CHAPTER V

THE CEH-12/HB9 TRANSCRIPTION FACTOR FUNCTIONS DOWNSTREAM OF UNC-4 IN THE SYNAPTIC SPECIFICITY PATHWAY

Introduction

As described in the Introduction, a transcriptional cascade regulates neural fate in the vertebrate spinal cord (reviewed in (Lee and Pfaff 2001). This cascade is set in place by the secreted morphogen Sonic Hedgehog (Shh). A gradient of Shh activates or represses expression of eh1-containing homeodomain proteins along the dorsal-ventral axis. Acting in combination with Groucho, these cross-repressive transcription factors specify progenitor domains in the ventral spinal cord. The combinatorial expression of different homeodomain proteins determines the subtype of neural progenitor. For example, motor neuron progenitors co-express Olig2, Nkx6.1, Nkx6.2, and Pax6 whereas interneuron progenitors express a different combination of transcription factors (i.e. Irx3 replaces Olig2 in the neighboring V2 interneuron progenitor domain) (Figure 1.3). The actions of these transcription factors define the post-mitotic motor neuron fate by keeping the wrong genes off (i.e. those that define interneuronal fate) and turning the right genes on (e.g. HB9 and Isl1/2).

HB9 is a critical determinant of vertebrate motor neuron differentiation. Loss of HB9 activity results in de-repression of the V0 interneuron gene *Chx10* (Thaler et al. 1999). Additionally, the pathfinding of some motor neurons is aberrant, and there is a complete loss of the phrenic nerve (Thaler et al. 1999). Similarly, in *Drosophila Hb9 (dHb9)* mutants, motor neurons display defective axonal projections and fail to synapse

with appropriate muscles (Broihier and Skeath 2002; Odden et al. 2002). These phenotypes are enhanced when *dHb9* mutants are combined with *Nkx6* mutants (Broihier et al. 2004). Thus, the coordinate roles of HB9 and Nkx6 factors in motor neuron differentiation are conserved.

Through parallel cell-specific genomic approaches outlined in Chapter IV, our lab has found that the nematode HB9 homolog, *ceh-12*, is upregulated in *unc-4* and *unc-37* mutant A-class neurons. Given the well-established role of HB9 in fly and vertebrate motor neuron specification, we hypothesized that CEH-12 might also specify motor neuron fate in *C. elegans*. This chapter presents our findings confirming that CEH-12 is a bona fide UNC-4 target gene and that it likely functions downstream of UNC-4 to control synaptic specificity.

Materials and Methods

Nematode strains

Nematodes were grown as described (Brenner 1974). *ceh-12 (gk391)* deletion mutants were obtained from the *C. elegans* Vancouver knockout consortium (Mark Edgley). These animals were backcrossed 2X against *unc-129*::GFP animals and 4X against *dpy-5* to create strain NC1018. The *ceh-12 (tm1619)* deletion mutant was obtained from the Japanese *C. elegans* knockout consortium (Shohei Mitani) and outcrossed 6X against *dpy-5* to create strain NC1019. The outcrossed deletion alleles were crossed into four *unc-4* mutants (*e120, e2320, e2322ts, wd1)* to test for suppression of the Unc-4 defect. These strains are: NC1036 (*gk391; wd1*), NC1037 *(tm1619; e2320*),

NC1038 (*tm1619; e2322ts*), NC1058 (*gk391; e2322ts*), NC1078 (*gk391; e120*). To test for *ceh-12* regulation of motor neuron markers, we generated NC1036 [*gk391; wdIs6* (*del-1*::GFP)], NC1057 [*tm1619*; *wdIs5* (*unc-4*::GFP)]. *ceh-12*::GFP strains are: NC802 [*unc-119 (ed3); wdEx310 (ceh-12*::GFP - *unc-119* minigene)], NC897 [*unc-119 (ed3); wdEx376 (ceh-12*::GFP - *unc-119* minigene)], NC898 [*unc-119 (ed3); wdEx377 (ceh-12*::GFP - *unc-119* minigene)], NC831 [*wdEx336* (*ceh-12*::GFP, pRF4 (*rol-6 (d)*)], NC942 [*wdEx404* (*ceh-12*::GFP, pRF4 (*rol-6 (d)*)].

Construction of ceh-12::GFP reporter lines

A 3kb region of promoter sequence upstream of the predicted *ceh-12* start was obtained by PCR amplification of genomic DNA (R. Fox). This fragment was cloned into TOPO and then subcloned into pPD95.75 using PstI and XmaI. This plasmid (*ceh-12*::GFP) was injected into N2 animals with the *rol-6* dominant marker (pRF4) to generate three independent transgenic lines (see strain names above). The *unc-119* minigene from MM051 (BamHI, filled in with T4 Polymerase, EcoRV) was blunt-ligated into the HindIII site (filled in with T4 polymerase) to make the vector suitable for biolistic transformation (see Materials and Methods, Chapter IV). Three additional transgenic lines were created using this method (see strain names above).

Transgenic expression of CEH-12

To test if ectopic expression of CEH-12 in *unc-4* motor neurons was sufficient to induce an Unc-4 defect, the *unc-4* promoter from pSV9-TOPO was subcloned in pSL1180 (Stratagene) using NheI and KpnI, to create pSV45. PCR was used to amplify

the *ceh-12* ORF (Open Biosystems) with a N-terminal KpnI site and a C-terminal PmeI site. I cloned this PCR product into TOPO (to create C12ORF-TOPO) and sequenced to insure there were no mutations in the coding sequence. pSV45 and C12ORF-TOPO were digested with KpnI and XhoI; ligation of these fragments created pSV46. This was digested with PmeI and blunt ligated to the *unc-54* 3' UTR (removed from pEML57-Rev using BamH1, filled in with T4 Polymerase, and SmaI) to generate pSV47 (*unc-4*::CEH-12::*unc-54* 3' UTR). This plasmid was injected with pJER1 (*myo-3*::dsRed2) and pCG9 (*acr-5*::YFP) into N2 animals; five lines were obtained and analyzed for Unc-4 defects (strain names listed above). The QuickChange site-directed mutagenesis kit (Stratagene) was used to mutate key residues in CEH-12 to create pSV51 (*unc-4*::CEH-12Q50S), pSV52 (*unc-4*::CEH-12W48A), pSV53 (*unc-4*::CEH-12W48F), and pSV54 (*unc-4*::CEH-12F3L). At least one line was obtained for all of the above plasmids (see strain names listed above).

