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The *C. elegans* NeuroD homolog *cnd-1* functions in multiple aspects of motor neuron fate specification

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SUMMARY

The basic helix-loop-helix transcription factor NeuroD (Neurod1) has been implicated in neuronal fate determination, differentiation and survival. Here we report the expression and functional analysis of *cnd-1*, a *C. elegans* NeuroD homolog. *cnd-1* expression was first detected in neuroblasts of the AB lineage in 14 cell embryos and maintained in many neuronal descendants of the AB lineage during embryogenesis, diminishing in most terminally differentiated neurons prior to hatching. Specifically, *cnd-1* reporter genes were expressed in the precursors of the embryonic ventral cord motor neurons and their progeny. A loss-of-function mutant, *cnd-1(ju29)*, exhibited multiple defects in the ventral cord motor neurons. First, the number of motor neurons was reduced, possibly caused by the premature withdrawal of the precursors from mitotic cycles. Second, the strict correlation between the fate of a motor neuron with respect to its lineage and position in the ventral cord was disrupted, as manifested by the variable expression pattern of motor neuron fate specific markers. Third, motor neurons also exhibited defects in terminal differentiation characteristics including axonal morphology and synaptic connectivity. Finally, the expression patterns of three neuronal type-specific transcription factors, *unc-3*, *unc-4* and *unc-30*, were altered. Our data suggest that *cnd-1* may specify the identity of ventral cord motor neurons both by maintaining the mitotic competence of their precursors and by modulating the expression of neuronal type-specific determination factors. *cnd-1* appears to have combined the functions of several vertebrate neurogenic bHLH proteins and may represent an ancestral form of this protein family.

Key words: *cnd-1*, NeuroD (Neurod1), bHLH, *Caenorhabditis elegans*, Neurogenesis, Motor neuron

INTRODUCTION

The basic helix-loop-helix (bHLH) proteins define a large family of structurally similar transcription factors that function in a variety of developmental processes including myogenesis (Jennings et al., 1994), haematopoiesis (Gering et al., 1998; Tomita et al., 1999), cardiogenesis (Ritter et al., 1999; Srivastava et al., 1995; Thomas et al., 1998), and neurogenesis (Brunet and Ghysen, 1999; Chitnis, 1999; Guillemot, 1999; Kageyama et al., 1997; Lee, 1997). bHLH proteins interact via the HLH domain to form homodimeric and heterodimeric complexes, and regulate gene expression by binding via the basic domain to E-box (CANNTG) sequences in the promoter regions of target genes (Murre et al., 1989). Within the same tissue, bHLH proteins of the same family often have overlapping functions and act sequentially. In the nervous systems of *Drosophila* and vertebrates, several subfamilies of bHLH proteins have been shown to convey neural competence and to specify sensory organ identity.

In *Drosophila* the selection of neural precursors from undifferentiated neuroectoderm is mediated by bHLH proteins encoded by the proneural genes (Ghysen and Dambly-Chaudière, 1988). Proneural bHLH proteins are grouped into two subfamilies based on sequence similarity within the bHLH domain: the Achaete-Scute Complex (AS-C) subfamily and the Atonal subfamily. Members of both subfamilies are transiently expressed in neuroectodermal clusters, and activate neurogenic programs upon dimerization with the ubiquitous bHLH protein Daughterless (DA). A feedback mechanism mediated by the Notch signaling pathway regulates the expression of proneural genes within a cluster such that only one cell acquires neural competence (Simpson, 1990). When ectopically expressed, AS-C and Atonal can convert undifferentiated ectodermal cells into neurons (Hinz et al., 1994; Jarman et al., 1993). Analyses of loss-of-function mutants have shown that AS-C conveys neural competency in the central nervous system (CNS) (Campos-Ortega, 1998; Chitnis, 1999), and both AS-C and members of the Atonal subfamily including Atonal and Amos are required for the generation of sensory organs in the peripheral nervous system (PNS) (Huang et al., 2000; Jan and Jan, 1994). AS-C and Atonal subfamilies also function to convey sensory neuron specificity. AS-C is required for the

generation of subsets of external sensory organs, Atonal for chordotonal organs and photoreceptors (Jarman et al., 1993; 1994), and Amos for a subtype of multiple dendritic organs and olfactory neurons (Goulding et al., 2000; Huang et al., 2000). TAP/Biparous, a third type of bHLH protein closely related to vertebrate Neurogenin and NeuroD, is expressed in neuronal and glial precursors, however, its role is not understood (Bush et al., 1996; Gautier et al., 1997; Ledent et al., 1998).

Analysis of vertebrate neurogenesis has revealed similar themes. The vertebrate bHLH proteins are divided into four subfamilies: MASH, also called Ascl (AS-C related), MATH, also called Atoh (Atonal-related), Neurogenin and NeuroD (or Neurod1; Brunet and Ghysen, 1999; Guillemot, 1999; Lee, 1997). Members of each subfamily are transiently expressed in overlapping regions and function in a redundant and sequential manner. Ectopic expression of MASH, MATH and Neurogenin converts non-neuronal ectoderm into neurons, and this conversion is sensitive to Notch signaling (Chitnis and Kintner, 1996; Jarman et al., 1993; Ma et al., 1996). Loss-of-function studies of MASH, MATH and Neurogenin suggest that each subfamily plays a proneural role in different parts of the developing nervous system in mouse. MASH1 is required for neurogenesis in hindbrain, ventral forebrain and olfactory epithelia (Cau et al., 1997; Guillemot and Joyner, 1993; Guillemot et al., 1993; Lo et al., 1991). MATH1 is required for the neurogenesis of granule cells in the cerebellum (Ben-Arie et al., 1997; Helms and Johnson, 1998). Neurogenins are necessary for the developing cranial and dorsal root ganglia where they regulate the expression of bHLH genes involved in later phases of neuronal determination (Ben-Arie et al., 1997; Fode et al., 1998; Ma et al., 1998, 1999, 1996; Sommer et al., 1996). Moreover, MASH1 also has a role in neuronal sub-type specification in both CNS and PNS as does the AS-C (Goridis and Brunet, 1999; Lo et al., 1998; Tuttle et al., 1999).

The NeuroD subfamily of bHLH genes has been proposed to function as neuronal differentiation factors. In addition to a bHLH domain, NeuroD subfamily members contain a 40 amino acid region, called the extended homology region, located C-terminal to the bHLH domain (Lee, 1997). The function of this extended homology region is not known. Ectopic expression of NeuroD in *Xenopus* oocytes converts non-neuronal ectoderm to fully differentiated neurons, and induces premature differentiation of neural precursors (Lee et al., 1995). In contrast to ectodermal conversion by MASH, MATH and Neurogenins, this NeuroD activity is insensitive to Notch signaling (Chitnis and Kintner, 1996). In the mouse CNS, NeuroD is transiently expressed in postmitotic neurons, outside the ventricular zone (Lee, 1997). NeuroD knock-out mice display major abnormalities in brain morphology, possibly arising from massive cell death of cerebellar and hippocampal granule cells unable to differentiate (Miyata et al., 1999). In the rodent retina, NeuroD has been implicated in neuron/glia fate determination, interneuron development and cell survival (Morrow et al., 1999).

The *C. elegans* nervous system has 302 neurons representing 118 classes based on morphology, connectivity and position (White et al., 1986). Several bHLH proteins expressed in the nervous system have been identified. The *hlh-2* gene encodes the *C. elegans* DA homolog, and its expression is restricted to neurons and their precursors during most of embryonic development (Krause et al., 1997). *hlh-3*, a *C. elegans* AS-C like gene, is coexpressed with *hlh-2* in many neuronal precursors, suggesting that the two proteins may heterodimerize (Krause et al., 1997). The in vivo roles of *hlh-2* and *hlh-3* are unknown. Mutations in *lin-32*, a *C elegans* Atonal homolog, cause the transformation of ray neuroblasts to hypodermal cells in the male tail. Ectopic LIN-32 expression results in the formation of additional ray papillae, supporting a proneural role for *lin-32* (Zhao and Emmons, 1995). A *C. elegans* NeuroD gene, named *cnd-1*, was identified based on sequence homology (Lee, 1997). We report here the isolation and characterization of a mutation in *cnd-1*.

MATERIALS AND METHODS

C. elegans genetics

All strains were generated from Bristol strain N2, and grown at 22.5°C as described by Brenner (1974). *cnd-1(ju29)* was induced by Methanesulfonic Acid Ethyl Ester (EMS) from CZ323 *sem-4(n1398); juIs1*. *juIs1* is an insertion of the [P*unc-25*-SNB::*GFP*; *lin15*(+)] extrachromosomal array (Hallam and Jin, 1998). The original *ju29* isolate was backcrossed three times to CZ333 *juIs1* prior to linkage mapping and phenotypic analysis. *ju29* was mapped to the *dpy-17 lon-1* interval of linkage group III based on the following data: from the progeny of animals of genotype *+/bli-4(e937)I; +/rol-6(e187)II; ju29/daf-2(e1368) vab-7(e1568) III; +/unc-39(e928) IV; +/dpy-11(e224)V; +/lon-2(e678)X*, 6/33 homozygous *ju29* animals segregated *daf-2(e1368) vab-7(e1562*), suggesting its linkage to chromosome *III;* from the progeny of animals of genotype *ju29 + +/unc-36(e251)dpy-18(e364am) III*, 7/10 homozygous *ju29* animals segregated *dpy-18*, and 1/10 segregated *unc-36;* from the progeny of animals of genotype *+ ju29 +/dpy-1(e1) lon-1(e185) III*, 9/34 homozygous *ju29* animals segregated *dpy-1*, 0/34 segregated *lon-1*; from the progeny of animals of genotype *+ ju29 +/dpy-17(e164) unc32(e189) III*, 2/5 *ju29* homozygous animals segregated *unc-32* and 1/9 segregated *dpy-17*.

