 2G, DB axonal polarity is restored to its normal posterior trajectory in *unc-37(e262)*; *vab-7(e1562)* mutant animals (Table 1). This finding indicates that the UNC-4 is likely to function as a negative regulator of DB genes that direct posterior axonal outgrowth and that this repression is sufficient to impose an anterior trajectory in *vab-7* mutant animals.

Table 2. DB motor axons adopt DA axonal polarity in the ventral nerve cord

Phenotype	Motorneurone	Wild type	*vab-7(e1562)*
 or	DA	100% (100/100)	100% (100/100)
 or	DB	100% (80/80)	7% (3/43)
	DB	0% (80/80)	93% (40/43)

DA and DB axonal phenotypes were scored in L3 and L4 larvae with the *unc-129::gfp* marker.

Schematic drawings show the polarity of axonal exits in the VNC as well as trajectories of axonal processes in the DNC.

In the wild type, commissures tend to exit posteriorly from the DA cell body, whereas commissures exit anteriorly from the DB soma.

DA and DB motoneurones for which the exit trajectory could not be reliably scored are depicted as motoneurone soma with vertically projecting commissures.

Twenty animals were scored in each case.

Numbers in parentheses refer to number of neurones with the indicated morphology/number of neurones examined.

vab-7 is required for *acr-5* expression in DB motoneurones

acr-5 encodes an acetylcholine receptor subunit that is normally expressed in B-class (DB, VB) but not in A-class (DA, VA) motoneurones (Winnier et al., 1999) (Fig. 4A). In

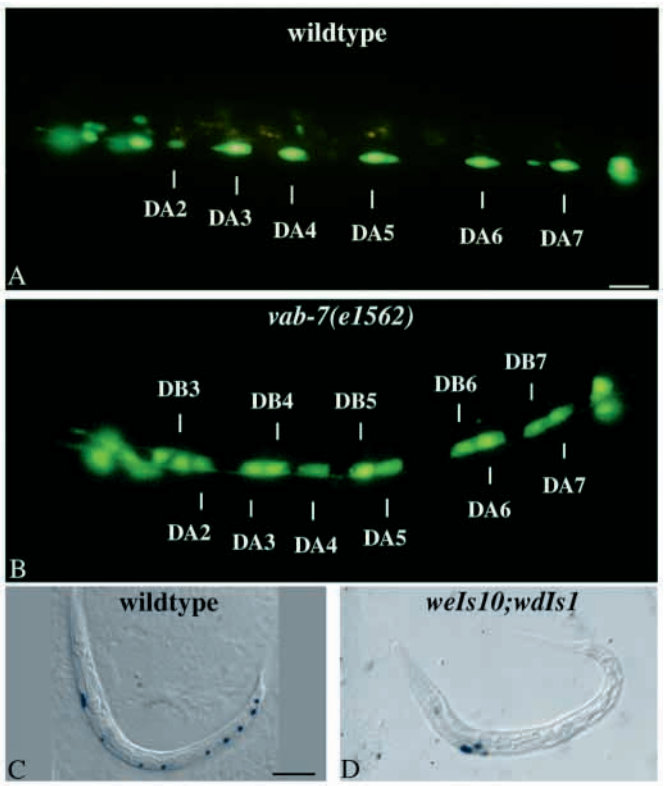


Fig. 3. *vab-7* represses *unc-4* in DB motoneurones. (A) Wild-type expression of *unc-4*::GFP in DA motoneurones of an L1 larva. (B) *unc-4*::gfp is ectopically expressed in the DBs in a *vab-7(e1562)* background. (C) Wild-type expression of *unc-4::lacZ* in DAs of an L1 larva (D) *unc-4::lacZ* is repressed in DAs of an L1 larvae by ectopic VAB-7 expession from *unc-3::vab-7* transgene (*weIs10*). β-Galactosidase staining is retained in a cluster of neurones at the anterior end of the VNC in which *unc-3::vab-7* is not expressed Anterior is towards the left in all panels. Scale bars: in A, 10 μm in A,B; in C, 2.5 μm in C,D.

