cnd-1/NeuroD1 Functions with the Homeobox Gene ceh-5/Vax2 and Hox Gene ceh-13/labial To Specify Aspects of RME and DD Neuron Fate in Caenorhabditis elegans

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ABSTRACT Identifying the mechanisms behind neuronal fate specification are key to understanding normal neural development in addition to neurodevelopmental disorders such as autism and schizophrenia. In vivo cell fate specification is difficult to study in vertebrates. However, the nematode Caenorhabditis elegans, with its invariant cell lineage and simple nervous system of 302 neurons, is an ideal organism to explore the earliest stages of neural development. We used a comparative transcriptome approach to examine the role of cnd-1/NeuroD1 in C. elegans nervous system development and function. This basic helix-loop-helix transcription factor is deeply conserved across phyla and plays a crucial role in cell fate specification in both the vertebrate nervous system and pancreas. We find that cnd-1 controls expression of ceh-5, a Vax2-like homeobox class transcription factor, in the RME head motoneurons and PVQ tail interneurons. We also show that cnd-1 functions redundantly with the Hox gene ceh-13/labial in defining the fate of DD1 and DD2 embryonic ventral nerve cord motoneurons. These data highlight the utility of comparative transcriptomes for identifying transcription factor targets and understanding gene regulatory networks.

Accurate control of gene expression is fundamental for the development and function of the central nervous system (CNS). Defects in CNS gene expression underlie many neurodevelopmental disorders, indicating a critical need for further study (Basu et al. 2009; Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014). Gene expression is controlled by combinations of transcription factors that work in conjunction with chromatin remodeling complexes to promote or inhibit RNA polymerase access to the genome (Clapier and Cairns 2009). According to Waddington’s model of cellular differentiation, cell fate progressively refines over multiple rounds of cell division, first to tissue-type progenitors, then exiting the cell cycle to take on a tissue-specific terminal fate (Waddington 1957). The transcription factors and chromatin remodeling complexes required for these narrowing rounds of fate specification are not well understood.

The basic Helix-Loop-Helix (bHLH) super family of proneural transcription factors includes Atonal, NeuroD, Neurogenin, and Achaete/Scute, and has broad roles in nervous system development (Baker and Brown 2018). This family of bHLH transcription factors function as either homodimers or heterodimers and bind to E-box sequences of the motif CANNTG. In Drosophila, the bHLH family acts in neural cell fate specification and neurogenesis, with different family members having roles in external sensory organ formation, chordotonal organ development, and others (Lee 1997).

Vertebrate neurogenic differentiation 1 (NeuroD1) is a bHLH transcription factor that has a role in the transcriptional activation of proneural genes (Wang and Baker 2015). In addition, NeuroD1 is expressed abundantly in the brain after terminal fate specification, which suggests a secondary role in nervous system homeostasis and/or neural maturation and survival (Miyata et al. 1999). Ectopic expression of NeuroD1 in Xenopus embryos can convert non-neural ectodermal cells into fully differentiated neurons, indicating the potential role of NeuroD1 as a neural differentiator factor (Lee et al. 1995; Lee 1997). Humans bearing homozygous NeuroD1 mutations showed severe cerebral hypoplasia and developmental delay, in addition to defects in pancreatic β-cell maturation and islet formation, demonstrating the importance of this gene in nervous system and pancreatic development (Rubio-Cabezas et al. 2010).
In the mouse, NeuroD1 is essential for the generation of granule cells in the hippocampus and the cerebellum (Miya et al. 1999; D’Amico et al. 2013). Despite extensive research on the role of NeuroD1 in cell fate specification and nervous system development (Seo et al. 2007; Pataskar et al. 2016), a comprehensive list of NeuroD1 targets has not been compiled and many questions on its role in neural development remain unanswered.

The nematode Caenorhabditis elegans, with its invariant cell lineage and well-defined nervous system, is an excellent model to study cell lineage determination and terminal fate specification (Sulston and Horvitz 1977; Sulston et al. 1983). Once a neuroblast exits the cell cycle, it needs to extend growth cones and axons through the extra-cellular matrix, find its appropriate pre- and post-synaptic partners, assemble a synapse, gap junction, or neuromuscular junction, then package the various proteins required for synaptic transmission (Chisholm et al. 2016; Jin and Qi 2018). This leads to two major questions. First, what cascade of transcription factors is required to specify interom cell fates, prior to final specification of a neuron? Second, does a single transcription factor control the fate of a single neuron, or is terminal fate specified in a combinatorial manner, with multiple transcription factors controlling different aspects of the final cell fate? Extensive work has identified a battery of transcription factors known as “terminal selectors”, which are required for terminal fate specification in C. elegans neurons (Hobert 2016 and references therein). These transcription factors generally act in a combinatorial fashion to specify cell fates, although individual transcription factors may specify the fate of multiple cells that are unrelated by cell lineage, type, or circuit. Terminal selectors typically have autoregulatory properties, in that they positively regulate their own transcription to maintain neuronal identity throughout the life of an organism. In addition, they either directly or indirectly control the expression of “terminal effector” genes, which are required for that neuron’s post-mitotic function, for instance neurotransmitter biosynthesis, packaging, and release. Despite this depth of knowledge, the “proneural” transcription factors that act up-stream of terminal selector genes are not well described.