Other strains used in this study include: CZ1197, containing an integrated P*unc-25::GFP* marker *juIs73*; CZ631, containing an integrated P*acr-2::GFP* marker *juIs14*; CZ1454 containing an integrated [P*unc-25::CFP*; P*acr-2::YFP*] marker *juIs96*; MT1522 *ced-3(n717)*; CB151 *unc-3(e151)*; CB596 *unc-30(e596);* and CB120 *unc-4(e120)*. GFP transgenes were introduced to *cnd-1(ju29)* by standard mating procedures and confirmed by GFP expression and noncomplementation tests to *ju29*. Double mutants of *cnd-1* with *ced-3, unc-30, unc-3*, and *unc-4* were confirmed by noncomplementation tests.

Molecular biology and germline transformation

Cosmids spanning the *dpy-17 lon-1* interval were obtained from the Sanger Centre, Cambridge, UK. pCZ171 was generated by subcloning a 10 kb *Not*I/*Nco*I fragment from ZC129 into the vector pSL1190 (Pharmacia), and pCZ171 was used for all subsequent subcloning experiments following standard procedures (Sambrook et al., 1989). pCZ175 was generated by *Nsi*I digestion followed by Klenow fill-in, resulting in a frame-shift at amino acid position 34. pCZ178 was generated by inserting a 6 kb *Hin*dIII-*Nsi*I fragment into pPD95.75. pCZ179 was generated by replacing the *Pvu*II to 3′ UTR portion of *cnd-1* in pCZ172 with GFP coding sequences along with *unc-54* 3′ UTR from pPD95.75. P*unc-25::CFP* and P*acr-2::YFP* constructs were generated by inserting a 1.4 kb *unc-25* promoter (Eastman et al., 1999) and a 3.4 kb *acr-2* promoter (Y. J., unpublished data) into pPD136.61 and pPD132.12 respectively. P*unc-30::GFP* was made by inserting a 2.3 kb *unc-30* promoter into pPD95.75 (Jin et al., 1994), and a P*unc-4::GFP* was made by inserting a 2.0 kb *unc-4* promoter into pPD95.75 (Miller et al., 1992). Heat-shock inducible expression

of *cnd-1* constructs were made possible by inserting, after the heat-shock inducible promoters in pPD49.78 and pPD49.83, a 1.7 kb *Xmn*I-*Eco*RV *cnd-1* genomic fragment beginning 170 bp 5′ to the predicted initiation ATG and ending 270 bp downstream of the stop codon, resulting in pCZ180 and pCZ181 respectively. To confirm the ectopic expression of *cnd-1* from these heat-shock inducible promoters, similar constructs were made by inserting GFP at the *Pvu*II site of *cnd-1* genomic DNA in pCZ180 and pCZ181.

*cnd-1(ju29); lin-15(n765*ts) or *lin-15(n765*ts) animals were used as hosts for germline transformation, following standard procedures using 50 ng/μl of p*lin-15(*EK), a plasmid containing a wild-type copy of the *lin-15* gene, as the coinjection marker (Mello et al., 1991; Clark et al., 1994). Cosmid and plasmid DNAs were injected at 5-10 ng/μl and 20-50 ng/μl respectively. *lin-15(n765ts)* animals are multivulva (Muv) at 22.5°C, thus transgenic animals were recognized as non-Muv. To clone *cnd-1*, transgenic animals were examined for the rescue of the Cnd-1 uncoordinated phenotype, and the number of ventral cord neurons were scored in selected transgenic lines. *juIs96* was generated by X-ray-induced chromosomal integration of an extrachromosomal array of [P*unc-25::CFP*; P*acr-2::YFP*].

Identification of the *cnd-1(ju29)* lesion

cnd-1 genomic DNAs encompassing all three exons and intron/exon boundaries were amplified from N2 and *cnd-1(ju29)* worm lysates by PCR. DNA sequences were determined using the Promega fmol DNA Cycle Sequencing System and [α-^{33}P]dATP (Amersham). The lesion in *cnd-1(ju29*) was confirmed in both strands using independently amplified genomic DNA products. Primer sequences are available upon request.

Nomarski, immunocytochemistry and reporter gene analysis

L1 larvae were collected by lysing gravid hermaphodites in hypochlorite the night before, mounted in M9 solution on 5% agar pad, and viewed under 63× or 100× magnification using Nomarski optics. The number and positions of ventral cord nuclei were scored as described by Sulston and Horvitz (1977).

Immunocytochemical analysis using anti-GFP, LIN-26, and UNC-86 antibodies was carried out as described by Finney and Ruvkun (1990), and at least three transgenic lines were examined**.** Embryos were collected by lysing gravid adults in hypochlorite, washed in 1× phosphate-buffered saline and fixed in 2% paraformaldehyde and methanol for 15-20 minutes at room temperature. Fixed embryos were permeabilized with one freeze-thaw cycle under liquid nitrogen. Embryos were stained with chicken anti-GFP (Chemicon) at 1:200 dilution, rabbit anti-LIN-26 at 1:100 dilution (Labouesse et al., 1996) or rabbit anti-UNC-86 at 1:50 dilution (Finney and Ruvkun, 1990). Stained embryos were visualized with FITC-conjugated donkey anti-chicken (Jackson) and Cy5-conjugated goat anti-rabbit (Jackson) secondary antibodies under a confocal microscope (Leica). Anti-GABA antibody staining was performed on mixed stage worms fixed in 1% glutaraldehyde and 4% paraformaldehyde and treated with collagenase (Sigma) as described by McIntire et al. (1992). GFP expression was visualized using a HQ-FITC filter set (Chroma), and CFP and YFP analysis were performed using a combination of CZ05 (for CFP) and W7/10C Dual excitation filters (for YFP) (Chroma) (Miller et al., 1999).

RESULTS

ju29 is a loss-of-function mutation in *cnd-1*, a *C. elegans* NeuroD homolog

ju29 homozygous animals showed kinky uncoordination when moving backward, but otherwise appeared normal in egg-laying, pharyngeal pumping, defecation, mechanosensation, and male mating behaviours. No zygotic or maternal embryonic lethality was observed in *ju29* homozygous animals. We mapped *ju29* to the *dpy-17 lon-1* interval on chromosome *III* (Fig. 1A). *ju29/+* heterozygotes were wild type, whereas the uncoordinated phenotype of *ju29* homozygotes was comparable to that of *ju29/sDf121* or *ju29/sDf123* heteroygotes, suggesting that *ju29* is a recessive loss-of-function mutation.

A pool of 11 cosmids spanning the *dpy-17 lon-1* interval were used to rescue *ju29* in germline transformation experiments. A single cosmid from this pool, ZC129, was found to contain the rescuing activity (Fig. 1A). A 10 kb *Not*I/*Nco*I fragment from ZC129 was fully capable of rescuing

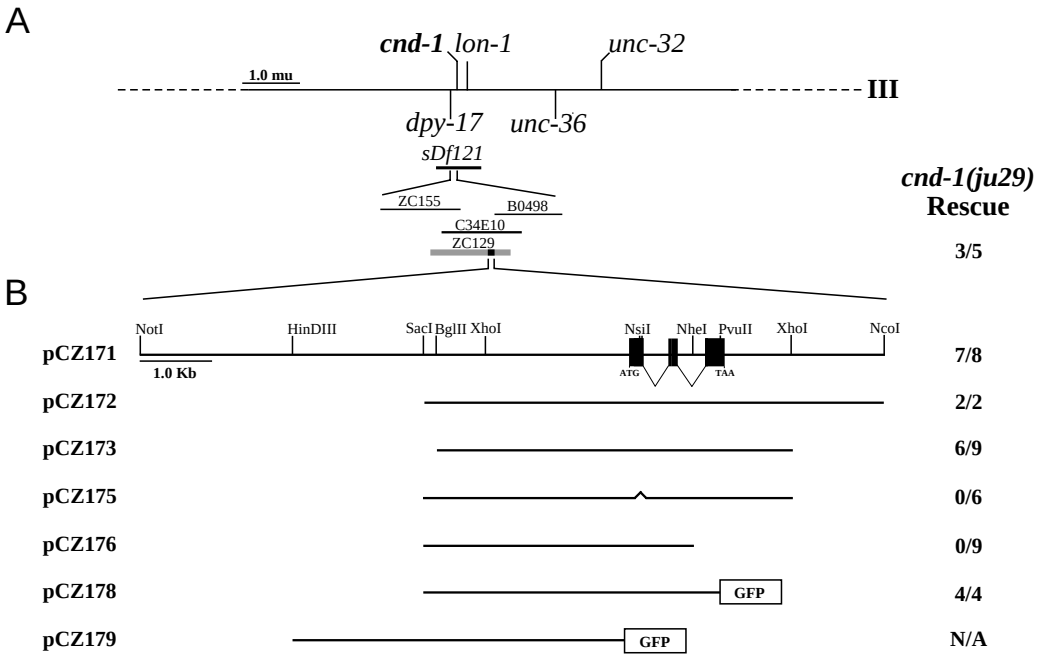
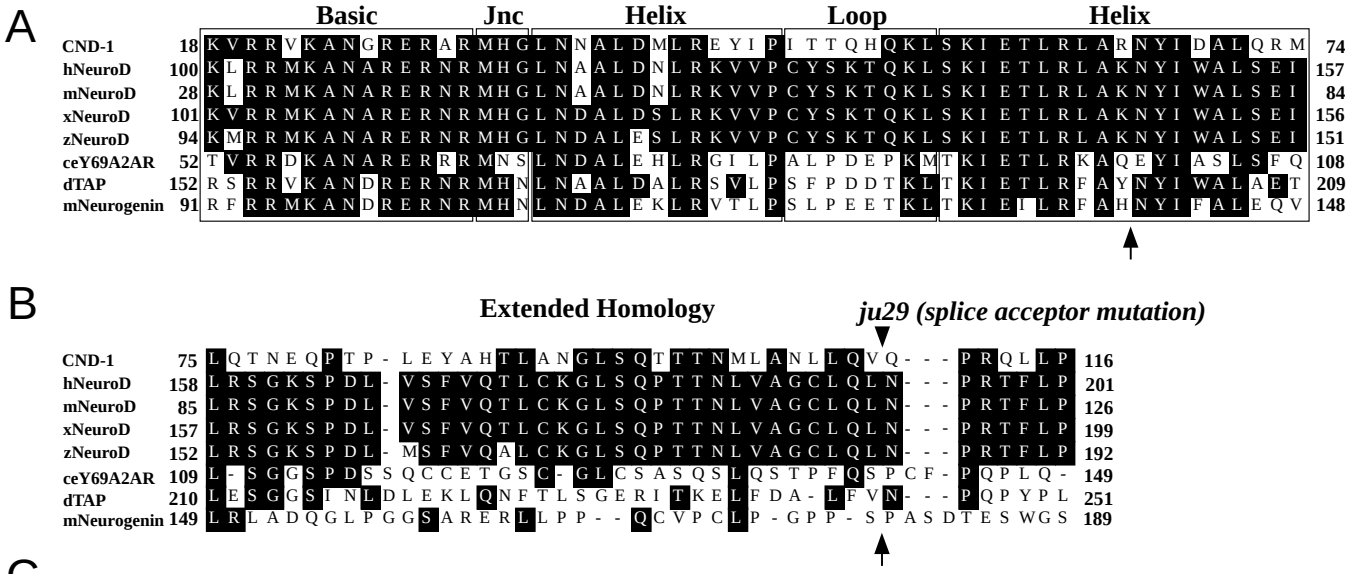