Table 3. Expression of *unc-4::gfp* in *vab-7(e1562)*

Genotype	DA	DB	*n*
Wild type	89% (144/162)	0% (0/135)	27
vab-7(e1562)	99% (143/144)	88% (105/120)	24

n, number of animals examined at the L 1 stage; the numbers of neurones examined are given in parentheses.

Only DA and DB embryonic motoneurones in the ventral nerve cord were scored (five DBs, six DAs).

The integrated trangene, *wdIs4*, was use to detect *unc-4::gfp* expression.

Table 4. Embryonic motor neuron expression of *acr-5:gfp* in mutant backgrounds

Genotype	DA	DB	*n*
Wild type	1%	92%	23
unc-4(e120)	91%	98%	22
vab-7 (e1562)	8%	9%	25
vab-7 (e1562); unc-4(e120)	83%	87%	24
unc-37(e262)	94%	96%	27
vab-7(e1562); unc-37(e262)	80%	90%	27

n, number of animals examined.

Only the DA and DB embryonic motoneurones in the ventral nerve cord were scored at the L1 stage (five DBs, six DAs).

A minimum of 120 total motoneurones were examined for each case.

The extrachromosomal array, *wdEx60*, was used to detect *acr-5::gfp* expression.

vab-7(e1562) mutants, however, we found that *acr-5::gfp* expression is specifically lost from the DBs (Fig. 4B; Table 4). It has previously been shown that *acr-5::gfp* is negatively regulated by UNC-4 and its co-factor UNC-37 in DA and VA motoneurones (Winnier et al., 1999) (Fig. 4C). Given that *unc-4* is derepressed in the DBs of *vab-7* mutants (Fig. 3B), loss of *acr-5::gfp* expression could be due to the ectopic expression of *unc-4*. This is indeed the case, as *acr-5::gfp* expression is restored to the DBs in *unc-4; vab-7* double mutants (Fig. 4D; Table 4). Repression of *acr-5::gfp* by ectopic UNC-4 also depends on *unc-37* as DB motoneurones express *acr-5::gfp* in *unc-37; vab-7* animals (Table 4). Therefore, in DB motoneurones, *vab-7* effectively promotes *acr-5::gfp* expression by repressing UNC-4 repressor activity.

Ectopic VAB-7 expression induces DB characteristics in embryonic and post-embryonic neurones

The anteriorly directed trajectory of DB motor axons in *vab-7(e1562)* mutants indicates that *vab-7* function is necessary to specify posterior axonal outgrowth in the DBs. To determine if *vab-7* function is also sufficient to specify a posterior axonal trajectory in other classes of motoneurones, we expressed VAB-7 ectopically under the control of the *unc-3* promoter. The *C. elegans unc-3* gene encodes a homologue of the O/E family of mammalian transcription factors. In the ventral nerve cord, *unc-3* is expressed in embryonic DA, DB neurones and in the post-embryonic VA, VB and AS motoneurones (Prasad et al., 1998) (T. Starich and J. Shaw, personal communication).

Animals carrying the *unc-3::vab-7* transgene *weIs10* can move forwards, but exhibit a strong backwards movement defect (data not shown). This striking UNC-4-like phenotype indicates that A-type motoneurones (DA and VA) may be affected. If the ectopically expressed VAB-7 protein retains wild-type function, then *unc-4* should be repressed in the DA and VA motoneurones. This is indeed the case: *unc-4::lacZ* expression is lost from A-type motoneurones in the *unc-*