The C. elegans bHLH transcription factor cnd-1 is orthologous to the human NeuroD1 gene and is one of the earliest proneural genes to be activated during C. elegans embryonic development (Hallam et al. 2000). However, the only reported defects seen in cnd-1 loss-of-function mutants are a relatively mild back-cooler phenotype caused by misspecification of 2-3 dorsal D (DD) motorneurons required for inhibitory GABAergic neuromuscular innervation, in addition to axon guidance and synapse remodeling defects in the remaining D neurons (Hallam et al. 2000). To gain a better understanding of CND-1’s role during C. elegans neural development, we performed an RNA-seq assay comparing embryonic wild type and cnd-1(ju29) mutant transcriptomes. We find that CND-1 positively regulates the expression of homeobox transcription factor ceh-5/Nax2 in the head RME and tail PVQ neurons. We also confirm that CND-1 is required for the generation of cnd-1 expressing cells during ventral nerve cord fate specification. Finally, we show that cnd-1 functions in parallel with the Hox gene ceh-13/Flabial to specify a subset of embryonic DD class ventral nerve cord motorneuron fates.

MATERIALS AND METHODS

Strains and maintenance

C. elegans strains were grown on nematode growth medium plates (NGM Lite) at 20° according to Brenner (1974). Bristol N2 strain was used as wild type and all analyses were conducted at 20°. The following alleles were used in this study: LGIII cnd-1(ju29), cnd-1(gk718) and ceh-13(sw1/) qC1 [dpy-19(e1259) glp-1(q339)]. Integrated transgenes used were jIs76 [unc-25p:GFP + lin-15(+)], kIs39 [sra-6p::GFP + lin-15(+)], bIs5 [unc-25p: mCherry], otIs356 [rab-3p(prom1):2xNLS::TagRFP], pkIs568 [gpa-9p::GFP + dpy-20(+)], and sIs10055 [cnd-1::his-24::mCherry + unc-119(ed3)]. Extra-chromosomal arrays used in this study were leEx2489 [ceu-5p::GFP + unc-119(+)]) and dbEx724 [f9p-6p::tax-2(CDNA);SL2::GFP + lin-15(+)]. The cnd-1(gk718) allele was identified by the C. elegans deletion mutant consortium (2012). All mutants were outcrossed at least twice prior to analysis. Table S1 shows details of strains generated during the course of this study including strain numbers and sources.

A cnd-1(gk718) ceh-13(sw1)qC1 line was built by crossing gk718/+ males into sw1/qC1, keeping lines that did not give rise to the qC1 dumpy/sterile phenotype (genotype gk718 /+ sw1), selecting for gk718 homozygous animals (uncoordinated phenotype) then screening for the embryonic lethal sw1 phenotype (parent genotype gk718 sw1/ gk718 +). Two recombinants were identified from 200 gk718 animals screened, consistent with the 1:1 map unit distance between cnd-1 and ceh-13 on LG III. These lines were rebalanced over the qC1 chromosomal inversion prior to further characterization.

RNA extraction

Embryos were isolated from gravid worms grown in liquid culture as described previously (Hudson et al. 2006). Total RNA was extracted using RiboZol (AMRESCO) and followed the vendor’s protocol except that embryos were frozen in liquid nitrogen prior to grinding. Embryonic tissue was added to 1 mL of RiboZol and 500 μL aliquoted into two 5 PRIME Phase Lock Gel Tubes. At the isopropanol stage, 20 ng/ml Glycogen was added to improve RNA pellet visualization. RNA quality control was assayed by measuring the A260/ A280 ratio using a Thermo Scientific NanoDrop and via an Agilent Bioanalyzer. All samples used for RNA-Seq had an RNA integrity number (RIN) above 9.2. Triplicate cnd-1(ju29) and N2 wild type samples were sent to the University of Kansas Genome Sequencing Core for RNA-seq library construction (Illumina TruSeq v2), barcode, pooled, and sequenced in a single lane on an Illumina HiSeq 2500 system (high output, single read 100bp sequencing).

RNA-Seq expression analysis pipeline

Gene expression abundance were obtained using previous published RNA-seq analysis workflows. Briefly, read quality was determined
Characterization of the ken2 deletion allele

2.0X Taq RED Master Mix Kit (Apex Bioresearch Products) was used to amplify the 3’ end of the ptrn-1 gene. Reverse primers at approximately 1kb intervals were used in combination with a single forward primer within the ptrn-1 coding region to amplify the ptrn-1 3’ untranslated region (UTR). Primer R9, located 10kb downstream of the F1 forward primer were the only pair which amplified cnd-1(ju29) genomic DNA, and gave a 3kb amplicon. Sanger sequencing was used to confirm the ken2 breakpoints, which corresponds to a 6980bp deletion/80bp insertion. Coordinates of the ken2 breakpoints along with insertion and flanking sequences are; LG X

AGAGATACACATGGTTGCTTTGTAGAAACCAG-TACCGCGATTTTCATCTACTCTTATTTTTTCTGTTTCTAATTGCAAGTT

CTAATTTTCGAGATTCCGGTGTTCTCGAGGT-(coelomocyte::GFP) L. F1 hermaphrodites that expressed the co-injection marker were single plated and screened to obtain stable lines.

cDNA synthesis and RT-qPCR

Total RNA from mixed staged embryos was treated with DNAase (New England Biolabs) and cleaned using the RNA Clean &amp; Concentrator kit (Zymo Research). First-strand synthesis was done using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). cdc-42 transcript levels were used to normalize for differences in input cDNA. Three or four biological samples, in triplicate, were run on a LightCycler 480 real-time PCR system and relative expression rations were calculated according to Pfaffl (2001).