Fig. 1. Genetic position and germline transformation rescue of *cnd-1(ju29)*. (A) *cnd-1(ju29)* maps between *dpy-17* and *lon-1* on chromosome III. The deficiency *sDf121* fails to complement *ju29*. The cosmid ZC129 rescues *ju29* animals and overlaps extensively with C34E10. (B) *cnd-1* genomic organization and germline transformation rescue with subclones of ZC129. pCZ175 contains a frameshift (^) in the first exon of *cnd-1*. *ju29* is a G→A transition in the 5′ splice junction of exon 3. Numbers to the right represent the number of rescued lines per total number of stable transmitting lines.



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% CND-1 Identity/Similarity

	Basic	HLH	ExHom
hNeuroD	**71**/85	**62**/77	**45**/63
mNeuroD	**71**/85	**62**/77	**45**/63
xNeuroD	**79**/85	**62**/79	**45**/63
zNeuroD	**71**/85	**60**/80	**43**/60
Y69A2AR	**71**/71	**46**/64	**17**/32
dTAP	**64**/78	**50**/70	**17**/32
mNeurogenin	**64**/78	**55**/63	**12**/20

Fig. 2. Sequence comparison between CND-1 and vertebrate bHLH proteins. (A) Alignment of the bHLH domain. GenBank accession: CND-1 (U10402), hNeuroD (AB018693), mNeuroD (U28068), xNeuroD (U28067), dTAP (AF022883), and mNeurogenin (U67776). (B) Alignment of the extended homology domain of CND-1 and vertebrate NeuroDs. *ju29* results in a frameshift after V109 within the extended homology domain. Arrows in A and B point to splicing junctions. (C) Percentage identities between CND-1 and vertebrate NeuroD bHLH and extended homology domains.

the uncoordinated phenotype of *ju29* mutants (Fig. 1B). This DNA fragment contains one predicted gene previously named *cnd-1* for *C. elegans* NeuroD-related protein, based on its homology to the bHLH transcription factor NeuroD (Lee, 1997 and Fig. 2). Further analysis of subclones obtained from this 10 kb fragment identified the minimal rescuing region to be a 5.0 kb *Bgl*II/*Xho*I fragment (Fig. 1B). Introduction of a frameshift within the predicted bHLH domain completely abolished rescuing activity, suggesting that *cnd-1* was the candidate gene for *ju29*.

The *cnd-1* locus is composed of 3 exons and encodes a 192 amino acid protein. The CND-1 bHLH domain shares 68% identity and 81% similarity to the vertebrate NeuroD subfamily, and 60% identity and 71% similarity to the Neurogenin subfamily (Fig. 2A,C). Additionally, CND-1 shares 45% identity and 63% similarity with vertebrate NeuroD proteins in the extended homology region (Fig. 2B,C; Lee, 1997). Complementary searches of the *C. elegans* protein database using the human NeuroD sequence also identified CND-1 as the closest relative in the *C. elegans* genome. A search of the *C. elegans* database using the CND-1 sequence further identified a closely related bHLH protein encoded by Y69A2AR. Y69A2AR is more similar to the *Drosophila* TAP and vertebrate neurogenins than to vertebrate NeuroD, however, both TAP and Y69A2AR have a region C-terminal to the bHLH domain, which is weakly similar to that of NeuroD (Fig. 2). It is interesting to note that *cnd-1* and Y69A2AR also share a conserved splice junction within the bHLH domain (Fig. 2A). While most vertebrate NeuroDs are encoded in a single exon, *cnd-1* contains two introns: one within the helix-loop-helix domain, the other within the extended homology region (Lee, 1997). Similar genomic organization exists in the bHLH domains of the *C. elegans hlh-2*, *hlh-3* and *lin-32* genes (Krause et al., 1997).

Sequence analysis of *cnd-1(ju29)* genomic DNA identified a single G to A transition in the splice acceptor of exon 3, which changes a typical 5′ splice acceptor sequence TTTCAG to TTTCAA (Figs 1, 2). Because the first nucleotide of exon 3 is guanine, the G to A transition in *ju29* mutants results in the formation of an atypical 5′ splice acceptor sequence TTTCAAG, which is found in approximately 0.3% of intron/exon boundaries in the *C. elegans* genome (A. Zahler, personal communication). Therefore, it is possible that the mutant splice acceptor sequence in *ju29* animals is recognized by the splicing machinery, producing a protein that is frameshifted after glutamine 107 within the extended homology region. To determine whether this potential translation product retains partial *cnd-1* function, we generated a construct (pCZ176) that is predicted to encode a truncated form of CND-1 containing exons 1 and 2 only (Fig. 1B). Transgenic lines containing this construct failed to rescue the uncoordinated phenotype of *cnd-1(ju29)* mutants. Although this transgenic analysis may not fully mimic the effect of *ju29* mutation, this observation, in conjunction with the phenotypic