ceh-12 is SL1-transpliced

RT-PCR using mRNA co-immunoprecipitated from NC714 (*unc-37*; wdEx257, see Chapter IV) animals was performed. mRNA (3 μg) was incubated with 1 μl of Affy 100 primer (see Chapter IV Materials and Methods) for 10 min at 70ºC, then cooled on ice for 2 min. The following was added: 4 μl for 1st strand buffer (Invitrogen), 2 μl 0.1M DTT, 1 μl 10 mM dNTP mix, and 1 μl rRNasin (Promega). This mixture was incubated at 42ºC for 2 min, 1 μl of SuperScript II was added, and continued at 42ºC for 1 hr. The reaction was terminated at 70ºC for 10 min. For the PCR reaction, 2 μl of this cDNA mixture was used as template with the SL1 (5' - GGTTTAATTACCCAAGTTTGAG - 3')

and ceh12p8-R (5' - GTTTAAACTCAAGAAGAGGAAGTTG - 3') primers. The CEH12-50 PCR program (95 ºC, 30s; 50 ºC, 30s; 72 ºC, 1 min; 30 cycles. 72 ºC 10 min. 4 ºC hold) was used. The resulting fragment was cloned into TOPO and sequenced. These data confirm that the *ceh-12* transcript is SL1-transpliced.

PCR detection of ceh-12 *deletion alleles*

Single worm PCR was performed as described in Chapter II Materials and Methods. To assay for the *ceh-12 (gk391)* deletion, the ceh-12 il (5' - TATTGCCA AGGAACAAAGGC - 3') and ceh-12 ir (5' - GCTTGCCATGCATTTACTGA - 3') primers were used. To assay for the *ceh-12 (tm1619)* allele, the F33Dll.4 IL (5' - GGG AATTGCCAAAACCATAG - 3') and F33D11.4 IR (5' - CAGTGAACCGAAATCAC GTA - 3') primers were used. The PCR program for both reactions was CEH-12KO (94ºC, 30s; 50 ºC, 30s; 72 ºC, 2 min 30s; 30 cycles. 4 ºC hold).

Results

ceh-12 is a member of the Mnx (HB9) homeodomain family and contains a conserved N-terminal eh1-like motif.

A phylogenetic analysis of amino acid sequences indicates that CEH-12 is the closest *C. elegans* relative of HB9 proteins (Figure 5.1A). The homeodomain of CEH-12 is ~70% conserved compared to other species. Additionally, CEH-12 includes a predicted N-terminal eh1 domain that is also conserved in other HB9 proteins (Figure 5.1B). The presence of this Groucho interaction domain suggests that CEH-12 and other HB9

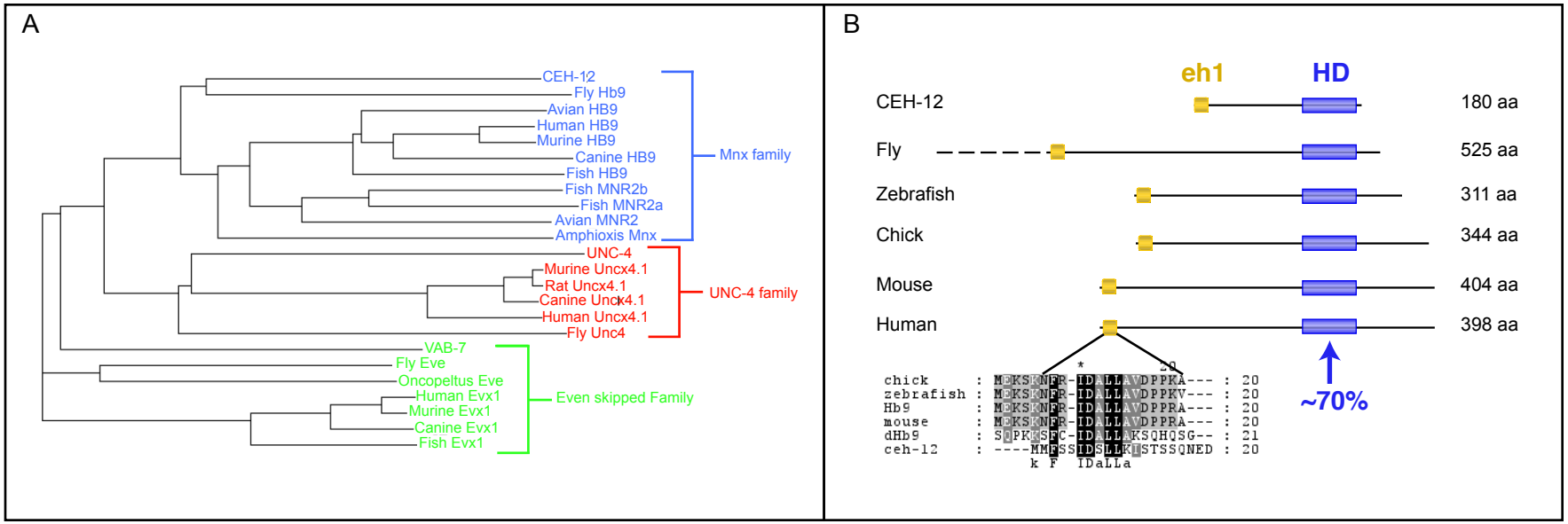

Figure 5.1 CEH-12 is a member of the Mnx (HB9) family of homeodomain proteins.
A. A phylogenetic tree showing that CEH-12 (top) is more similar to Mnx (HB9) proteins than to other classes of homeoproteins.
B. HB9 family members include a highly conserved homeodomain (70% compared to CEH-12) and an N-terminal eh1-like domain.