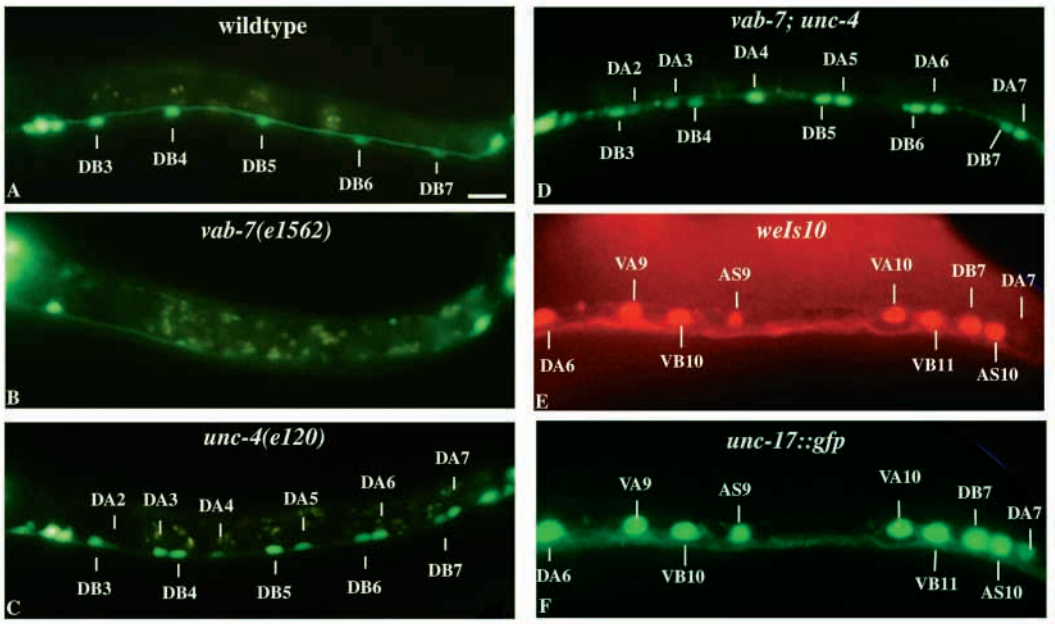


Fig. 4. *acr-5* expression in DB neurones is induced by VAB-7 repression of *unc-4*. (A-D) *acr-5::gfp* in lateral views of L1 larvae. (A) *acr-5::gfp* is expressed in wild-type DB motoneurones but is absent (B) from DBs in a *vab-7(e1562)* mutant. (C) *acr-5::gfp* is ectopically expressed in DA neurones of an *unc-4(e120)* mutant. (D) *acr-5::gfp* expression is restored to the DBs by genetic removal of *unc-4* in the *unc-4(e120);vab-7(e1562)* double mutant. (E) *acr-5::gfplacZ* detected by anti-β-galactosidase antibody in an adult hermaphrodite expressing ectopic VAB-7 from *weIs10*. *acr-5::gfplacZ*, which is normally only in DBs and VBs, is induced in DAs, VAs and ASs. (F) *unc-17::gfp* expression of animal in E used to identify the neurones. Anterior is towards the left. Scale bar: 10 μm.

Table 5. Ectopic *vab-7* activates *acr-5::lacZ*

Genotype	DA	VA	DB	VB	AS	*n*
Wild type *(Exacr-5:lacZ)*	0% (66/66)	0% (132/132)	100% (88/88)	100% (176/176)	0% (154/154)	22
weIs10;Exacr-5::lacZ	70% (29/41)	85% (51/60)	100% (52/52)	91% (62/68)	93% (63/68)	47

n, number of adult hermaphrodites examined.
lacZ expression was detected by antibody staining.
Expression of the *unc-17::gfp* marker in the *weIs10 (unc-3::vab-7 unc-17::gfp)* strain and DAPI staining aided in the identification *acr-5::lacZ*-positive postembryonic neurons (VA, VB, AS). In parentheses are the number of neurones with *acr-5::lacZ* expression/number of neurones examined.

3::vab-7 background (Fig. 3D; data not shown). Furthermore, we also found that *acr-5::gfp* is now ectopically expressed in the DAs and VAs in this transgenic line as would be expected from the inhibition of *unc-4* activity in these cells by ectopic VAB-7 (Fig. 4E; Table 5).

We next asked whether ectopic VAB-7 could direct a posterior axonal polarity in neurones that normally have anteriorly directed processes. Neuronal processes were visualized with the *unc-17/cha-1*::*gfp* reporter, which is expressed in DA, DB, VA, VB, AS and VC motoneurones (Lickteig et al., 2001) (J. Rand, personal communication). We first examined DA neurones and found that ectopic VAB-7 induces the DA axons to adopt a posterior trajectory in the DNC (Fig. 5B; Table 1). Although the polarity reversal of the DA motor axons is correlated with *unc-4* repression, it is unlikely that this DB-like trait depends on VAB-7 inhibition of *unc-4*, because DA motor axons retain their normal anterior trajectory in *unc-4* mutants (White et al., 1992; Miller and Niemeyer, 1995) (Table 1). Thus, VAB-7 expression appears to be sufficient to direct posterior axonal outgrowth.