Dye-filling assay

Animals were washed off plates, washed three times with M9 buffer then incubated for one hour in 100 ng/µl Vybrant DiD cell-labeling solution (Invitrogen) as described previously (Schultz and Gumienny 2012). After destaining for one hour on NGMLite plates seeded with OP50 E. coli, animals were imaged by confocal microscopy at 40x magnification for DiD uptake (647nm excitation) in the amphid neurons and GFP expression (488nm excitation).

Microscopy

Well-fed worms grown under standard conditions were used for expression pattern characterization. Images were captured on either a Zeiss LSM 700 confocal microscope, Zeiss Axiovision compound microscope, or Olympus BX61 compound microscope. Expression patterns in L1 larvae were imaged within 1 hr of hatching. For quantitative imaging of cnd-1p::his-24::mCherry expression in wild type and cnd-1 mutant backgrounds, 2-4 cell embryos were isolated from gravid hermaphrodites and mounted on a bead pad (Murray et al. 2008). After 6-7 hr, when the embryos reached comma stage, a single image stack (to eliminate possible photobleaching) was captured for each embryo using a Zeiss LSM 700 confocal microscope at 40x magnification under identical settings, ensuring no detector saturation. Images were processed using Fiji (Schindelin et al. 2012) using a Z-project - Sum Slices workflow which rendered the summated stacks as 32-bit images. Pixel values and counts for the whole image (1024 × 1024 pixels) were obtained using the Analyze - Histogram function (using the pixel value range) then processed in Microsoft Excel (sum [pixel value x pixel count]).

Individual DD neuron identities was inferred by imaging unc-25p::GFP in L1 larvae then measuring the nose-to-neuron, neuron-to-neuron, or neuron-to-tail distances along the anterior-posterior body axis using the segmented line function in Fiji. DD neuron identity in cnd-1(gk718) and ceh-13(sw1) mutant combinations were mapped to the closest relative location in wild type L1 larvae. Total ventral
nerve cord cells were determined by double-labeling with juIs76 [unc-25p::GFP] and otlIs356 [rab-3p[prom1];z2xNLS::TagRFP] then counting from DD1 or the posterior end of the terminal pharyngeal bulb (if DD1 was absent) to the anus. In wild type, this included most of the 22 DA, DB and DD class motorneurons, plus some cells in the retrovesicular ganglion and tail region.

**Neuroanatomy**

Cell identity was confirmed by crossing transgenic arrays into previously characterized strains then imaging as above. Table S1 shows the strains used for cell identification.

**Statistical analysis**

Student t-tests were performed in Microsoft Excel, Mann-Whitney tests were performed in R, and two-tailed Fisher Exact tests were performed using GraphPad QuickCalcs. Graphs were generated in Excel or SAS. RNA-seq significance values reported in the text are not adjusted for false discovery. Benjamini-Hochberg corrections for multiple comparisons are available in the supplemental data. Bonferroni corrections were applied to other data where appropriate.

**Data availability**

All reagents are available on request. Table S1 shows strains generated in this study. Tables S2 and S3 show lists of significantly down-regulated and up-regulated genes in the cnd-1(ju29) comparative transcriptome. Figure S1 shows a volcano plot summarizing the comparative transcriptome data. Figure S2 shows DEXseq hits identified in this work along with any expression validations. Figure S3 compares predicted CND-1 wild type and ju29 mutant protein sequences. Figure S4 shows cnd-1p::his-24::mCherry and unc-25p::GFP co-localization. Raw and processed transcriptome files generated in this study are publicly available via the Gene Expression Omnibus (Edgar et al. 2002), accession number GSE125051 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE125051). Supplemental material available at figshare: https://doi.org/10.25387/g3.12567827.