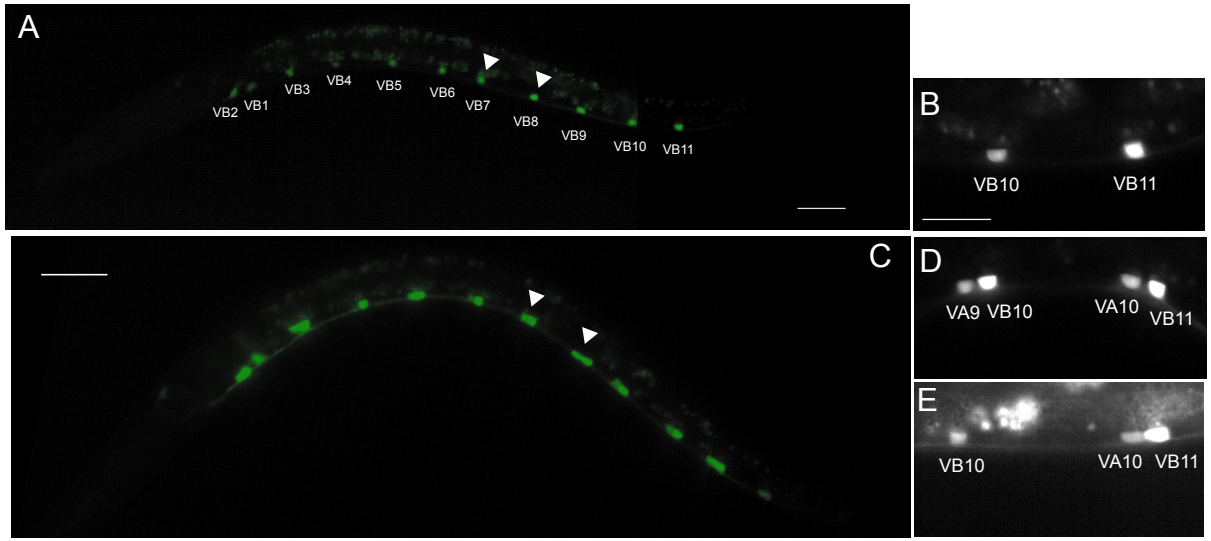

Figure 5.2 *ceh-12*::GFP is expressed in VB motor neurons and is negatively regulated by UNC-4 and UNC-37 in A-class neurons.

A, B. Wildtype expression

A. An L2 larva showing *ceh-12*::GFP expression in all VB motor neurons along the ventral nerve cord (VNC). Arrowheads point to single cells.

B. Close-up of the posterior VNC showing single VB motor neurons expressing GFP.

C, D, E. Expression in *unc-4* and *unc-37* mutants.

C. An *unc-4 (e120)* L1/L2 transition larva showing *ceh-12*::GFP in VA and VB motor neurons. Arrowheads point to adjacent pairs of VA and VB motor neurons.

D. Close-up of the posterior VNC in an *unc-4 (e120)* L2 larvae showing derepression of *ceh-12*::GFP in VA motor neurons.

E. An *unc-37 (e262)* L2 larvae displays ectopic *ceh-12*::GFP expression in VA motor neurons.

Anterior to left and ventral down in all panels. Scale bars - A, C. 20 µm, B, D, E, 10 µm.

proteins are likely to function as transcriptional repressors (Thaler et al. 1999; Broihier and Skeath 2002; William et al. 2003).

ceh-12::GFP is expressed in VB motor neurons

Our model of UNC-4 function (Fig 1.14) predicts that an UNC-4 target gene should be normally expressed in VB motor neurons. This prediction is confirmed for *ceh-12*. All six *ceh-12*::GFP transgenic lines (see Materials and Methods) display fluorescence in VB motor neurons (Figure 5.2A, B). Expression is highly specific to VBs, although we also observe occasional weak expression in the pharyngeal/intestinal valve and in a few head neurons (data not shown).

ceh-12::GFP is negatively regulated by *unc-4* and *unc-37*

To assay for *unc-4* regulation, *ceh-12*::GFP was crossed into *unc-4 (e120)* and *unc-37 (e262)* mutant backgrounds. Our model (Figures 1.14, 1.15) predicts that GFP should be ectopically expressed in VA motor neurons in these mutant backgrounds. The results of this experiment support this prediction (Figure 5.2C-E). We also detect variable ectopic expression in DA motor neurons, thus validating the MAPCeL data obtained from embryonic *unc-4* motor neurons (data not shown). We conclude that *ceh-12* is a strong candidate *unc-4* target gene.

CEH-12 expression in VA motor neurons induces an Unc-4-like backward movement defect

If CEH-12 is sufficient to miswire VA motor neurons, then ectopic expression of CEH-12 in these cells should induce a backward movement defect. We used the *unc-4*

promoter to drive CEH-12 expression in A-class motor neurons. As shown in Figure 5.3, *unc-4*::CEH-12 transgenic animals were unable to execute backward movement and instead coiled dorsally, similar to *unc-4* mutants. Because CEH-12 is predicted to act as a repressor, this phenotype could have resulted from CEH-12-dependent repression of *unc-4*. I examined *unc-4*::GFP expression in *unc-4*::CEH-12 transgenic animals, however, and determined that *unc-4*::GFP was expressed normally (data not shown). Therefore, CEH-12 must be acting on other genes in VA motor neurons to induce the backward Unc defect.

The homeodomain is required for CEH-12 function

I next performed structure-function experiments to determine what domains of CEH-12 were important for this phenotype. Point mutations in CEH-12 were generated to disrupt residues important for DNA binding and to ablate predicted interactions with Groucho (Figure 5.4). Transgenic animals expressing CEH-12 proteins with point mutations that are likely to perturb the folding of the homeodomain do not show the Unc-4 phenotype (e.g. W48A and W48F); this result rules out the possibility that the CEH-12 induced backward movement defect is due to non-specific overexpression of CEH-12 in VA motor neurons. In contrast, a mutation in amino acid 50 of the homeodomain (Q50S) that controls DNA binding specificity still induces a backward Unc defect. I postulate that while this mutation decreases the affinity of CEH-12(Q50S) for its normal DNA binding sites, it does not abolish binding altogether; thus, the level of CEH-12 expression in A-class neurons must be sufficient to allow CEH-12(Q50S) to bind to *ceh-12* regulated genes. Mutation of the conserved phenylalanine residue in the N-terminal eh1 domain