This conclusion is substantiated by the observation that ectopic expression of VAB-7 in AS motoneurones imposes posterior axonal outgrowth in the DNC. The post-embryonically derived AS motoneurones do not express UNC-4 (Miller and Niemeyer, 1995) but are morphologically similar to the DAs with anterior axonal projections (White et al., 1986). The *unc-3::vab-7* transgene, however, reverses AS axonal polarity (Fig. 5B; Table 1). AS motoneurones also show ectopic expression of *acr-5::gfp* (Fig. 4E; Table 5) and *unc-129::gfp* (data not shown), which indicates that these postembryonic motoneurones have assumed other aspects of DB fate in addition to the posteriorly directed axonal trajectory. Therefore, ectopic expression of VAB-7 is sufficient to induce a spectrum of DB-like traits in both embryonic and postembryonic motoneurones.

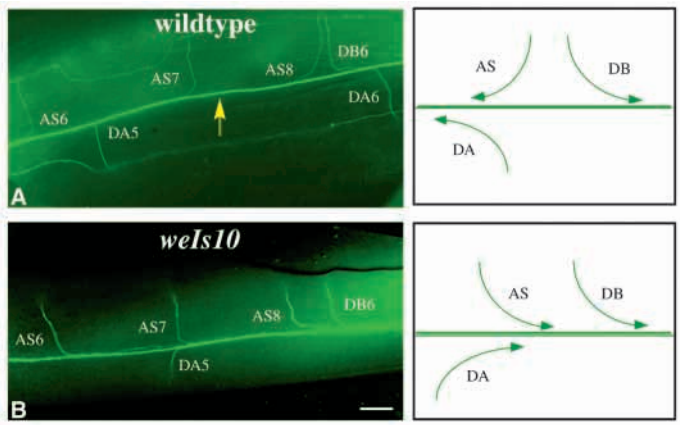


Fig. 5. Ectopic VAB-7 is sufficient to promote posterior axonal outgrowth in cholinergic motoneurones. Dorsal views of *unc-17/cha-1::gfp* staining of DA, DB and AS axons entering the DNC of adult animals. (A) DA and AS motor axons project anteriorly in the wild type; DB axons are posteriorly directed. (B) An *unc-3::vab-7* transgene (*weIs10*) drives ectopic VAB-7 expression in ventral cord motoneurones and redirects AS and DA axons to adopt a posterior trajectory. Yellow arrow points to DNC. Anterior is towards the left. Scale bar: 15 μm.

vab-7 function is necessary for fasiculation of dorsal as well as ventral nerve cords

In addition to the axonal polarity reversals of DB motoneurones, *vab-7(e1562)* mutants also show significant disorganization of neuronal processes in both the dorsal and ventral nerve cords (Fig. 6B,D). Although the polarity reversal of *vab-7* mutants is rescued by removal of *unc-4* activity, this defasiculation defect remains (Fig. 2F), indicating that abnormal polarity is not the cause of defasiculation. In addition, abnormal expression of *unc-4* in DB motoneurones does not account for the disrupted fasicular organization of the axial nerve cords. We conclude that *vab-7* must mediate some other DB-trait that in turn is necessary for proper process placement in both the dorsal and ventral nerve cords. To test this idea, we examined nerve cord fasiculation in *vab-7* mutants ectopically expressing VAB-7 from the *unc-3::vab-7* transgene. We found that the *unc-3::vab-7* transgene rescues both the fasiculation (Fig. 6E) and forward movement defects of *vab-7* mutants (data not shown). Interestingly, although the DNCs and VNCs of *vab-7* mutants appear defasiculated when viewed using the *unc-17::gfp* reporter (expressed in all cholinergic neurones; Fig. 6B,D), fasiculation appears normal in the VNC but not the DNC when viewed with *unc-129::gfp*, a reporter expressed only in DA and DB neurones (Fig. 6F). This suggests that neurones other than DAs and DBs are defasiculated in the VNC. Our results indicate that DB neurones might have an important role in bundling in the nerve cords.