**RESULTS**

**cnd-1 controls the expression of multiple genes During embryogenesis**

Previous studies demonstrated that the pronuclear transcription factor cnd-1 is active early in embryogenesis, with expression first being seen at the 14-cell stage and persisting until just prior to hatching (Hallam et al. 2000; Murray et al. 2012). cnd-1 reporter gene expression decreases rapidly beyond the L1 stage but persists at low levels in adult head and ventral neurons (Kratsios et al. 2011). To gain a better understanding of how CND-1 controls gene expression during early nervous system development, we performed RNA-seq on RNA isolated from three samples each of N2 wild type and cnd-1(ju29) mutant mixed stage embryos and analyzed the data using the DSeq2 package (Love et al. 2014). cnd-1(ju29) is a G-to-A transition in the splice acceptor of intron 2 and behaves as a strong loss-of-function recessive allele (Hallam et al. 2000). cnd-1(ju29) mutant embryos show 105 genes with significantly lower transcript levels ($P < 0.05$) when compared to wild type (Table S2) and 46 genes with significant higher transcript levels (Table S3, Figure S1). Table 1 shows the top 40 most significant hits sorted by up-regulation and p-value. Surprisingly, only a single transcription factor gene, ceh-5, was identified in the down-regulated dataset, whereas three transcription factors genes (nhr-68, nhr-77, and cnd-1 itself) were found in the up-regulated dataset. This suggests that cnd-1 functions close to

the end of a transcriptional regulatory cascade during *C. elegans* embryogenesis. This is in contrast to *nhr-1*neurogenin, which controls expression of at least eight downstream transcription factors (Christensen et al. 2020).

We also analyzed our data using DEXseq, a variant of the DSeq2 workflow (Anders et al. 2017). This analysis compares data sets by aligning individual sequence blocks (exons, alternative transcriptional start sites, and alternative splice sites) and is a sensitive way to identify splice or transcriptional variants between two datasets. Using this approach, aak-2, strw-85, and ptrn-1 were found to have at least one significantly different transcript block in cnd-1(ju29) mutants when compared to wild type (Figure S2). aak-2 belongs to the AMP-activated protein kinase (AMPK) family and has roles in DAF-2-mediated insulin signaling, lifespan, and temperature-dependent dauer larva formation (Apfeld et al. 2004; Hardie 2014). In cnd-1 mutants, the first exon of an internally transcribed aak-2c variant is expressed at a significantly higher level ($P < 0.05$), suggesting that CND-1 may repress this internally transcribed variant in wild type animals (Figure S2A).

DEXseq identification of ptrn-1 transcript differences were resolved by visual inspection of the ptrn-1 genomic locus using Integrated Genome Viewer (Robinson et al. 2011; Thorvaldsdottir et al. 2013). This revealed loss of all gene transcription in the 7-8kb region immediately downstream of ptrn-1. Genomic PCR coupled with Sanger sequencing confirmed this to be a novel 690bp deletion/80bp insertion allele that removes most of the ptrn-1 3’ UTR including the polyadenylation signal, along with three downstream genes (F35B3.1, F35B3.4 and F35B3.10), of which F35B3.1 and F35B3.4 were also differentially expressed in our transcriptome (Figure S2I and Table S2). F35B3.10 codes for a predicted snRNA whose transcript was not represented in either wild type or cnd-1(ju29) datasets. ptrn-1 codes for a known neuronal microtubule stabilizing protein (Chuang et al. 2014; Marcette et al. 2014; Richardson et al. 2014), so it is possible that this ptrn-1(ken2) deletion may enhance the cnd-1(ju29) uncoordinated phenotype and be selected for during out-cross. To control for this possibility, we performed validation assays using the cnd-1(gk718) mutation, which is a large deletion allele, predicted to be a null mutant, and was shown by genomic PCR not to contain the ken2 deletion (Figure S2K).

**cnd-1 controls ceh-5 expression in a subset of neurons**

Our comparative transcriptome showed that ceh-5 was significantly down-regulated in cnd-1(ju29) mutants when compared to wild type (Table 1). ceh-5 is the *C. elegans* ortholog of the mammalian transcription factor ventral anterior homeobox 2 gene (Vax2), which is required for correct dorsoventral patterning of the eye (Take-uchi et al. 2003; Liu et al. 2008; Alfano et al. 2011). A sequence search across the ceh-5 locus found a single candidate cnd-1 CATAAT E-box binding site about 50bp 5’ to the ceh-5 translational start site (Figure 1A).

To better understand the role of cnd-1 in controlling ceh-5 expression, we used a ceh-5p::GFP reporter gene to compare expression patterns in wild type and cnd-1 mutants (Figure 1, Table 2) (Reece-Hoyes et al. 2007). In wild type L1 larvae, ceh-5p::GFP showed robust expression in head muscles, a subset of head neurons (including the RME neurons), and five or six cells in the tail including the PVQ/R neurons (Figure 1B). Weak ceh-5p::GFP expression was also seen in the coelomocytes (asterisks, Figure 1B) and the pharyngeal terminal bulb. In cnd-1(gk718) mutants, ceh-5p::GFP expression was lost from many head neurons including the RMEs, and also the tail neurons (Figure 1C), but was retained in some head muscles and also the coelomocytes. We used quantitative PCR to further validate ceh-5 transcript levels; these were significantly lower in
Table 1  Top 40 differentially expressed genes in the cnd-1(ju29) RNA-seq dataset based on P-value. (A) Down-regulated genes; (B) up-regulated genes. Gene ID: gene identity; gene name, commonly used gene name/cosmid name; base mean, mean of normalized counts with Benjamini-Hochberg adjustment for false discovery rate.