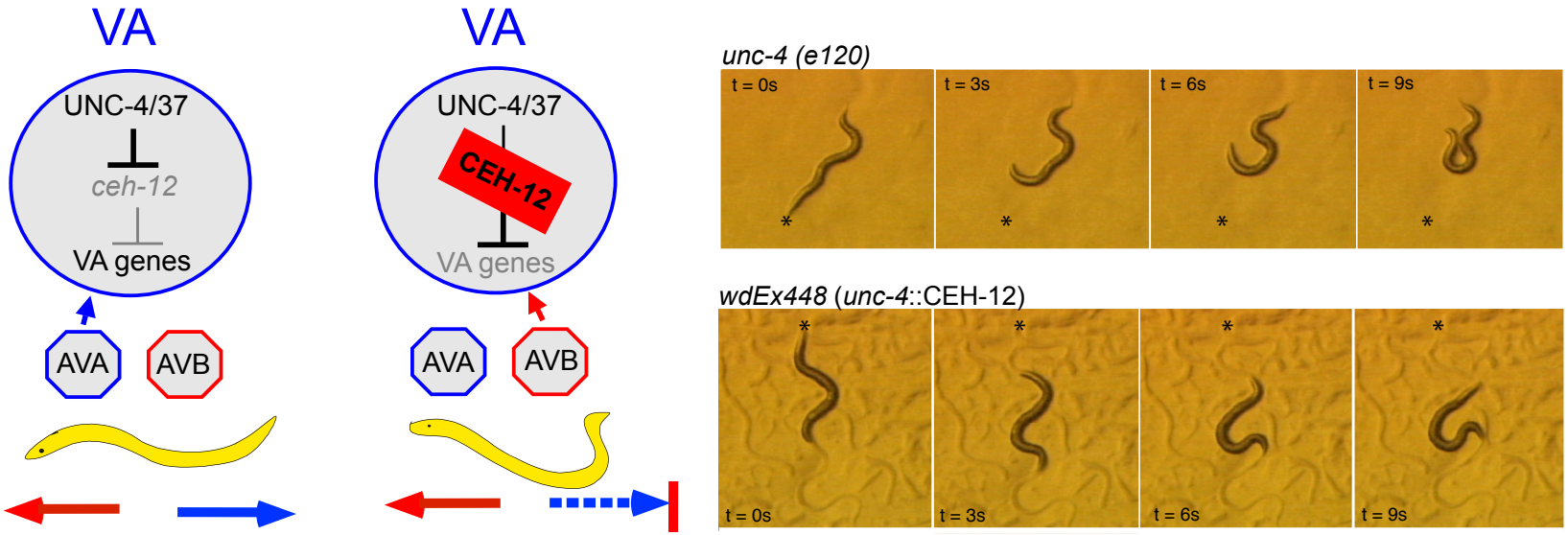

Figure 5.3 Expression of CEH-12 in A-class motor neurons induces a backward Unc phenotype.
We propose that UNC-4 and UNC-37 repress *ceh-12* expression in VAs to allow inputs from command interneuron partners that drive backward locomotion (e.g. AVA). This model predicts that ectopic CEH-12 expression in VAs should induce a backward movement defect resulting from the miswiring of VA motor neurons with inputs normally reserved for VBs (e.g. AVB). Following a tap on the head, *unc-4 (e120)* mutants are unable to crawl backward and instead coil dorsally into an omega (Ω) shape (e.g. t = 9s panel). Ectopic expression of CEH-12 (*wdEx448*) in VAs is sufficient to induce a backward Unc phenotype that is strikingly similar to that of *unc-4* mutant animals.

Asterick denotes initial location of the head in movement assays.

CEH-12 mutation	Predicted to disrupt:	Induce Unc-4 phenotype?
unc-4::CEH-12 (F3L)	Groucho binding	Yes
unc-4::CEH-12 (W48F)	DNA binding	No
unc-4::CEH-12 (W48A)	DNA binding	No
unc-4::CEH-12 (Q50S)	DNA binding specificity	Yes

Figure 5.4 CEH-12 is required to bind DNA but not Groucho to induce an Unc-4 phenotype when ectopically expressed in A-class neurons.
Top. Domain structure of CEH-12, depicting the mutations made in ectopic expression constructs.
Bottom: Chart detailing the mutations, what they are predicted to disrupt, and the affect on inducing a backward Unc phenotype when expressed in A-class neurons (see text for details).

also did not disrupt CEH-12 activity. This result suggests that the CEH-12-induced Unc-4 like phenotype may not depend on interactions with Groucho.

ceh-12 deletion mutants suppress *unc-4* and *unc-37* mutants

We have shown that ectopic expression of CEH-12 in VA motor neurons is sufficient to induce the Unc-4 phenotype. If de-repression of *ceh-12* is also responsible for VA miswiring in *unc-4* mutants, then the loss of CEH-12 activity in an *unc-4* mutant should suppress the backward Unc phenotype. We obtained the *ceh-12 (gk391)* deletion allele from the North American *C. elegans* knockout consortium (Mark Edgley, Vancouver) and *ceh-12 (tm1619)* from the Japanese *C. elegans* knockout center (Shohei Mitani, NRBP, Tokyo) to test this model. Both of these alleles remove the ATG start codon, while *tm1619* also deletes part of the homeodomain (Figure 5.5). Thus, both *gk391* and *tm1619* are likely null alleles.