DISCUSSION

Coordinated movement depends on the integration of distinct functions provided by separate classes of motoneurones. In the nematode, *C. elegans*, DA and DB class motoneurones innervate dorsal muscles but adopt axonal trajectories of opposite polarity (DA, anterior; DB, posterior) and express separate sets of genes. We have shown that the differentiation of these motoneurone subclasses depends on the antagonistic actions of the VAB-7 and UNC-4 homeodomain proteins.

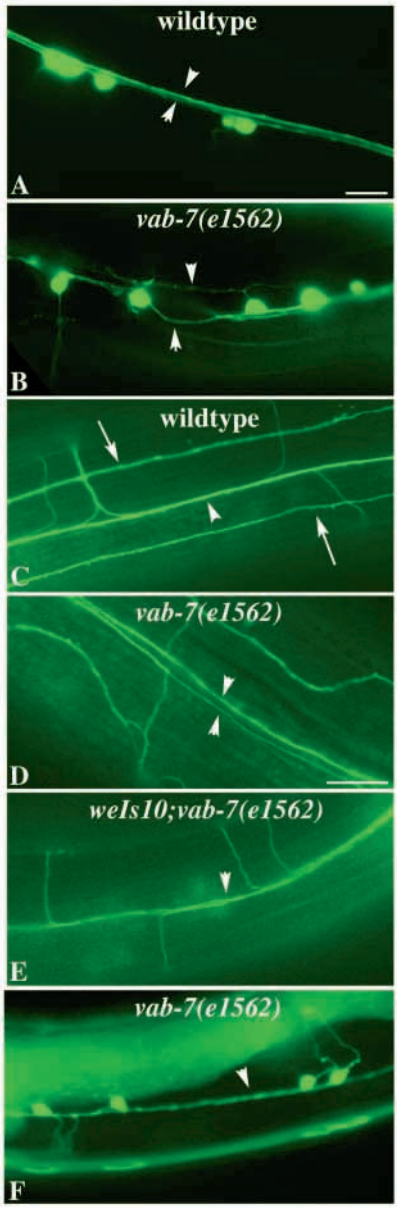


Fig. 6. Fasciculation of both dorsal and ventral nerve cords depends on *vab-7* activity. (A-E) *unc-17/cha-1::gfp* was used to visualize cholinergic motoneurone processes in adults. (A) Ventral view of wild type. Arrows point to normal right and left process bundles in the VNC. (B) Ventral view of *vab-7(e1562)* showing highly defasciculated VNC (arrows). (C) Dorsal view of wild type. Arrowhead points to the DNC; arrows point to dorsal sub-lateral nerve cords. (D) Dorsal view of defasciculated DNC in *vab-7(e1562)* mutant (arrowheads). (E) Ectopic expression of VAB-7 in *weIs10* restores normal fasiculation to the DNC in a *vab-7(e1562)* mutant. (F) Expression of *unc-129::gfp* in *vab-7(e1562)*, showing normal fasiculation of DA and DB neurones in the VNC. Anterior is towards the left. Scale bars: in A, 15 μm in A-C,E,F.

The DB motoneurone fate

VAB-7 appears to have two roles in DB fate determination. First, VAB-7 blocks expression of the A-class gene, *unc-4*, in DBs (Fig. 7). In *vab-7* mutants, ectopic expression of UNC-4 represses B-class genes and induces DB motoneurones to adopt the anteriorly directed axonal trajectory of DA motoneurones. Second, VAB-7 promotes DB characteristics independently of UNC-4 repression. Ectopic expression of VAB-7 in cholinergic motoneurone classes that do not express UNC-4 is sufficient to induce expression of the B-class genes (i.e. *acr-5*, *unc-129*) and to impose the posterior polarity characteristic of DB motor axons. Posterior DB polarity also appears to be controlled by another, as yet unknown pathway, as this trait is normal in *unc-4; vab-7* double mutants. Finally, as discussed below, the defasciculation defects observed in *unc-4; vab-7* animals reveal an independent *vab-7* function that is necessary for proper bundling of processes in both the dorsal and ventral nerve cords.