| Gene_ID         | Gene Name | Base mean | log2 fold change | P-value | P-adj  |
|-----------------|-----------|-----------|------------------|---------|--------|
| A. cnd-1(ju29) transcriptome down-regulated genes (most significant p-value) |
| WBGene00018031  | F35B3.4   | 402       | -1.7             | 6.5E-22 | 1.76E-17 |
| WBGene00005832  | srv-85    | 428       | -1.2             | 3.7E-20 | 4.96E-16  |
| WBGene00014955  | Y102ASC.6 | 158       | -1.0             | 2.6E-10 | 1.19E-06  |
| WBGene00010212  | fbxn-192  | 262       | -0.9             | 2.6E-08 | 7.00E-05  |
| WBGene00044213  | Y102ASC.36| 131       | -0.9             | 1.2E-07 | 2.96E-04  |
| WBGene00014954  | Y102ASC.5 | 66        | -0.9             | 7.1E-09 | 2.40E-05  |
| WBGene00007454  | C08F11.7  | 54        | -0.9             | 3.6E-10 | 1.38E-06  |
| WBGene00015990  | C18H2.3   | 120       | -0.9             | 1.3E-16 | 1.15E-12  |
| WBGene00014454  | MTC.7     | 1917      | -0.7             | 8.3E-06 | 0.016   |
| WBGene00010958  | ndf-4     | 70        | -0.7             | 8.4E-06 | 0.016   |
| WBGene00010209  | fbxn-191  | 31        | -0.7             | 1.1E-08 | 3.38E-05  |
| WBGene00007201  | exos-4.1  | 947       | -0.7             | 3.1E-05 | 0.056   |
| WBGene00014472  | MTC.33    | 2190      | -0.6             | 6.5E-04 | 0.93    |
| WBGene00016953  | C55C3.3   | 65        | -0.5             | 1.1E-03 | 1.00    |
| WBGene00015044  | cyp-34A9  | 82        | -0.5             | 1.0E-03 | 1.00    |
| WBGene00016506  | abhd-5.1  | 95        | -0.5             | 1.9E-03 | 1.00    |
| WBGene00006650  | ttf-1     | 1948      | -0.5             | 2.8E-04 | 0.42    |
| WBGene00000754  | col-181   | 39        | -0.5             | 3.1E-03 | 1.00    |
| WBGene00000430  | ceh-5     | 332       | -0.5             | 6.2E-03 | 1.00    |
| WBGene00014672  | C08F11.6  | 27        | -0.4             | 7.8E-04 | 1.00    |
| WBGene000077585 | T01G5.8   | 7         | -0.3             | 5.7E-05 | 0.096   |
| WBGene00020498  | Y60C6A.2  | 35        | -0.3             | 2.0E-03 | 1.00    |
| WBGene00022013  | Y60C6A.1  | 59        | -0.3             | 2.1E-03 | 1.00    |
| WBGene00020213  | hsp-12.6  | 164       | -0.2             | 4.4E-03 | 1.00    |
| WBGene00012790  | Y43D4A.4  | 6         | -0.2             | 1.4E-04 | 0.22    |
| WBGene00015549  | C08G3.3   | 24        | -0.2             | 2.9E-03 | 1.00    |
| WBGene00008396  | D106B.9   | 27        | -0.2             | 7.2E-03 | 1.00    |
| WBGene00017371  | sre-39    | 4         | -0.2             | 2.2E-03 | 1.00    |
| WBGene00020178  | T02H6.8   | 4         | -0.1             | 3.5E-03 | 1.00    |
| WBGene00045311  | Y57G11C.57| 3         | -0.1             | 5.7E-03 | 1.00    |
| WBGene00044293  | K08D12.7  | 3         | -0.1             | 6.2E-03 | 1.00    |
| WBGene00044390  | ZK177.11  | 3         | -0.1             | 7.0E-03 | 1.00    |
| WBGene00011429  | T04C12.7  | 7         | -0.1             | 7.8E-03 | 1.00    |
| B. cnd-1(ju29) transcriptome up-regulated genes (most significant p-value) |
| WBGene00018031  | lgc-34    | 1214      | 1.3              | 4.3E-16 | 2.91E-12 |
| WBGene00005832  | F35B3.3   | 41        | 0.9              | 2.37E-11| 1.28E-07 |
| WBGene00014955  | cto-3     | 66934     | 0.7              | 6.72E-06| 0.015   |
| WBGene00010212  | ZC21.10   | 882       | 0.5              | 0.003  | 1.00    |
| WBGene00044213  | cnd-1     | 958       | 0.5              | 0.004  | 1.00    |
| WBGene00008677  | col-171   | 8         | 0.1              | 0.007  | 1.00    |
| WBGene00004567  | C32E8.4   | 3         | 0.1              | 0.007  | 1.00    |

Both cnd-1(ju29) and cnd-1(gk718) RNA samples, with gk718 showing lower ceh-5 transcript levels when compared to ju29, suggesting that CND-1(ju29) may retain some function (Figure 1D). Together, these data indicate that CND-1 is necessary for ceh-5p::GFP expression in a subset of cells including the RME head and PVQ tail neurons, although it is not known if these cells are lost, changing fate, or are merely losing ceh-5 reporter gene expression.