We chose four *unc-4* alleles for this study, ranging from weak (i.e. temperature-sensitive) to strong (i.e. null). We found that *ceh-12* deletions can fully suppress the *unc-4 (ts)* allele but only partially suppress strong *unc-4* null alleles (Figure 5.6, data not shown). In addition, *ceh-12* suppresses *unc-37 (e262)* in an incompletely penetrant manner. These data are consistent with a model in which *unc-4* functions to repress two parallel pathways in VAs (Figure 5.7). In *unc-4* null alleles, these two pathways are fully de-repressed and turn VA genes off, thus leading to the miswiring event. The loss of *ceh-12* activity in these strong *unc-4* alleles only partially restores expression of VA genes, thereby leading to an incomplete suppression of the Unc-4 movement phenotype. However, the weak allele, *unc-4 (ts),* retains some function such that the two downstream

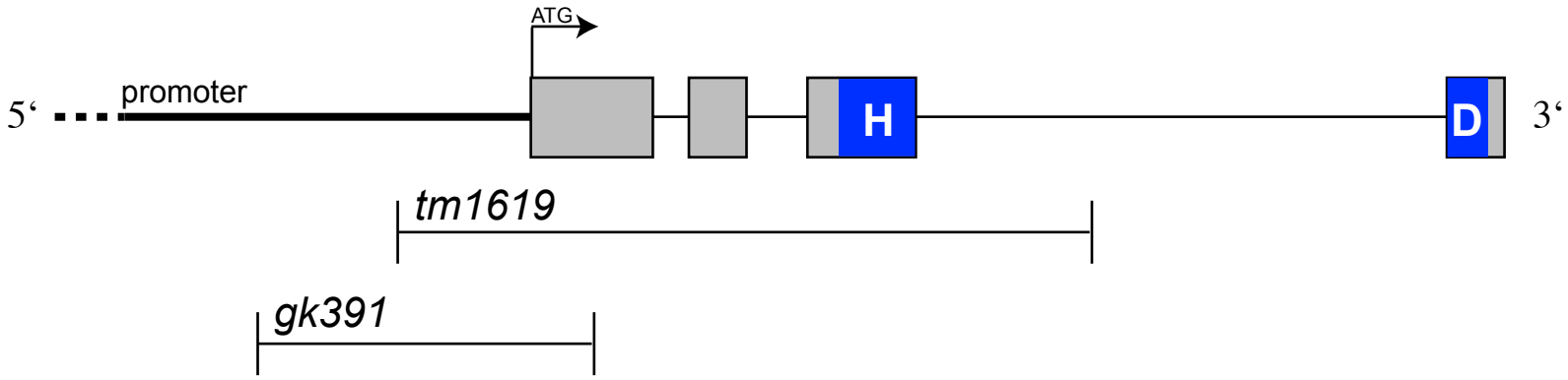

Figure 5.5 *ceh-12* deletion alleles.
ceh-12 is encoded by four exons, the last two covering the homeodomain (HD). Two deletion alleles, *tm1619* and *gk391*, remove 5’ sequence (promoter) and the ATG start codon. The *tm1619* deletion extends into the HD. Both deletions are predicted null alleles.

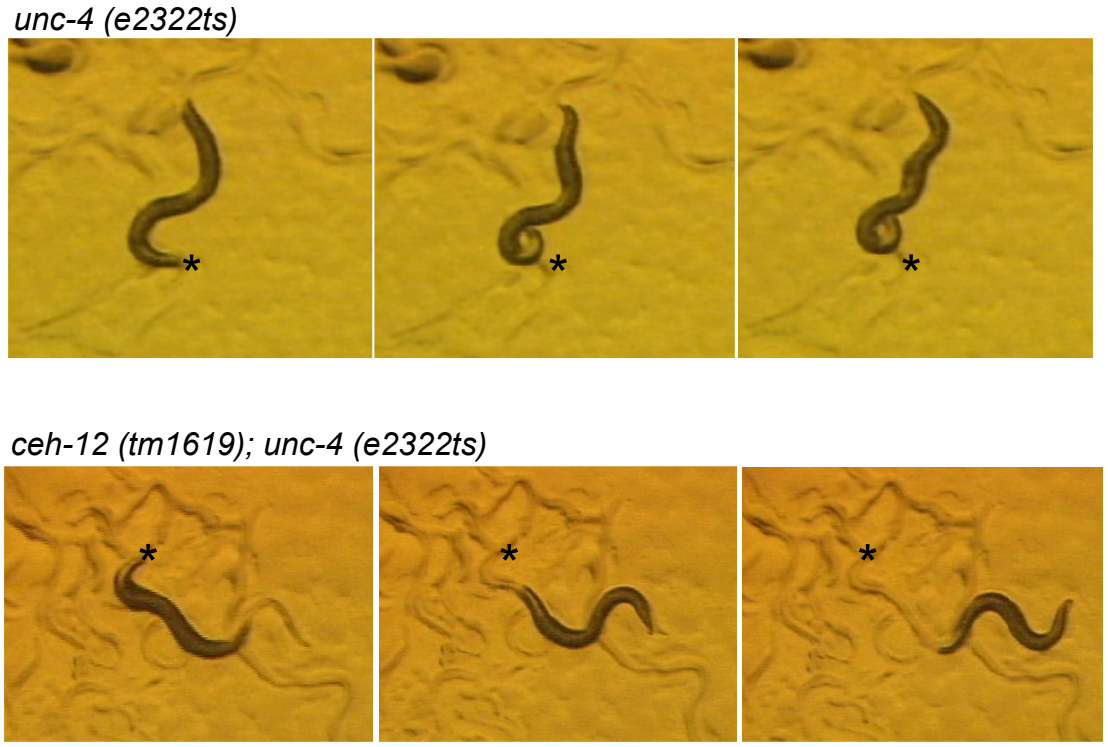

Figure 5.6 *ceh-12 (tm1619)* fully suppresses a weak *unc-4* allele.
We predict that the loss of *ceh-12* function in *unc-4* mutants should restore backward locomotion. This model is true for weak *unc-4* alleles. *unc-4 (e2322ts)*, at the non-permissive temperature, cannot crawl backward. The introduction of a *ceh-12* deletion allele in *unc-4 (ts)* mutants fully suppresses the *unc-4* backward movement defect.

Asterick denotes initial location of the head in movement assays.