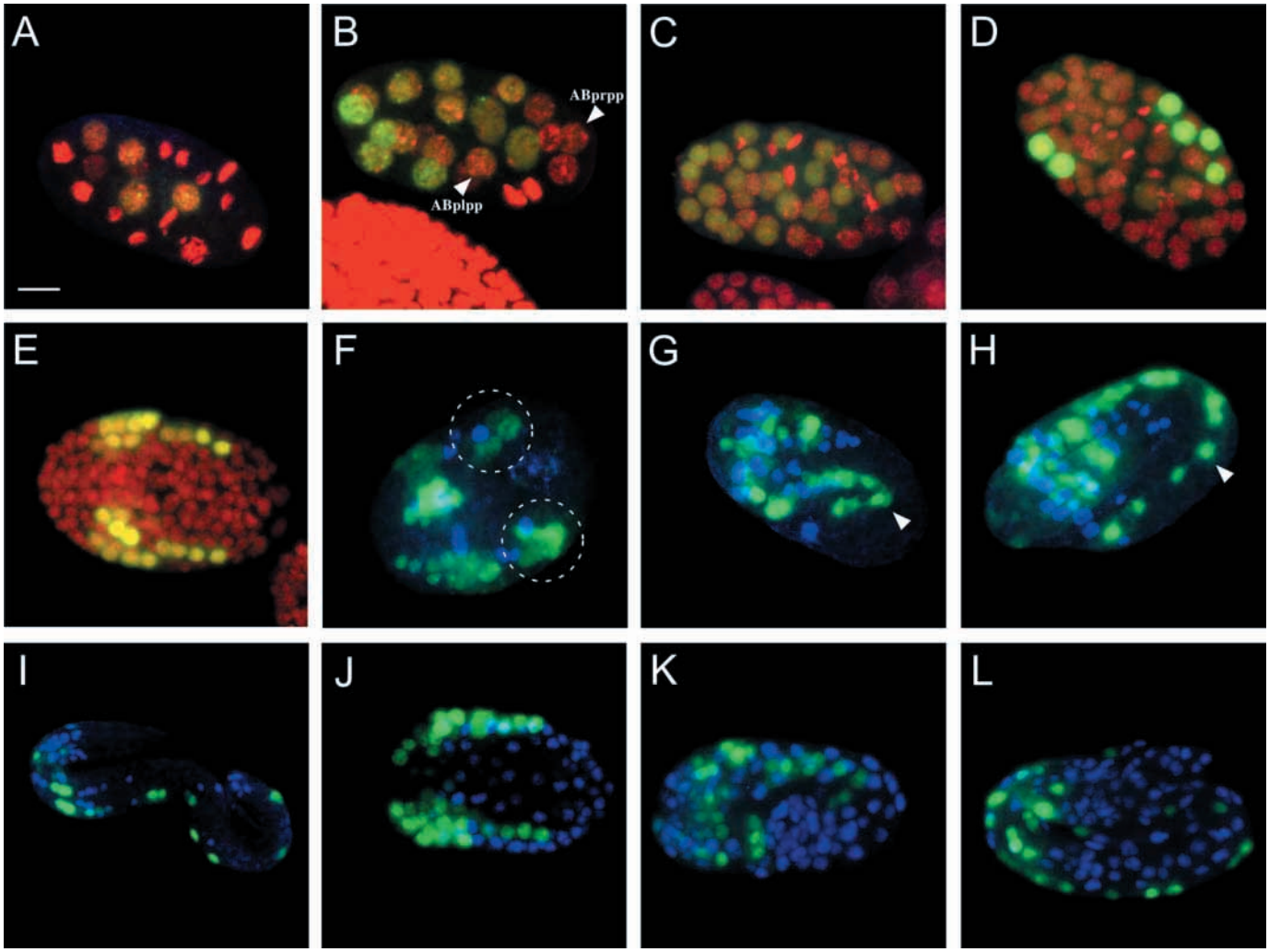

Fig. 3. *cnd-1* is expressed in embryonic neuroblasts and ventral cord motor neurons. Confocal images of embryos carrying a transgene of *juEx244* [pCZ179; Rol-6]. In A-E, Anterior is left, and dorsal is up. Green is CND-1::GFP, and red is propidium iodine. (A) 14-cell stage embryo (approximately 50 minutes). (B) 24-cell stage embryo (approximately 75 minutes). Arrowheads indicate ABplpp and ABprpp neuroblasts. (C) Early gastrulation, 48-cell stage embryo (approximately 100 minutes). (D) Mid-gastrulation, 110-cell stage embryo (approximately 150 minutes). (E) Late gastrulation, 250-cell stage embryo (approximately 200 minutes). (F-I). *cnd-1* is expressed in neural precursors and differentiating ventral cord neurons. Green represents CND-1::GFP, blue is UNC-86. (F) Approximately 230 minute embryo. CND-1::GFP is observed in the ABplppap and ABprppap derived neuroblasts (circled), non-overlapping with UNC-86-expressing cells. (G) Early comma stage embryo, approximately 360 minutes. CND-1::GFP is expressed in anterior and ventral nerve cord neurons (arrowhead). (H) Two-fold stage embryo, approximately 450 minutes. CND-1::GFP expression persists in differentiating ventral nerve cord motoneurons (arrowhead). (I) Newly hatched L1 larva, approximately 800 minutes. (J-L) *cnd-1* expression (green) does not overlap with LIN-26 (Blue). (J) Late gastrulation, 250-cell stage, approximately 200 minutes. (K) One and half-fold stage embryo, approximately 425 minute. (L) Three-fold stage embryo, approximately 500 minutes. The merge is due to LIN-26-expressing nuclei directly below the focal plane of CND-1::GFP-expressing nuclei. Scale bar: 10 μm.

analysis described below, suggests that *ju29* causes a strong loss of *cnd-1* function.

cnd-1 is expressed in neuroblasts and differentiating neurons during embryogenesis

We analyzed the expression pattern of *cnd-1* using two reporter constructs: pCZ179 containing GFP driven by 6 kb of *cnd-1* regulatory sequence, and pCZ178 containing GFP fused in-frame at Ser163 of CND-1 (Fig. 1). Transgenic lines containing pCZ178 rescued both the uncoordination and cellular defects of the ventral cord motor neurons of *cnd-1(ju29)* mutants, and GFP was localized to the nucleus (Fig. 1B, and see later). To minimize misrepresentation caused by the mosaicism of the extrachromosomal arrays, we collected data from three independent transgenic lines containing pCZ179 or pCZ178 respectively. pCZ178 and pCZ179 were expressed in similar sets of cells, however, GFP expression from pCZ178 was much weaker than from pCZ179 in most cells.

CND-1::GFP expression was first detectable in four descendants of the AB lineage of 14 cell embryos (Fig. 3A). AB derived neuroblasts give rise to most of the *C. elegans* nervous system. By the 24-cell stage, approximately 75

minutes postfertilization, CND-1::GFP was found in 15 AB derived blastomeres, two of which, ABplpp and ABprpp, are precursors of many embryonic ventral cord motor neurons (Fig. 3B). CND-1::GFP expression was observed in numerous unidentified nuclei throughout gastrulation and epidermal enclosure (Fig. 3C-E). By early comma stage, approximately 360 minutes postfertilization, CND-1::GFP was found in many postmitotic neurons in the head and in the ventral cord (Fig. 3G). The expression of CND-1::GFP in the ventral cord neurons was maintained until hatching (Fig. 3H-I), but disappeared completely by the end of the first larval stage. CND-1::GFP was not observed in postembryonically derived motor neurons.

To determine whether *cnd-1* was expressed in non-neuronal cells of the AB lineage, embryos carrying *cnd-1::GFP* transgenes were double labeled with anti-GFP and anti-LIN-26 antibodies. LIN-26 is expressed in non-neuronal ectoderm including epidermal and glial-like cells (Labouesse et al., 1996). In all embryonic stages examined the expression of CND-1::GFP and LIN-26 did not overlap (Fig. 3J-L). To confirm the identity of specific AB-derived blastomeres during later stage embryogenesis, embryos carrying CND-1::GFP transgenes were double labeled with anti-GFP and anti-UNC-86 antibodies (Fig. 3F-I). CND-1::GFP and UNC-86 are primarily expressed in different subsets of mitotic and postmitotic neurons throughout embryogenesis. At 230 minutes postfertilization, UNC-86 is expressed in ABplaaaaaa, ABarpapaaa, ABplapaaaa, ABprapaaaa, ABarppaaap, and ABarpppapp blastomeres (Finney and Ruvkun, 1990). At the same stage, CND-1::GFP was found in a non-overlapping set of neuroblasts derived from the ABplppap and ABprppap, including ABplppaap, ABplppapp, ABplpppaa, ABprppapa, ABprppaap, ABprppapp and ABprpppaa, which give rise to the embryonic ventral cord motor neurons (Figs 3F, 4A). In summary, our reporter transgene analysis reveals that CND-1::GFP is expressed in both mitotically active neuroblasts throughout embryogenesis, and in subsets of postmitotic neurons including the ventral cord motor neurons.

The *cnd-1(ju29)* mutation affects multiple aspects of embryonic ventral cord motor neurons

In vertebrate nervous systems, neurogenins are transiently expressed in neural precursors and are necessary for neuronal fate determination in peripheral ganglia, whereas NeuroD is predominantly expressed in postmitotic and differentiating neurons and is required for neuronal differentiation following terminal mitosis. To elucidate the function of *cnd-1* in *C. elegans*, we analyzed the effect of the *cnd-1(ju29)* mutation on the ventral cord motor neurons. The ventral cord of a wild-type first stage larva (L1) contains six GABAergic motor neurons called DDs, and 16 cholinergic motor neurons that are subdivided into nine DAs and seven DBs (White et al., 1986). All the ventral cord motor neurons except DB2 are derived from the ABp lineage (Fig. 4A). Six DDs and seven DAs are born as sisters from ABplpp and ABprpp precursors, and six DB and two DAs are derived from the posterior daughters of ABplp and ABprp precursors (Fig. 4A). During elongation of the embryo, these motor neurons intercalate, resulting in the intermixed distribution of the GABAergic and cholinergic motor neurons along the length of the ventral nerve cord. The identity of each motor neuron can be unambiguously determined based on the position of its nucleus in the ventral cord, under Nomarski optics.

The number of ventral cord motor neurons is reduced in *cnd-1* mutant L1s

In a newly hatched wild-type L1, 15 nuclei are positioned in the ventral cord between the retrovesicular and the preanal ganglia, corresponding to DA_{2-7}, DB_{3-7}, and DD_{2-5} (Fig. 4B). In *cnd-1* mutant L1s, 11-15 nuclei were present in this region (Table 1 and Fig. 5). Moreover, the stereotyped arrangement of the ventral cord nuclei was disrupted. These defects were more obvious in the anterior portion of the nerve cord than the

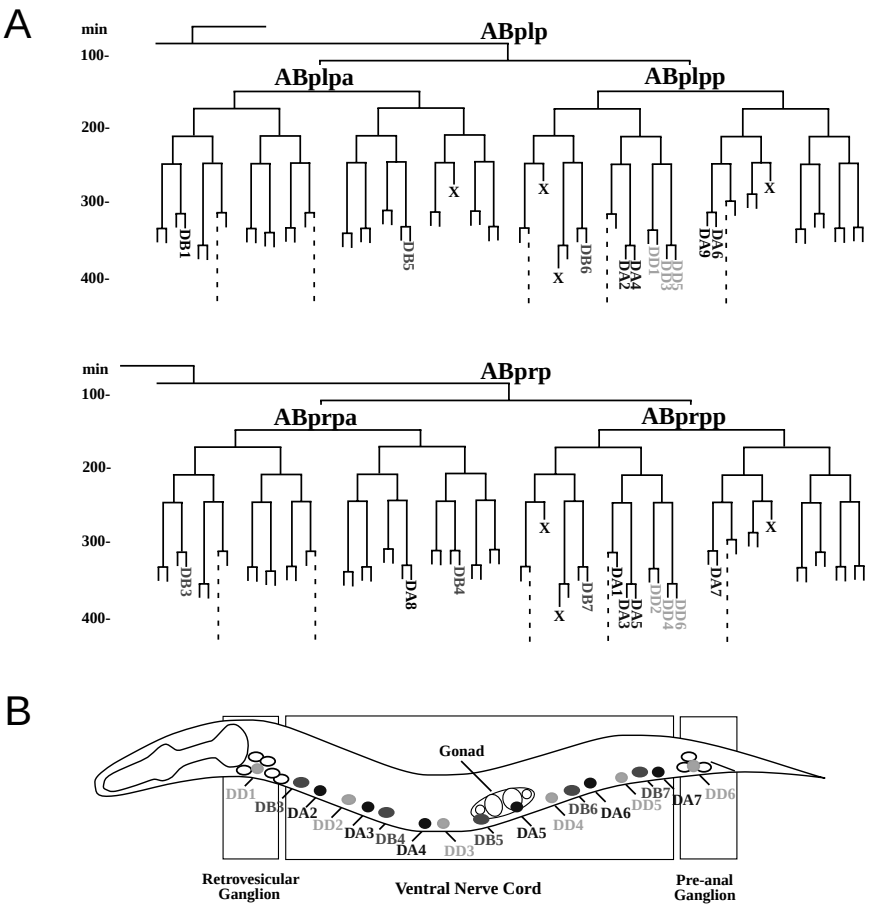

Fig. 4. Embryonic lineage and position of DD, DA and DB motor neurons in first stage larva. (A) Lineage diagram (Sulston et al., 1983). (B) Schematic of the position of DD, DA and DB nuclei in the ventral nerve cord of a wild-type L1 larva. The 15 nuclei referenced in the text include DB_3 to DA_7.