CND-1 controls the fate of some cnd-1-expressing cells during embryonic nervous system development

The cnd-1 locus is comprised of three exons spanning a 1.5kb region of chromosome III (Figure 2A). As mentioned previously, the cnd-1(ju29) allele used in our transcriptome analysis is a G-to-A transition in the splice acceptor of intron 2 and was predicted to force a splice onto a non-canonical splice acceptor leading to a frame shift (Hallam et al. 2000). When cnd-1 RNA-seq data were viewed using Integrated Genome Viewer, we confirmed that cnd-1(ju29) transcripts showed the G-to-A transition at the ju29 base change and also a 1bp shift in the splice acceptor (Figure 2B, red column at the start of exon 3; Figure 2C; Figure S3). In addition, around 20% of transcripts show inclusion of intron 2, presumably because the ju29 mutation creates a weak splice acceptor. Figure 2B shows representative read depth across the cnd-1 locus. In terms of raw reads and when normalized to Fragments per Kilobase Million (FPKM), cnd-1 transcript levels are
almost twice as high in *cnd-1(ju29)* compared to wild type, suggesting that CND-1 may be partially responsible for regulating its own transcript levels via transcriptional repression.

To further explore these data, we performed quantitative confocal microscopy on wild type and *cnd-1* mutant comma stage embryos carrying an integrated *cnd-1p:psia-2:mCherry* transgenic reporter gene (Murray et al. 2012). The comma stage is easily identified during embryonic development and provides a defined time point to quantitatively compare mCherry expression levels. Contrary to what RNA-seq revealed, we found that *cnd-1p:*mCherry reporter gene expression was significantly lower in *cnd-1* mutants when compared to wild type (Figure 2D, *P* < 0.005, *n* = 34, 25, and 30 embryos analyzed for wild type, *cnd-1(ju29)* and *cnd-1(gk718)* mutants respectively). The reason for this discrepancy was unclear but may be assay dependent. For instance, inclusion of intron 2 within the *cnd-1(ju29)* mRNA transcript may increase its representation within the RNA-seq dataset, causing it to appear up-regulated. Alternatively, it may increase the stability of *cnd-1(ju29)* transcripts, causing an apparent up-regulation of RNA levels.

To clarify the role of *cnd-1* during nervous system development, we also analyzed *cnd-1* reporter gene expression in L1 larvae. Only three types of ventral cord motorneuron (cholinergic DA and DB, and GABAergic DD class) are born during embryogenesis and can be easily assayed in young L1 larvae using genetically encoded reporter genes. Previous data showed that *cnd-1(ju29)* mutants exhibit variable loss of all three embryonic motor neuron types (Hallam et al. 2000). We examined *cnd-1p:*mCherry expression in early L1 larvae, within an hour of hatching, counting nuclei in the retrovesicular ganglion and also the ventral nerve cord (Figure 3). The retrovesicular ganglion is a linear cluster of cells located on the ventral midline of the worm immediately posterior to the pharynx, and contains the anterior most DA, DB and DD motorneurons (DA1, DB1, DB2 and DD1), along with eight additional cells. Wild type animals showed an average of nine *cnd-1p:*mCherry nuclei in the retrovesicular ganglion and 10 in the ventral nerve cord (*n* = 26). As there are 22 motorneurons in early L1 larvae, this suggests that only a subset express the *cnd-1* reporter gene. Based on their location along the ventral nerve cord, co-labeling with *unc-25p:GFP* (a known DD neuron marker), and by corroborating against single cell RNA-seq expression data (Packer et al. 2019), we tentatively conclude that *cnd-1p:*mCherry is expressed in DA1-5, DB1, DB3, and DD1-6 (Table 3 and Figure S4). In *cnd-1(ju29)* mutants, the average number of *cnd-1* positive cells in the retrovesicular ganglion increased to 10 (*P* < 0.005), although the number of cells in the ventral nerve cord dropped dramatically to around six (*n* = 19, *P* < 0.001). In *cnd-1(gk718)* mutants, the number of *cnd-1* positive cells dropped to an average of seven in the retrovesicular ganglion and four in the ventral nerve cord (*P* < 0.0005). When comparing *cnd-1p:*mCherry expression between the *cnd-1(ju29)* and *cnd-1(gk718)* backgrounds, both retrovesicular ganglion and ventral nerve cord cell counts are significantly different (*P* < 0.0005 for retrovesicular ganglion cells, and *P* < 0.005 for ventral nerve cord cells). Based on these reporter gene studies, we conclude that CND-1 is required for the fate specification of a subset of embryonic ventral nerve cord neurons, confirming data reported by Hallam et al. (2000). In addition, the above data suggests that the *cnd-1(ju29)* protein retains some activity, or in some contexts behaves in a neomorphic manner to affect the developmental outcome of CND-1-dependent cell fates (Figure S4). For this reason, all remaining analyses were performed using the *cnd-1(gk718)* allele.