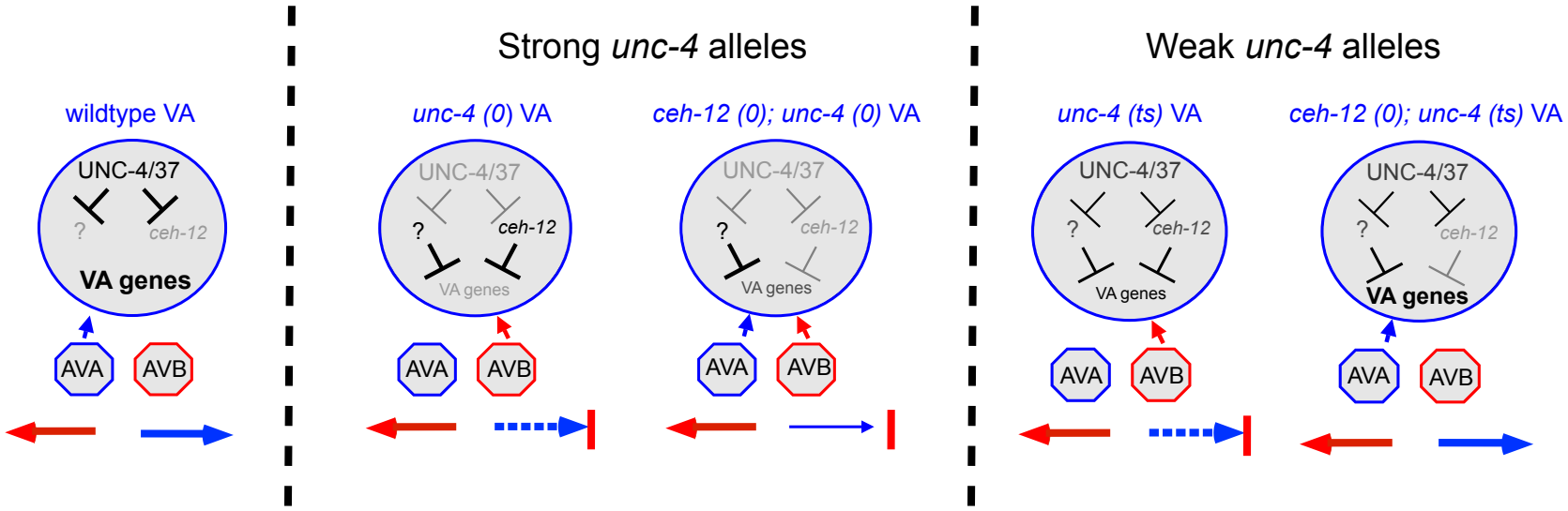

Figure 5.7 Model of UNC-4 synaptic specificity pathway.
In wildtype VA motor neurons, UNC-4 and UNC-37 corepress *ceh-12* and an unknown gene (?) to ensure full expression of VA genes and normal synaptic inputs (AVA). In *unc-4* null *(0)* mutants, these two pathways are fully derepressed, leading to a complete repression of VA genes, abnormal synaptic inputs (AVB), and disabled backward movement (dashed blue arrow).When *ceh-12* deletions *(0)* are combined with *unc-4 (0)* alleles, a partial restoration of VA gene expression is coupled with an incomplete suppression of the backward movment defect (thin blue arrow). In weak *unc-4* alleles (*ts*), *ceh-12* and ? are partially repressed. However, the combined activity of these two repressor pathways is sufficient to reduce the levels of VA genes and promote inputs from AVB command interneurons. Removing the function of the *ceh-12* branch in *unc-4 ts* mutants tips the balance toward VA gene expression and normal interneuronal inputs; thus, these animals are fully suppressed and can crawl backward (thick blue arrow).

pathways are not fully de-repressed and VA genes are expressed at a low level. In this case, the loss of the *ceh-12* branch in *unc-4 (ts)* mutants is sufficient to elevate VA gene expression above a necessary threshold for induction of VA-type inputs and thus normal movement.

ceh-12 deletion mutants have no obvious movement defect and do not regulate motor neuron markers

Our results indicate that *ceh-12* is **sufficient** in VA motor neurons to induce B-type inputs. We analyzed the *ceh-12* mutants to determine if CEH-12 was also **necessary** in VBs for normal synaptic connections. Close examination reveals a subtle movement defect, which we plan to assess with quantitative motility assays (see Chapter VI). It seems unlikely, however, that VB motor neurons are miswired in *ceh-12* mutants, because overall forward movement in these animals is robust.

To assess the role of *ceh-12* in VB motor neuron fate, we crossed several motor neuron markers into *ceh-12* mutants. From this analysis we conclude that *ceh-12* activity in VBs is not required to activate *del-1*::GFP and *acr-5*::GFP expression (Figure 5.8). Additionally, *unc-4*::GFP is not ectopically expressed in VBs in *ceh-12* mutants, suggesting that CEH-12 does not repress *unc-4* in these cells (Figure 5.8). These results are consistent with our model that *ceh-12* acts in parallel to another gene to repress VA genes while yet another pathway is responsible for activating expression of VB genes (Figure 5.7).

The potential redundant function of CEH-12 is consistent with the observed phenotypes in *Drosophila*. *dHb9* mutants have a weak axon guidance/fasciculation defect; these phenotypes are enhanced by mutations in *Nk6* (Broihier et al. 2004). In