Table 1. Loss of *cnd-1* function results in motor neuron loss and cell fate specification defects

Genotype	Number of ventral cord neurons	*n*	Number of ventral cord neurons expressing			
			acr-2::GFP	*n*	*unc-25::GFP*	*n*
Wild type	15.0(±0.0)	200	11.0(±0.0)	100	4.0(±0.0)	100
cnd-1(ju29)	12.9(±0.5)	110	8.0(±1.2)	37	2.8(±0.9)	56
sDf121/cnd-1(ju29)unc-36(e251)	12.6(±0.4)	53	7.3(±1.2)	20	3.2(±0.9)	33
ced-3(n717)	15.0(±0.0)	100	11.0(±0.0)	100	4.0(±0.0)	100
cnd-1(ju29); ced-3(n717)	12.5(±0.5)	97	7.2(±1.0)	23	3.4(±0.9)	41

Synchronized L1 animals were examined under Nomarski optics with epifluorescence. Wild-type animals are those of *lin-15(n765)ts* carrying transgenic markers *juIs14* [P*acr-2::GFP;lin-15(+)*], *juIs73* [P*unc-25::GFP;lin-15(+)*]. Numbers in parentheses are the standard deviation.

posterior portion. In wild-type L1s, the region of the ventral cord posterior to the gonad contains six cells in two groups of three, corresponding to DD_4 DB_6 DA_6 and DD_5 DB_7 DA_7 (Fig. 4B). 80% of *cnd-1* L1s examined exhibited a wild-type distribution of these six posterior motor neurons (*n*=110). In contrast, 90% of *cnd-1* L1s had lost one or more nuclei in the ventral cord anterior to the gonad (*n*=110). These defects in *cnd-1(ju29)/sDf121* heterozygotes was slightly stronger than *cnd-1(ju29)* homozygous, resulting in a total of 10-14 nuclei (Table 1). However, the frequency of losing 1, 2, 3 or more neurons was comparable in *cnd-1(ju29)* homozygotes and *cnd-1(ju29)/sDf121* heterozygotes (Table 1), supporting the conclusion that *ju29* causes a strong reduction in *cnd-1* activity.

The neuronal loss in *cnd-1* is not due to programmed cell death or epidermal fate transformation

The loss of ventral cord motor neurons in *cnd-1* mutants could be caused by cell death, early withdrawl of neuronal precursors from the cell cycle, or cell fate transformation. To distinguish between these possibilities, we first constructed *cnd-1(ju29); ced-3(n717)* double mutants in which programmed cell death is blocked due to the loss of CED-3 caspase activity (Yuan et al., 1993). We observed no difference in the number and the pattern of ventral cord neurons in *cnd-1(ju29); ced-3(n717)* double mutant L1s, compared to *cnd-1(ju29)* mutants alone (Table 1 and Fig. 5C). We then examined the expression of LIN-26 to determine if there was an increase in epidermal cells. The number and pattern of LIN-26-expressing cells were indistinguishable between *cnd-1* and wild-type embryos and L1s (data not shown). We have found no alternations in the nuclei positions of muscle, hypodermal or intestinal cells in *cnd-1* mutant L1 animals (*n*=20) under Nomarski microscope, nor did we find extra nuclei at inappropriate positions in *cnd-1* mutant L1s. Moreover, we never observed cells expressing P*unc-25::GFP* and P*acr-2::GFP* at improper locations (see later). Thus, it is unlikely that we failed to count the postmitotic ventral cord neurons that end in inappropriate positions. Rather, we infer from our data that the motor neuron loss in *cnd-1* mutants may be caused by early withdrawal of precursors from the mitotic cell cycle.

The expression of motor neuronal fate specific markers is altered in *cnd-1*

To determine which class of ventral cord motor neuron was affected in *cnd-1* mutants, we examined the expression of GABAergic and cholinergic fate markers. In wild-type L1s, the DD neurons express the glutamic acid decarboxylase, *unc-25* (Jin et al., 1999) and the neurotransmitter, GABA (Fig. 6A), and can be visualized by a P*unc-25::GFP* reporter gene. The cholinergic DA and DB neurons express a GFP reporter driven by the *acr-2* promoter. *acr-2* encodes a non-alpha acetylcholine receptor subunit (Y. J. and H. R. Horvitz, unpublished results) (Fig. 6D). We observed a reduction in the number of ventral cord motor neurons expressing GABA or P*unc-25::GFP* and

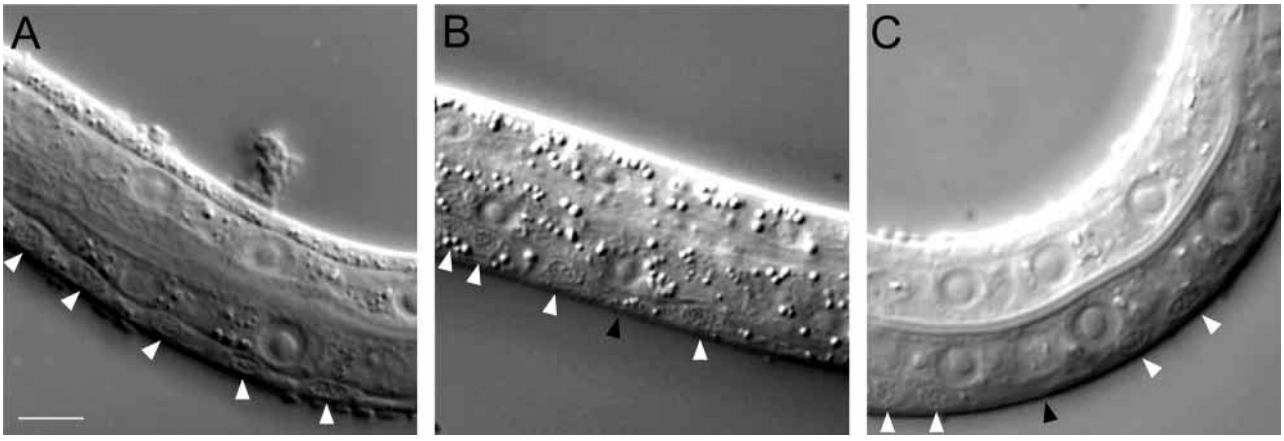

Fig. 5. Ventral cord motor neuron loss in *cnd-1* mutants does not result from programmed cell death. Nomarski photographs of anterior ventral nerve cords. (A) Wild-type L1, arrowheads from left to right indicate stereotyped positioning of DB_3, DA_2, DD_2, DA_3 and DB_4. (B) *cnd-1* L1, black arrowhead indicates position of a missing nucleus. The two nuclei at the presumptive DB_3 and DA_2 positions are closer together than in the wild type. (C) *cnd-1; ced-3* L1, black arrowhead indicates position of a missing nucleus; the positions of the remaining nuclei are also altered. Scale bar: 5 μm.