**cnd-1 and ceh-13 are co-expressed in a subset of ventral cord motorneurons**

Previous work using RNA-seq analysis of FACS-isolated *cnd-1p:*mCherry-positive cells showed that Hox gene *ceh-13* (labial transcription) were enriched in *cnd-1* expressing cells when compared to negative controls (Burwick et al. 2016). The *ceh-13* locus has multiple consensus CND-1 E-box binding sites (Figure 4A), raising the possibility that *cnd-1* may control aspects of *ceh-13* transcription. In addition, *ceh-13* mutants exhibit loss of ventral cord motorneurons similar to that seen in *cnd-1* mutants (Stefanakis et al. 2015). This led us to investigate the relationship between *cnd-1* and *ceh-13* in controlling embryonic motorneuron cell fate specification.

![Figure 1](https://example.com/figure1.png)

**Figure 1** *cnd-1* controls *ceh-5* expression in a subset of neurons. (A) Schematic of the *ceh-5* genomic region showing predicted CND-1/HHL-2 binding site (Grove et al. 2009), and structure of the *ceh-5* reporter gene used in this study. (B, C) *ceh-5p::GFP* reporter gene expression in wild type (B) and *cnd-1(gk718)* mutants (C). Filled arrows, head muscles; open arrows, PVQL/R neurons; asterisks, coelomocytes. Bracketed regions show RME plus other head neurons. Scale bar = 25 μm. (D) Box and whisker plot showing average quantitative RT-PCR levels of *ceh-5* mRNA transcript in wild type, *cnd-1(ju29)* and *cnd-1(gk718)* mutants. Open diamond, average; box shows median, first, and third quartiles. Whiskers show data extreme in 1.5 × interquartile range. Data are relative to *cdc-42* mRNA. *P* < 0.025, Student’s t-test with Bonferroni correction for multiple comparisons.

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We examined *ceh-13p::GFP* and *cnd-1p::mCherry* expression in the retrovesicular ganglion and ventral nerve cords of wild type, *cnd-1(gk718), ceh-13(sw1)/qC1* heterozygotes, and *ceh-13(sw1)* homozygous mutant L1 larvae (Figure 4B-G). *ceh-13(sw1)* is a 1.5kb deletion of that removes most of intron 1, and all of exons 2 and 3 (Figure 4A) and behaves as a recessive null allele (Brunschwig et al. 1999). *ceh-13(sw1)* homozygous animals are 97% embryonic lethal, with the remaining 3% of surviving larvae showing strong body morphology defects. We used this phenotype to identify L1 stage *sw1* homozygotes to analyze for *ceh-13p::GFP* and *cnd-1p::mCherry* expression. In wild type and *ceh-13(sw1)/qC1* heterozygotes, *cnd-1p::mCherry* and *ceh-13p::GFP* showed a complex and partially overlapping expression pattern in the retrovesicular ganglion and ventral nerve cord, with around two cells co-expressing *cnd-1* and *ceh-13* in the ganglion and an average of 4.1 (wild type) and 4.7 cells (*ceh-13/qC1*) co-expressing in the ventral nerve cord (Figure 4B-O, Tables 4 and 5). In *cnd-1(gk718)* and *ceh-13(sw1)* homozygotes, the average number of cells co-expressing each marker in the ventral nerve cord dropped significantly to 3.0 and 2.8 for *cnd-1* and *ceh-13* respectively. Both *cnd-1* and *ceh-13* homozygous mutants also showed a significant reduction in cells that expressed *cnd-1p::mCherry* only, but not *ceh-13p::GFP*. Based on the similarity in phenotypes shown, we conclude that both *cnd-1* and *ceh-13* have roles in controlling a subset of ventral nerve cord cell fates during embryogenesis. We note that the ganglion and ventral cord cell counts in this double reporter gene assay were slightly different from the data reported in Figure 3. However, the data in Figure 3 was captured on a confocal microscope whereas the data in Figure 4 was captured using epifluorescence.

**Table 2** Summary of *ceh-5p::GFP* expression in L1 larvae. (A) Average number of head neurons, head muscle bundles, and tail neurons observed in wild type and *cnd-1(gk718)* mutants respectively (+/− standard error of the mean).  **P < 0.01, Mann-Whitney U-test with continuity correction.** (B) Percentage of animals showing expression in other tissues. Note that the GFP reporter strain used in this assay was an extra-chromosomal array and showed some expression variability between animals.  **P < 0.01, Fisher Exact test**

| Strain | # head neurons | # head muscles | # tail neurons |
|--------|----------------|---------------|---------------|
| wild type (n = 11) | 12.5 (1.6) | 4.0 (0) | 2.2 (0.3) |
| *cnd-1(gk718)* (n = 5) | 4.8 (0.8)** | 2.2 (0.7)** | 0.0 (0)** |

| Strain | pharynx | coelomocytes | gut |
|--------|---------|--------------|-----|
| wild type (n = 11) | 100% | 73% | 100% |
| *cnd-1(gk718)* (n = 5) | 20%** | 60% | 100% |