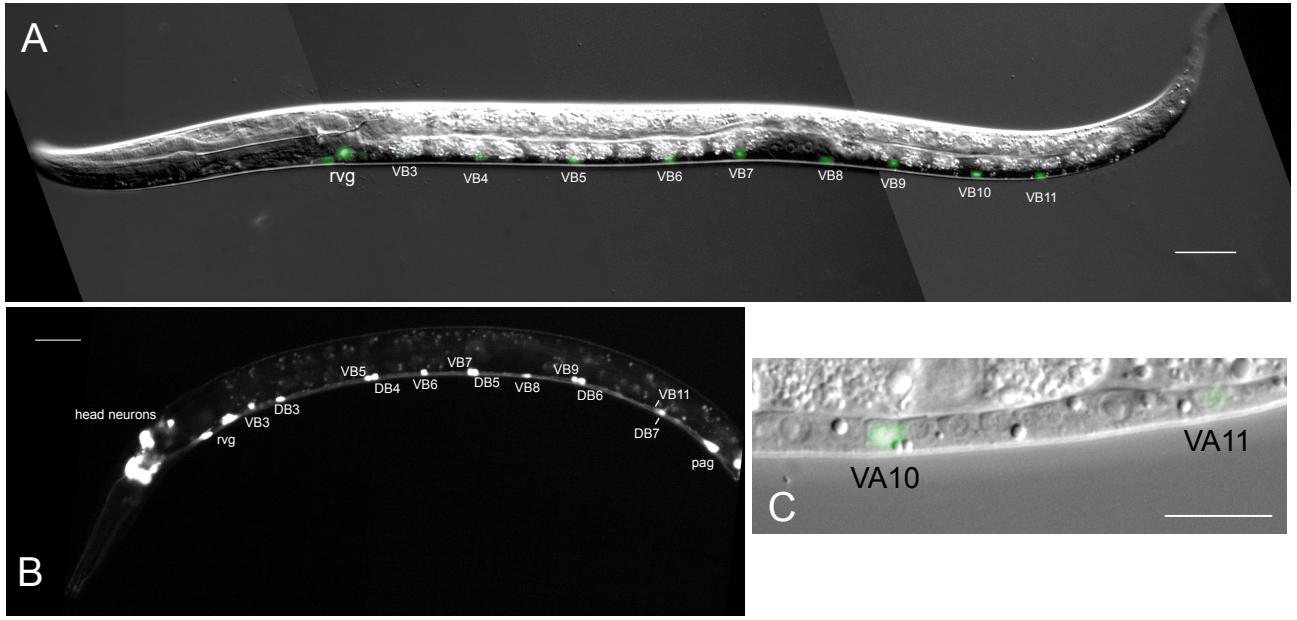

Figure 5.8 *ceh-12* does not regulate markers of motor neuron fate.
All animals are L2 larvae. Anterior is to the left.
A. *del-1*::GFP expression is unaffected in *ceh-12 (gk391)* mutants. GFP is seen in VB motor neurons in the ventral nerve cord and in SAB motor neurons in the retrovesicular ganglion (rvg).
B. *acr-5*::GFP expression is similarly unaffected in *ceh-12 (tm1619)* mutants. GFP is expressed in VB and DB motor neurons in the ventral nerve cord. Other neurons expressed in the head, rvg, and pre-anal ganglion (pag).
C. *ceh-12 (gk391)* animals express *unc-4*::GFP solely in VA motor neurons, demonstrating that *unc-4* is not negatively regulated by *ceh-12* in VB motor neurons.

Scale bars. A, B = 20 μm, C = 10 μm.

mice, HB9 has weak effects on motor neuron specification; axonal projections from the spinal cord are normal but cell migrations are perturbed. Thus, *ceh-12* mutants might exhibit subtle defects in VB axonal fasciculation, which has yet to be examined.

Discussion

ceh-12 is negatively regulated by UNC-4 and UNC-37 and functions downstream to control synaptic specificity

Our parallel genomics approaches from DA and VA motor neurons identified a single common *unc-4* and *unc-37* regulated gene, the HB9 homolog *ceh-12*. We have now confirmed that CEH-12 functions downstream of UNC-4. We established that *ceh-12*::GFP is VB-specific and ectopically expressed in VA (and DA) motor neurons in *unc-4* and *unc-37* mutants (Figure 5.2) . These results are consistent with our model of UNC-4 function (Fig 1.14).

What are the consequences of ectopic *ceh-12* expression in VA motor neurons in *unc-4* mutants? To address this question, we first drove expression of CEH-12 in A-class neurons to 'over-ride' the negative regulation by UNC-4. These transgenic animals display a backward movement defect that is strikingly similar to that of *unc-4* mutants (Figure 5.3). As evidence from *Drosophila* and vertebrates suggest that HB9 proteins are transcriptional repressors, we favor the model that CEH-12 is acting to turn VA genes off and thereby switch synaptic connections to B-type interneurons. We established that this phenotype is dependent on CEH-12 interactions with DNA but not on the function of the eh1-like domain. Thus, *ceh-12*, like other HB9 proteins, may not depend on Groucho to repress gene expression (William et al. 2003).

The above data indicate that CEH-12 is **sufficient** to induce the Unc-4 phenotype when ectopically expressed in A-class neurons. Our next experiments aimed to test if CEH-12 was also **necessary**. To accomplish this goal, we constructed double mutants between *ceh-12* deletion alleles and *unc-4* mutants. We determined that *ceh-12* mutants strongly suppress a weak *unc-4* allele but only partially suppress null *unc-4* mutants. We interpret this finding to rule out a simple linear model in which UNC-4 controls synaptic specificity strictly through CEH-12. We propose a model in which at least two parallel pathways act downstream of UNC-4 and UNC-37, both branches of which repress VA gene expression. We postulate that normal VA inputs depend on a minimum level of VA gene expression; if this expression level exceeds this threshold then the animal will have proper wiring and crawl backward. Expression levels below this threshold, however, result in the Unc-4 miswiring event and the consequent defect in backward locomotion. To support this model we must identify VA genes that are not expressed in *unc-4* mutants. An analysis of the genes downregulated in *unc-4* and *unc-37* mutants (as determined by our microarray experiments) will aid in our search for these genes.

COG-1/Nkx6 is a candidate *unc-4* target gene

In *Drosophila* the Nkx6 homeodomain protein works in conjunction with dHb9 to define motor neuron subtype identity (Figure 5.9) (Broihier et al. 2004). In vertebrates, Nkx6. factors are also co-expressed with HB9 in motor neuron progenitors (Lee and Pfaff 2001). Thus, the evolutionarily conserved roles of HB9 and Nkx6 factors strongly argue for a similar mechanism in nematodes. Indeed, we have obtained preliminary evidence that is consistent with this prediction; the *cog-1* transcript is elevated in the mRNA-