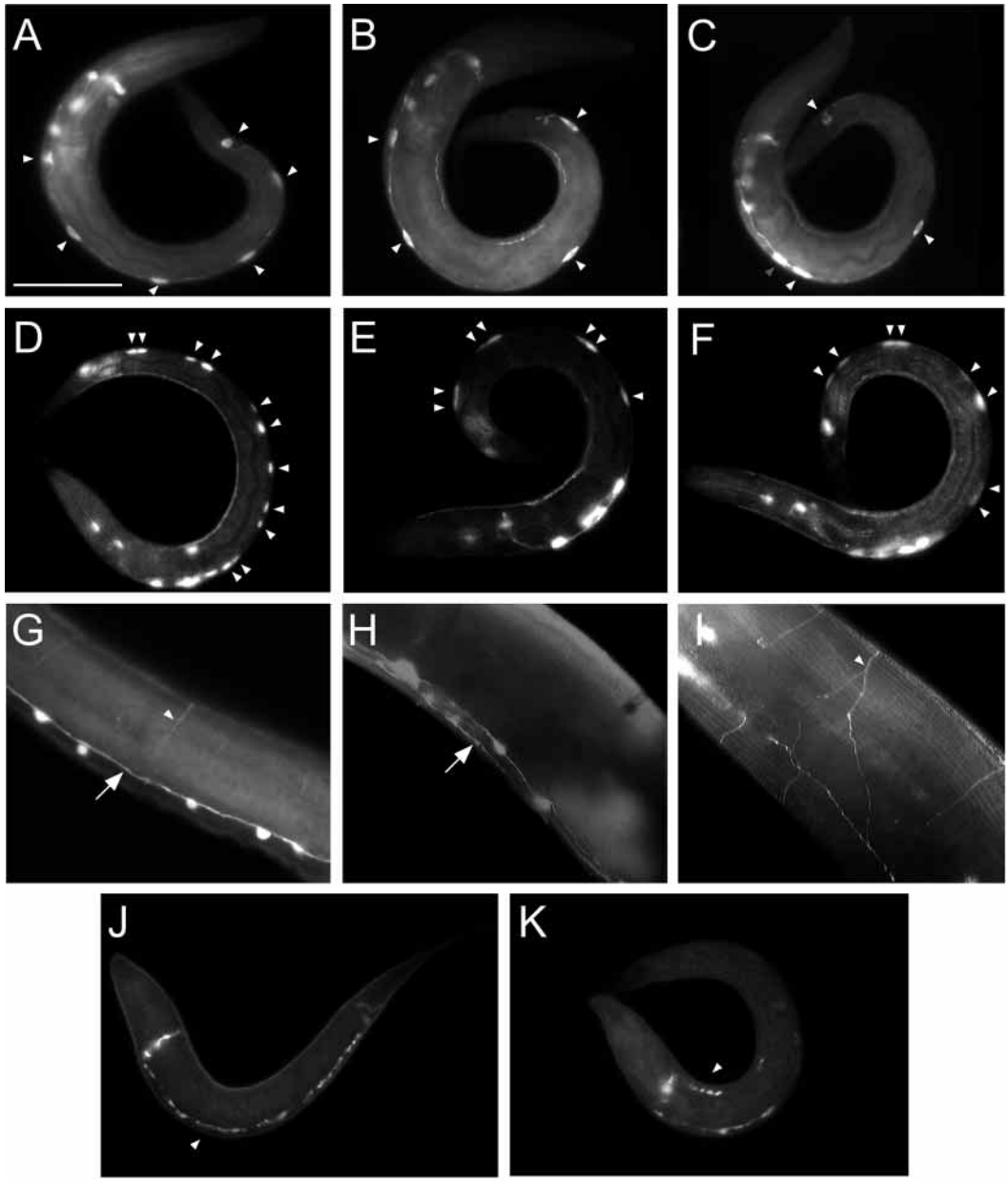

Fig. 6. *cnd-1(ju29)* mutants have multiple defects in motor neuron fate and differentiation. (A) Six DD neurons in a wild-type L1 express GABA (arrowheads). (B-C) *cnd-1* mutant L1s exhibiting a reduction in the number (arrowheads) and altered positions of GABA-expressing neurons (grey arrowhead). (D) Eleven neurons in a wild-type L1 express P*acr-2::GFP*, a cholinergic marker (arrowheads). *cnd-1(ju29)* (E) and *cnd-1(ju29)/sDf121* (F) mutant L1s exhibited a similar reduction in the number of cholinergic motoneurons (arrowheads). (G) The ventral nerve cord of a wild-type adult visualized by anti-GABA antibodies. Note the tight axon bundle within the ventral cord (arrow) and branchless commisure (arrowhead). In *cnd-1* mutants, axons are defasciculated (arrow in H) and commissures branched and wandering (arrowhead in I). (J,K) Ventral cord motor neurons in *cnd-1* mutants exhibit defects in synapse formation. (J) Wild-type L1 expressing the P*unc-25 SNB::GFP* marker in DD motor neurons. GFP is localized to the ventral cord corresponding to DD synapses onto ventral body wall muscles (arrowhead). (K) P*unc-25 SNB::GFP*-expressing neurons in a *cnd-1* mutant L1 exhibiting aberrant dorsal GFP localization (arrowhead). Scale bar: 50 μm.

P*acr-2::GFP* in *cnd-1* L1s (Table1 and Fig. 6B,C,E,F). In the ventral cord between the retrovesicular and preanal ganglia, the number of motor neurons expressing GABA or P*unc-25::GFP* in *cnd-1* mutants ranged from 2 to 4, compared to 4 in wild-type L1s (Table 1); and the number of ventral cord neurons expressing P*acr-2::GFP* ranged from 5 to 11 in *cnd-1* mutants, compared to 11 (six DA and five DB) in wild-type L1s (Table 1). These defects in *cnd-1(ju29)*, *cnd-1(ju29)/sDf121* and *cnd-1(ju29); ced-3(n717)* double mutants were similar (Table 1 and Fig. 6E,F).

The strict correlation between the identity of a motor neuron and its position appears disrupted in *cnd-1* mutants because the spatial pattern of GABA, P*unc-25::GFP* and P*acr-2::GFP* expression was altered, even in animals that had no neuronal loss (Fig. 6C,E,F). To further analyze this defect, we examined the expression of P*unc-25::GFP* or P*acr-2::GFP* in the six neurons in the posterior ventral cord, corresponding to the cells DD_4 DB_6 DA_6 and DD_5 DB_7 DA_7 (Fig. 4B). In 71% of *cnd-1* mutants (*n*=31), we observed P*acr-2::GFP* expression in neurons located in presumptive DD positions. Likewise, in 35% of *cnd-1(ju29)* mutants (*n*=26), we observed P*unc-25::GFP* expression in neurons located in presumptive DA and/or DB positions. Our analysis thus indicates that both GABAergic and cholinergic neurons are affected by the *cnd-1* mutation. Moreover, alterations in the spatial patterns of motor neuron fate-specific markers in *cnd-1* do not simply reflect the disorganization of the ventral cord as the result of motor neuron loss. Rather, the altered spatial expression of GABAergic and cholinergic fate markers may reflect defects in neuronal fate determination, or in cell migration and intercalation.

The fate of some embryonic ventral cord motor neurons is misspecified in *cnd-1*

The disorganization of the ventral cord motor neurons in *cnd-*

Table 2. Abnormal expression of GABAergic or cholinergic fate markers in *cnd-1* mutants

Genotype	No. of ventral cord neurons	*n*	No. of ventral cord neurons expressing either				No. of ventral cord neurons co-expressing		No. of ventral cord neurons expressing neither	
			acr-2::GFP	*n*	*unc-25::GFP*	*n*	*acr-2* and *unc-25*	*n*	*acr-2* or *unc-25*	*n*
juIs96	15.0(±0.0)	100	11.0(±0.0)	100	4.0(±0.0)	100	0.0(±0.0)	100	0.0(±0.0)	100
juIs96; cnd-1(ju29)	12.1(±1.0)	71	6.4(±1.2)	71	2.4(±1.1)	71	0.0(±0.0)	71	3.2(±1.5)	71

Synchronized L1 animals were examined under Nomarski optics with epifluorescence. Wild type refers to *lin-15(n765)ts* carrying the transgenic marker *juIs96* [P*unc-25::GFP* P*acr-2::YFP lin-15(+)*]. Numbers in parentheses are the standard deviation.

1 mutants created ambiguity in the assignment of neuronal fate by position. To examine definitively how motor neuron fate is affected in *cnd-1* mutants, we needed to observe both GABAergic and cholinergic fates simultaneously in the same animal. We thus generated transgenic animals that coexpressed a P*unc-25::CFP* and a P*acr-2::YFP* marker (see Materials and Methods). In wild-type L1s the distribution of CFP- and YFP-expressing motor neurons faithfully reflected the number and position of DD, DA and DB motor neurons in the ventral cord (Table 2). In *cnd-1* L1s, 1 to 4 neurons expressed P*unc-25::CFP* and 4 to 9 expressed P*acr-2::YFP* (Table 2). The number and pattern of CFP and YFP expressing neurons were consistent with those observed in *cnd-1* mutants containing either marker alone (Table 1). Further analysis of the six neurons in the posterior ventral cord revealed a diverse distribution pattern of GABAergic and cholinergic neurons (Table 3). Out of 57 *cnd-1* L1s, only three animals showed a wild-type pattern, and five displayed a pattern that would be consistent with a complete anterior-posterior inversion of the spatial order of the GABAergic and cholinergic neurons. In 86% of *cnd-1* mutant L1s (*n*=57), the six posterior motor neurons exhibited a diverse pattern of P*unc-25::CFP* and P*acr-2::YFP* expression, giving rise to 34 types, each represented by one to four animals (Table 3). However, no single neuron coexpressed P*unc-25::CFP* and P*acr-2::YFP*. Nor was there a tendency for GABAergic or cholinergic neurons to cluster. Approximately 20% of the cells (*n*=342) expressed neither marker, however, they expressed a pan-neuronal marker F25B3.3-GFP (*edIs20*) (D. Pilgrim, personal communication), but not LIN-26 (data not shown), suggesting that they were neuronal cells. Some of the data presented in Table 3 may be attributed to a defect in cell migration or intercalation during the formation of the ventral cord while the fates of the cells are intrinsically intact. However, the fact that a significant portion of the cells expressed neither fate-specific marker suggests that these neurons are either misspecified or not specified in the absence of *cnd-1* function.

Some embryonic ventral cord motor neurons exhibit delays in neuronal fate determination in *cnd-1*

In wild-type animals, the embryonic ventral cord motor neurons begin to express the two fate specific markers as soon as they are born. In *cnd-1* mutants, we noticed that the expression of these two markers was not turned on in some neurons until mid- to late-L1. We followed the expression of P*unc-25::GFP* and P*acr-2::GFP* in the same animal from early L1 (1-6 hours post-hatching) to late L1 (8-14 hours post-hatching). In 31% of *cnd-1* mutant L1s (*n*=61), we observed a 3-6 hours delayed onset of the expression of either marker in one to two neurons per animal. This observation suggests that *cnd-1* may play a role in the temporal regulation of a subset of neuronal fate determination events in ventral cord motor neurons.