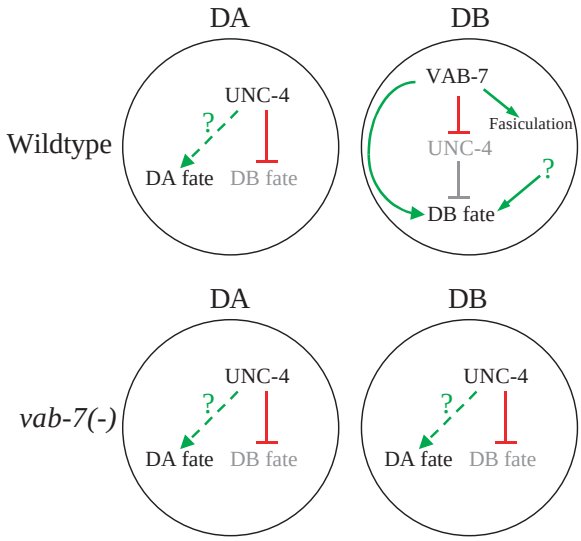


Fig. 7. Specification of DA versus DB motoneurones fate. UNC-4 blocks expression of DB genes in DA motoneurones. It is not known if UNC-4 also acts to promote expression of DA-specific genes. VAB-7 specifies DB traits (posterior axonal polarity, *acr-5* expression) by repressing UNC-4 and by acting in a parallel UNC-4-independent pathway. The posterior trajectory of DB motoneurones may also depend on another unknown pathway (?) that does not involve VAB-7. Nerve cord fasciculation is uniquely dependent on VAB-7 function.

General models of neuronal fate determination

vab-7 directs the DB motoneurone fate. Are there functional counterparts of *vab-7* for other motoneurone classes? Two genes with properties in common with *vab-7* are *unc-4* and *unc-30*. These genes encode paired-class homeodomain proteins important for A-type and D-type motoneurone fate, respectively (Miller et al., 1992; Jin et al., 1994). *unc-4* is predominately expressed in DA and VA motoneurones, and is required for proper VA synaptic inputs and for repression of *acr-5* in these neurones (White et al., 1992; Winnier et al., 1999). *unc-30* controls GABA expression, axonal pathfinding and synaptic connections in DD and VD motoneurones (Jin et al., 1994). It is not yet known whether *unc-4* or *unc-30*, like *vab-7*, are sufficient for a range of A-type or D-type fates, respectively, although UNC-30 has been shown to control genes for both the synthesis and packaging of the D-type neurotransmitter GABA (Eastman et al., 1999). It is interesting that *unc-4* is not required for the maintainance the anterior polarity of A-type motor axons (White et al., 1992; Miller and Niemeyer, 1995). Our finding that ectopic *unc-4* can reverse the polarity of DB neurones argues that UNC-4 may have addtional A-type specifying roles that may be masked in *unc-4* loss-of-function mutants by partial redundancy with an as yet unknown factor. Because VAB-7, UNC-4 and UNC-30 control the fates of different motoneurone classes, but do not control their production, these proteins are likely to function largely in postmitotic cells to define subsets of neurone-specific traits.

Fasciculation and eve in *Drosophila* and *C. elegans*

In *vab-7* mutants, both dorsal and ventral nerve cords are defasciculated. This defect is not rescued by restoring proper posterior polarity of DB neurones (by removing *unc-4*), but is

rescued by ectopic VAB-7 expression, suggesting that *vab-7*, and possibly DB neurones promote process bundling. Interestingly, Even-skipped in *Drosophila* also has a role in fasciculation (Landgraf et al., 1999). Axonal growth of Eve-expressing neurones (aCC and RP2) in the ISN nerve trunk and their subsequent innervation of dorsal muscles is dependent on Even-skipped. Furthermore, ectopic expression of Even-skipped in the nervous system promotes SN and ISN nerve trunk fasciculation. Landgraf et al. (Landgraf et al., 1999) have provided indirect evidence that *eve* activity is required for expression of an unknown neuronal adhesion molecule. Mutations in a number of genes are known to cause nerve bundle defasciculation in *C. elegans* (McIntire et al., 1992; Wightman et al., 1997; Bloom and Horvitz, 1997). One candidate for a downstream target of *vab-7* is the α-integrin INA-1, which is expressed in DB (and other) neurones and is required for nerve bundle fasciculation (Baum and Garriga, 1997).