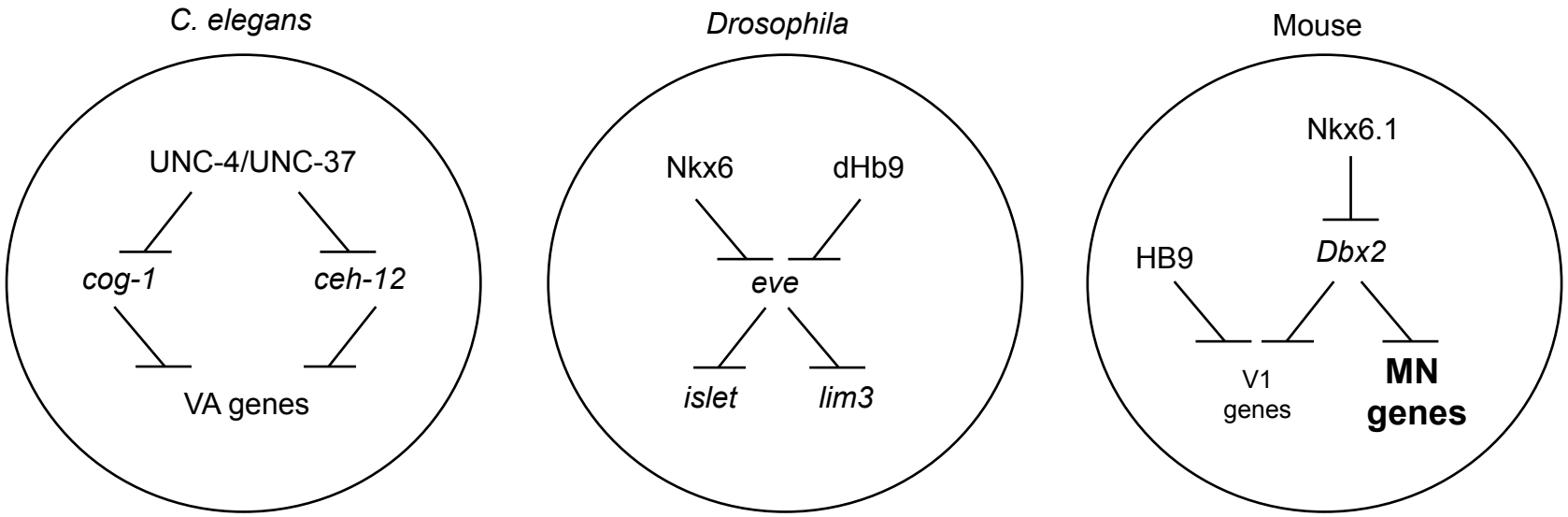

Figure 5.9 Nkx6 and HB9 factors function together to promote motor neuron differentation.
In *C. elegans*, our hypothetical model suggests that UNC-4/UNC-37 mediated repression of *cog-1*/*Nkx6* and *ceh-12*/*HB9* allows for VA gene expression. In *Drosophila*, Nkx6 and dHb9 work together in ventrally-projecting motor neurons to repress *eve* expression, thus promoting expression of the neural-specifiers *islet* and *lim3* (Broheir, et al., 2004). In mice, Nkx6.1 acts in MN and V1 interneuron progenitors to repress *Dbx2* expression (Sander, et al., 2000). In motor neuron progenitors, HB9 induces motor neuron differentation by repressing V1 genes promoted by Nkx6.1-mediated repression (Thaler, et al., 1999).

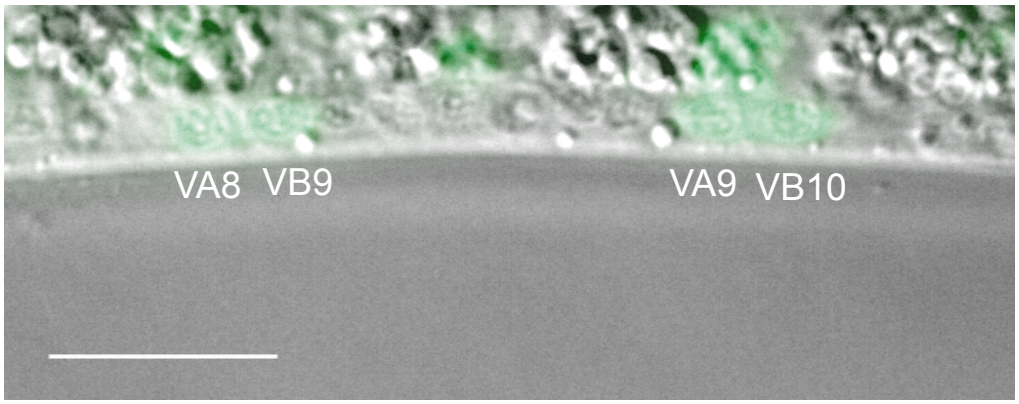

Figure 5.10 ***cog-1*****::GFP is expressed in VA and VB motor neurons.**
Scale bar = 10 μm.

tagging data obtained from *unc-37* mutant VA motor neurons. Thus, *cog-1* expression is presumptively repressed by UNC-37/Groucho in the VAs. Repression is likely to be incomplete, however, as a *cog-1*::GFP reporter gene is normally expressed in VA and VB motor neurons (Figure 5.10). It will be interesting to determine if *cog-1*::GFP is 'brighter' in the VAs in *unc-37* and *unc-4* mutants. In addition, *cog-1* mutant alleles can be tested for enhancement of CEH-12-dependent suppression of *unc-4* null mutants. This result would support the idea that *cog-1* functions in parallel to *ceh-12* downstream of *unc-4* in the neural specificity pathway (Figure 5.9).

ceh-12 may not be necessary in VB motor neurons to establish normal synaptic connections

Since *ceh-12* plays a strong role controlling synaptic specificity in VA motor neurons, we analyzed the *ceh-12* deletion alleles to determine if CEH-12 functioned similarly in VB motor neurons. However, *ceh-12* mutants do not display an obvious movement defect, suggesting that VB motor neurons are not miswired. Additionally, known A-class and B-class markers are expressed normally in *ceh-12* mutants. These data are consistent with a model in which *ceh-12* functions in parallel with another VB gene to repress VA gene expression. As mentioned above, *cog-1*/*Nkx6* is a strong candidate as it is normally expressed in VB (and VA) motor neurons (Figure 5.10). A new student in the lab, Judsen Schneider, has made a *ceh-12; cog-1* double mutant and discovered that it displays a synthetic Unc phenotype. This result could be indicative of parallel functions for *ceh-12* and *cog-1* in ventral cord motor neuron differentiation. Judsen is now testing the *ceh-12; cog-1* double mutant for misexpression of VA and VB GFP markers.