The axonal morphology of the embryonic ventral cord motor neurons is abnormal in *cnd-1*

To determine if *cnd-1* mutants exhibit defects in other events of neuronal differentiation, we examined the axonal pathfinding using the GFP markers. In wild-type animals, the nerve processes of DD, DA and DB motor neurons form a single, tight nerve bundle or fascicle in the ventral cord,

Table 3. Misspecification of ventral cord motor neuron fate in *cnd-1* mutants

Genotype	Expression pattern	*n*
juIs96	●○○ ●○○	100
juIs96; cnd-1(ju29)	○○● ○○●	5
	○○◍ ○○◍	4
	○○◍ ○◍○	4
	○○◍ ●○○	3
	●○○ ●○○	3
	○○○ ○○◍	2
	○○○ ◍○○	2
	○○○ ◍○◍	2
	○○● ○●○	2
	○○◍ ○◍●	2
	○◍◍ ○○◍	2
	●○○ ◍○○	1
	◍○◍ ○○●	1
	○○○ ○●◍	1
	○○● ○○○	1
	○○● ◍○●	1
	○○◍ ○◍◍	1
	○○◍ ●◍○	1
	○○◍ ◍○○	1
	○○◍ ◍○●	1
	○●◍ ○◍○	1
	○◍○ ○●○	1
	○◍● ◍○○	1
	●○○ ◍○●	1
	●○● ○○○	1
	●○◍ ●○○	1
	●◍○ ●○○	1
	●◍○ ●○○	1
	◍○○ ○○●	1
	◍○○ ○○◍	1
	◍○○ ○●○	1
	◍○○ ○○◍	1
	◍○○ ●○◍	1
	◍○○ ◍○○	1
	◍○◍ ○○○	1
	◍◍◍ ○○●	1
		57

● P*unc-25::GFP*
○ P*acr-2::GFP*
◍ Non-expressing

Patterns of P*unc-25::GFP* and P*acr-2::GFP* expression in the six posterior ventral cord neurons ranked by frequency of occurrence.

punctuated by circumferential branches called commissures (Fig. 6G). Each commissural branch reflects a single DD, DA or DB process extending into the dorsal cord. Within the dorsal cord, DDs extend their processes in both anterior and posterior directions, while DAs extend their processes anteriorly and DBs posteriorly. In *cnd-1* mutants, the axons of the GABAergic and cholinergic motor neurons often defasiculated in the ventral cord, some wandered to the wrong place, and others had additional commissural projections (Fig. 6H,I). In some neurons, the commissures were completely absent (data not shown).

The synaptic connectivity of the DD neurons is abnormal in *cnd-1*

In wild-type L1 larvae, DD neurons initially innervate ventral body wall muscles, but later remodel their synaptic connectivity by removing ventral synapses and forming new synapses onto dorsal body wall muscles (White et al., 1978, 1986). Using a synaptic vesicle associated GFP marker, P*unc-25 SNB::GFP*, to visualize DD presynapic terminals we have found that DD remodeling occurs in a 3-5 hour time period at the end of the L1 stage (Hallam and Jin, 1998). In wild-type L1s of 0-13 hours posthatching, this GFP marker is exclusively localized to the ventral cord (Fig. 6J). In *cnd-1* L1s of 3-5 hours posthatching, we often observed GFP localization to the dorsal cord, indicating that the neurons formed synaptic contacts with dorsal body wall muscles (Fig. 6K). Moreover, in three animals, the neurons that had initially formed ventral synaptic connections remodeled to form dorsal synapses prior to P nucleus migration events (9 hours posthatching), in contrast to the wild-type DDs that begin remodeling several hours later.

In conclusion, our phenotypic analysis has revealed that loss of *cnd-1* function results in several defects in the ventral cord motor neurons: neuronal loss likely resulting from premature precursor withdrawal from the cell cycle; loss of neuronal identity; misspecification of neuronal fate; temporal delay in the onset of neural-fate-specific markers, and alteration in axonal morphology and synaptic connectivity.

cnd-1 modulates the spatial expression of *unc-30*, *unc-4* and *unc-3* in the ventral cord motor neurons

The early expression of CND-1::GFP in neuronal precursors suggests that some of the defects in *cnd-1* mutants may result from the failure of *cnd-1* to act on its downstream genes. Three transcription factors have been shown to control specific aspects of neuronal identity in DA, DB and DD motor neurons. The homeodomain protein UNC-4 is expressed in cholinergic DAs (Miller and Niemeyer, 1995; Miller et al., 1992). UNC-3, a *C. elegans* O/E-like zinc-finger transcription factor, is expressed in both DA and DB motor neurons. In *unc-3* mutants DAs and DBs exhibit abnormal axonal morphology (Prasad et al., 1998). In addition, we found that the expression of P*acr-2::GFP* in DA neurons was absent in *unc-3(e151)* mutants, suggesting that *unc-3* may regulate the expression of *acr-2* (Fig. 7C). The homeodomain protein UNC-30, controls the fate of the GABAergic DD motoneurons (Jin et al., 1994). In *unc-30* mutants, DD neurons do not express GABA, and exhibit defects in axonal morphology and synaptic connectivity. UNC-30 directly controls the transcription of *C. elegans* glutamic acid decarboxylase, *unc-25*, and the GABA vesicular transporter, *unc-47* (Eastman et al., 1999).

To address how *cnd-1* interacts with these genes, we examined the expression of *unc-30*, *unc-4* and *unc-3* in *cnd-1* mutants using reporter constructs. Consistent with the reduction of GABAergic and cholinergic motor neurons in *cnd-1* mutants, the number of neurons that expressed GFP reporters

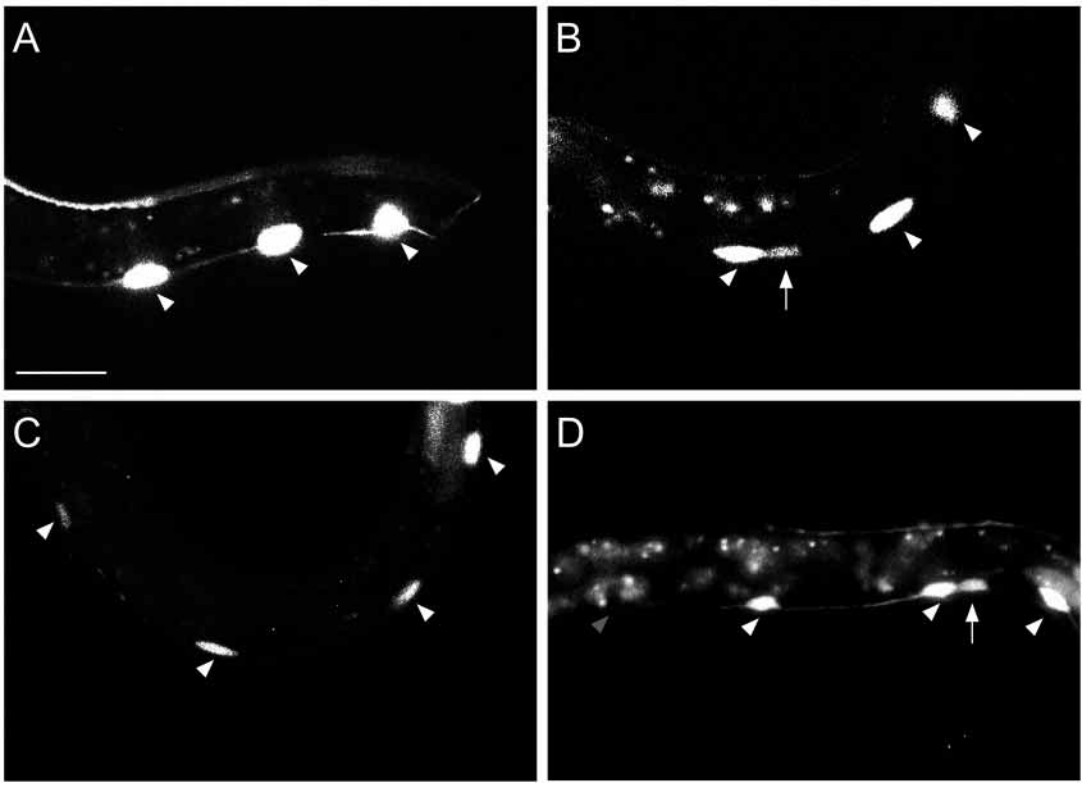

Fig. 7. *unc-4 unc-30* and *unc-3* are downstream of *cnd-1*. *(A)* P*unc-4::GFP* expression in the posterior region of the ventral cord of a wild-type L1. GFP is seen in three cells corresponding to DA_{5-7} (arrowheads). (B) P*unc-4::GFP* expression (arrowheads) in the same region of a *cnd-1* L1, an additional motor neuron is expressing GFP (arrow). Reduction of P*unc-4::GFP* expression is also observed (not shown). The expression of P*unc-30::GFP* and P*unc-3::GFP* was similarly affected (not shown). (C) P*acr-2::GFP* expression in the DB neurons is absent in *unc-3* mutants. Arrowheads are DA neurons. (D) P*acr-2::GFP* expression is spatially altered in *unc-3(e151); cnd-1(ju29)* double mutant L1s. Scale bar: 20 μm.

driven by the promoters of *unc-30, unc-4* or *unc-3* respectively was reduced by 2-3 cells for P*unc-30::GFP* and P*unc-4::GFP* and 4-5 cells for P*unc-3::GFP* (50 to 100 animals were scored for each transgenic line, and two to three independent lines were analyzed). Consistent with the altered distribution of GABAergic and cholinergic neurons in *cnd-1* mutants (Table 3), the cells expressing P*unc-30::GFP,* P*unc-4::GFP* and P*unc-3::GFP* showed no correlation with their lineage and positions in the ventral cord (Fig. 7A, B, and data not shown for P*unc-30::GFP* and P*unc-3::GFP*). Furthermore, the expression of *Punc-25::GFP* was completely abolished in *unc-30(e596); cnd-1(ju29)* double mutants, supporting the hypothesis that *cnd-1* acts through *unc-30* to specify aspects of GABAergic motor neuron fates. In addition, although P*acr-2::GFP* expression in DBs is normal in *unc-3(e151)* single mutants, the number and pattern of motor neurons expressing P*acr-2::GFP* was altered in *unc-3(e151); cnd-1(ju29)* double mutants (Fig. 7D), suggesting that *cnd-1* affects the fate of DB neurons via additional factors. Taken together, our data indicate that *unc-30*, *unc-4*, and *unc-3* act downstream of *cnd-1*. However, ubiqutious and constitutive expression of *cnd-1* induced by heat-shock promoter was unable to induce ectopic expression of P*unc-30::GFP,* P*unc-4::GFP* or P*unc-3::GFP* markers (data not shown). The constitutive expression of *cnd-1* caused no detectable abnormality in the ventral cord neurons and overall animal behaviors (data not shown). Therefore, we speculate that *cnd-1* may function as a modulator to coordinate the spatial expression of neuronal-fate-specific transcription factors.