Conservation of Even-skipped function

Genetic studies in *C. elegans*, *Drosophila*, and the mouse have shown that Even-skipped homologues function to distinguish alternative fates in the motoneurone circuit. In each case, Eve prevents one class of neurone from adopting traits normally reserved for another. In *Drosophila*, Eve is expressed in motoneurones that project along the ISN nerve to innervate dorsal muscles. In *eve* mutants, these motoneurones adopt the axonal trajectory of a different class of ISN motoneurones that synapse onto ventral muscles (Landgraf et al., 1999). Similarly, in *vab-7* mutants in *C. elegans*, DB motoneurones reverse their normal posterior axonal polarity and instead assume the anteriorly directed trajectory of DA motor axons (this work). In the spinal cord of mouse *Eux1* mutants, V0 interneurones are apparently transformed into V1 interneurones (Moran-Rivard et al., 2001). At least in *C. elegans* and in *Drosophila*, ectopic expression of Even-skipped is also sufficient to impose axonal trajectories normally associated with *eve*-expressing motoneurones (Landgraf et al., 1999) (this work).

A common element of Eve function in all three species is the repression of a downstream HD protein, which is normally expressed in the alternative neurone. In *C. elegans*, *vab-7* prevents expression of the DA gene, *unc-4*, in DB motoneurones. Evx1 functions in mouse V0 neurones as a negative regulator of the engrailed homologue, En1, a marker for V1 cells (Saueressig et al., 1999; Moran-Rivard et al., 2001). In *Drosophila*, ectopic expression of Eve is sufficient to inhibit Islet in ISN motoneurones (Landgraf et al., 1999).

In addition to these similarities in Eve function, our work shows that VAB-7 functions within a reciprocally inhibitory network: VAB-7 inhibits the DA fate and UNC-4 inhibits the DB fate. Thus, one way that VAB-7 promotes DB differentiation is by blocking expression of a HD transcription factor that antagonizes DB traits. By extension, we propose that HD transcription factors in *Drosophila* and mouse are likely to antagonize fates promoted by Eve. For example, EN1 might exert a negative effect on V0 interneurone differentiation when ectopically expressed in V1 cells in *Evx1* mutants just as UNC-4 inhibits DB fates in *vab-7* mutant animals. In this case, normal V0 cell migration and axonal trajectory might be restored in *Evx1 En1* double mutant mice (Saueressig et al., 1999; Moran-Rivard et al., 2001). Both UNC-4 and EN1 include EH-1 domains that have been shown to recruit the transcriptional co-repressor protein, Groucho (Jimenez et al., 1997; Winnier et al., 1999). In the case of UNC-4, interactions with the nematode Groucho homologue, UNC-37, repress B-class motoneurone traits (Pflugrad et al., 1997; Winnier et al., 1999). Reciprocal inhibition by EH-1-containing HD proteins that recruit Groucho might be common, as recent work has revealed that such a mechanism in the vertebrate spinal cord defines distinct domains of neural progenitor cells (Muhr et al., 2001). Thus, our work demonstrates that important elements of both the logic and molecular mechanisms employed by HD proteins in the specification of neuronal fates in the motor circuit have been preserved in evolution from nematodes to mammals.

We thank D. Frisby and J. Rand for *unc-17/cha-1::gfp*, B. Prasad and R. Reed for the *unc-3* promoter, J. Culotti for *unc-129::gfp*, A. Fire for *gfp* expression vectors, and R. Smith for monoclonal antibody help. We are also grateful to members of the Ahringer and Miller laboratories for helpful discussions. Some strains used in this study were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, St Paul) which is funded by the NIH NCRR. B. E was supported by the BBSRC; C. N. and J. A. were supported by a Wellcome Trust Career Development Award (No. 045515) and a Senior Research Fellowship (No. 054523) to J. A.; J. R. was supported by an HHMI predoctoral fellowship; and D. M. was funded by NIH (NS 26115).

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