DISCUSSION

cnd-1 functions as a neuronal modulator to specify motor neuron fate

In *C. elegans*, CND-1 has the most similar sequence to NeuroD. Our analysis of a *cnd-1* loss-of-function mutation suggests that *cnd-1* primarily functions to maintain neuroblast identity and to specify neuronal fates. This conclusion is based on the following observations. First, CND-1::GFP is expressed in neural precursor cells in early embryogenesis. Second, *cnd-1* mutants exhibit a reduction in the number of embryonic ventral cord motor neurons. This reduction does not result from programmed cell death because the number of ventral cord neurons in *cnd-1* mutants is unchanged when cell death is blocked. The positions of other types of cells are normal, and no extra cells appeared at inapproapriate positions. Moreover, we observed no difference in the number of LIN-26-expressing nuclei between wild-type and *cnd-1* embryos, nor did we observe ectopic LIN-26 expression in L1 ventral cord nuclei indicative of neuronal to non-neuronal fate transformations. Thus, we infer that one function of CND-1 is to maintain the mitotic competence of neuroblasts until terminal mitosis. Third, in *cnd-1* mutants, individual neurons express GABAergic- or cholinergic-specific markers seemingly at random. Some neurons show a delay in the marker's expression, or express neither marker. The fact that the expression of three neuronal fate-specific transcription factors were similarly affected in *cnd-1* mutants further supports fate misspecification of the motor neurons. However, we did not detect a clear pattern of fate transformation in ventral cord motor neurons in *cnd-1* mutants, suggesting that *cnd-1* does not act as a binary switch gene. Nor does *cnd-1* specify a particular type of motor neurons because DA, DB and DD neurons are equally affected. We did not observe a clustering of a particular neuronal type, nor was there any bias in favor of one neuronal type over another, suggesting that *cnd-1* functions as a modulator for neuronal fate specification, and a balance of inhibitory and excitatory neurons may be achieved by default. Fourth, many motor neurons exhibited defects in terminally differentiated traits including axonal morphology and synaptic connectivity, suggest that *cnd-1* may play multiple roles in neuronal fate specification and differentiation. Finally, we have shown that *cnd-1* may direct neuronal fate specification and differentiation by acting as an upstream regulator of three sub-type-specific neuronal transcription factors.

Although CND-1::GFP is expressed in many neuronal precursors during embryogenesis, loss of *cnd-1* function does not have dramatic phenotypic consequences. We do not believe the relatively moderate phenotypes observed in *cnd-1* mutants stem from residual activity because both genetic and cellular analysis suggest that *ju29* has lost most wild-type *cnd-1* activity. The mild phenotypes of *cnd-1* mutants may reflect redundant functions with additional factors.

The hypothesis that adoption of neuronal fates is coupled with cell cycle exit has been widely recognized (Edlund and Jessell, 1999). Recently, Deadpan and Asense, two neural bHLH proteins, have been shown to regulate the expression of a cyclin dependent kinase inhibitor, Dacapo, in the developing optic lobes of *Drosophila* larvae (Wallace et al., 2000). MyoD bHLH protein has been shown to regulate cell cycle progression during myogenesis. The initiation of myogenic programs of gene expression follows cell cycle exit and reflects increased levels of CDK inhibition and reduced MyoD activity. The expression of p21, a Cip/Kip class CDK inhibitor, depends in part on MyoD expression (Halevy et al., 1995), suggesting a form of feed-back inhibition coupling the level of MyoD activity with the onset of terminal mitosis and differentiation. Perhaps *cnd-1* may function in a similar manner by regulating both the mitotic competence of neuronal precursors and certain temporal aspects of ventral cord motor neuron fate determination including the onset of neurotransmitter synthesis and the developmental remodeling of synaptic connectivity patterns.

Evolutionary comparison of *cnd-1* with NeuroD and other bHLH proteins

CND-1 is a member of the NeuroD subfamily of bHLH transcription factors based on overall sequence similarity and conserved protein motifs. NeuroD has been proposed to function as a neural differentiation factor largely based on the following evidence (Lee, 1997). In *Xenopus* and mouse embryos NeuroD is primarily expressed in postmitotic neurons. Unlike early-acting bHLH neuronal determination genes, NeuroD is insensitive to lateral inhibition mediated by Notch and Delta (Chitnis, 1999). Moreover, in several CNS regions, including cortex, hippocampus, cerebellum and olfactory bulb, NeuroD expression persists into adulthood, suggesting unknown late-acting functions in terminally differentiated neurons (Lee, 1997). NeuroD loss-of-function studies in vertebrate retina, hippocampus and cerebellum have

provided in vivo support for this function (Miyata et al., 1999; Morrow et al., 1999). In addition, retinal cells from NeuroD knockout mice exhibited a three- to four-fold increase in the number of Muller glia within the developing retina, suggesting that NeuroD plays a critical role in the neuron-glial fate determination step. Based on this observation, Morrrow et al. (1999) raised the possibility that NeuroD may function in both fate determination and differentiation steps of postmitotic retinal neurons.

Our analysis has revealed similarities and differences between CND-1 and vertebrate NeuroD. By GFP reporter gene analysis, *cnd-1* is expressed in a subset of postmitotic motor neurons where it appears to regulate axonal outgrowth and synaptic connectivity. However, *cnd-1* appears to be expressed primarily in mitotically active neuronal precursors and may regulate both the mitotic competence of neuroblasts and neuronal sub-type selection such as neurotransmitter identity. In this regard, CND-1 shares some functional characteristics with vertebrate MASH1 and *Drosophila* Asense and Tap. MASH1 expression in both the CNS and PNS appears to play a dual role, coordinating generic programs of neuronal fate determination with sub-type-specific programs including neurotransmitter identity (Guillemot, 1999). Asense is detected in neural precursors and their progeny but is not expressed in proneural clusters (Brand et al., 1993; Jarman et al., 1993). Tap, although more closely related to vertebrate Neurogenins, contains 33% sequence similarity in the extended homology region common to CND-1 and NeuroD (Fig. 2). At present no *Drosophila* NeuroD homolog has been identified. CND-1 shares most sequence homology with vertebrate NeuroD proteins, however, the bHLH domain of CND-1 is also closely related to members of the vertebrate Neurogenin subfamily. The expression pattern of *cnd-1* appears to resemble features of both neurogenins and NeuroD. A hybrid kind of homology is also evident in Y69A2AR, a *C. elegans* neurogenin-like gene that shows significant sequence similarity and conservation in the genomic structures to *cnd-1*. CND-1, Y69A2AR, Asense and Tap may therefore resemble ancestral types of bHLH proteins that later diverged into two or more subfamilies that function during sequential steps or in specific subprograms of neuronal fate determination and differentiation. Neurogenins function as neuronal determination factors in neural precursors and activate NeuroD expression in mouse and *Xenopus* (Ma et al., 1996). By analogy, *cnd-1* may resemble an ancestral bHLH protein combining the functions of vertebrate Neurogenin and NeuroD.

Downstream signaling of *cnd-1*

In mouse and *Xenopus*, Neurogenins have been shown to activate NeuroD (Ma et al., 1996); however, the downstream targets of NeuroD remain unknown. We have identified *unc-30, unc-4* and *unc-3* as potential targets of CND-1, because the expression of these three genes is reduced and spatially altered in *cnd-1* mutants. Mammalian homologs of *unc-30, unc-4* and *unc-3* have been identified, and some aspects of their function appear to be conserved. The homeodomain of *unc-30* is 83% identical to those of the vertebrate Pitx family (Drouin et al., 1998), and *Pitx-2* can transcriptionally activate GAD67 (Condie, 1999). UNC-3 is a member of vertebrate O/E family, and has multiple roles in cholinergic motor neuron differentiation and sensory neuron function (Prasad et al., 1998). O/E transcription factors have been implicated in the terminal differentiation of olfactory neurons (Tsai and Reed, 1997). PHD1, a vertebrate paired homeodomain protein closely related to *C. elegans unc-4*, directly follows the expression of MASH1 and precedes the expression of terminal differentiation markers in the dorsal spinal cord (Saito et al., 1996). Several consensus E box sequences are present in the promoters of *unc-3, unc-4* and *unc-30*. However, the fact that some cells still express these three genes in *cnd-1(ju29)* mutants and that ectopic *cnd-1* expression fail to induce the expression of these three genes suggest that either CND-1 is an activator that is necessary but not sufficient for the activation of these neuronal-type-specific genes, or that *cnd-1* may not act as a direct transcriptional activator or repressor. In the latter case, CND-1 may function as a transcriptional modulator to ensure the correct spatial and temporal expression of neuronal-sub-type-specific differentiation factors. Studies of MyoD and Myf5 bHLH proteins have shown that these bHLH proteins can influence chromatin structure at the target sequences to activate myogenic-specific genes (Gerber et al., 1997). It will be of interest to see if neurogenic bHLH proteins employ similar functional strategies.

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