**Figure 2** *cnd-1(ju29)* transcript levels are significantly up-regulated when compared to wild type, although embryonic *cnd-1p::mCherry* fluorescence is reduced in *cnd-1* mutants. (A) Schematic of the *cnd-1* genomic region showing location of predicted *CND-1*/HLH-2 binding sites, and the *stIs10055 [cnd-1p::his-24::mCherry]* reporter gene used extensively in this study. (B) Integrated Genome Viewer output of wild type and *cnd-1(ju29)* RNA-seq reads showing raw transcript depth and average Fold Per Kilobase Million coverage. Arrowhead shows location of the *ju29* mutation. (C) Inset of boxed region in (B), showing representative reads, the *ju29* G-to-A mutation, and the non-canonical 3’ splice acceptor used in the *ju29* mutant. (D) Quantitative fluorescence of *cnd-1p::his-24::mCherry* expression in comma-stage embryos in wild type, *cnd-1(ju29)* and *cnd-1(gk718)* mutant embryos. Error bars show standard error of the mean.  **P < 0.005, ***P < 0.0005, Student’s t-test with Bonferroni correction for multiple comparisons.**
which gives slightly lower resolution and may have led to an undercount of cells that were directly adjacent to or behind each other.

cnd-1 and ceh-13 function redundantly to induce DD1 and DD2 motorneuron fate

The similarity in cnd-1 and ceh-13 loss-of-function phenotypes and their effect on each other's reporter gene expression suggest that they may function together, either to cross-regulate each other or to specify ventral nerve cord motor neuron fate. To clarify this, we used an unc-25p::GFP reporter gene (Jin et al. 1999) to examine DD motorneuron fate in cnd-1(ju29) and ceh-13(sw1) single mutants, and cnd-1(gk718) ceh-13(sw1) double mutant L1 larvae (Figure 5). unc-25p::GFP is expressed in the six DD neurons (annotated in color, Figure 5A), in addition to the four RME head neurons (asterisk, Figure 5A). We also plotted the cell body location of each DD neuron relative to the nose and tail tip, to establish which cells were more sensitive to loss of cnd-1 or ceh-13. Wild type animals showed GFP expression in all six DD neurons (Figure 5B, H), in agreement with previous studies (Jin et al. 1999). However, cnd-1(gk718) mutants showed an average of 2.5 DD neurons (Figure 5C, H, n = 25, P < 0.001), with DD1 and DD2 being retained and DD3-6 being lost. L1 larvae of ceh-13(sw1)/qC1 or qC1 genotype (i.e., those with wild type morphology) showed GFP expression in all 6 DD neurons (n = 21 larvae scored). In contrast, ceh-13(sw1) homozygous animals (identified by body morphology defects) showed on average four DD neurons, with DD1 generally being present but with variable loss of DD2-6 (Figure 5E, H, n = 18, P < 0.001). cnd-1 ceh-13/qC1 balanced double mutants again showed a wild type DD neuron induction proportion.

| Table 3 | cnd-1 and unc-25 markers co-express in sub-sets of ventral cord motorneurons. N = 21 wild type animals scored. Cells are listed from left-to-right in the anterior-to-posterior order they appear in L1 larvae. *In two animals, we saw an additional cell body (tentatively identified as RIGL) between DB3 and DA2. #Three animals had cnd-1-positive cells at the DB5 location. However, we suspect these are animals where DA4 and DB5 switched position during development |

| Ventral cord neuron | DB2 | DD1 | DB1 | DA1 | DB3 | RIGL* | DA2 | DD2 | DA3 | DB4 | DA4 | DD3 |
|--------------------|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|-----|-----|
| unc-25p::GFP        | 0   | 21  | 0   | 0   | 0   | 0     | 0   | 21  | 0   | 0   | 0   | 21  |
| cnd-1p::mCherry     | 0   | 21  | 20  | 21  | 18  | 2     | 21  | 21  | 21  | 0   | 18  | 21  |

| Ventral cord neuron | DB5 | DA5 | DD4 | DB6 | DA6 | DD5 | DA7 | DB7 | DA8 | DA9 |
|--------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| unc-25p::GFP       | 0   | 0   | 21  | 0   | 0   | 21  | 0   | 0   | 21  | 0   |
| cnd-1p::mCherry    | 3*  | 21  | 21  | 0   | 21  | 0   | 21  | 0   | 21  | 0   |

Figure 3 cnd-1 controls the fate of some cnd-1-expressing cells in the retrovesicular ganglion and ventral nerve cord. (A-F) Confocal and DIC micrographs of cnd-1p::his-24::mCherry expression in (A) wild type, (C) cnd-1(ju29), and (E) cnd-1(gk718) mutants. (B, D, F) DIC images of the above. Arrowheads show cnd-1-positive nuclei in the ventral nerve cord. Bracket shows retrovesicular ganglion. Inset in (E) is contrast-stretched to highlight the two most posterior cnd-1-positive neurons. Scale bar in (F) = 25µm. (G, H) Box and whisker plots showing average number of cnd-1-positive cells in the retrovesicular ganglion and ventral nerve cord respectively. n = 26, 19, and 17 animals for wild type, cnd-1(ju29), and cnd-1(gk718) respectively. Open diamond shows the average; box shows median, first, and third quartiles; whiskers show data extremes in 1.5 × interquartile range with outliers shown beyond. ** P < 0.005; *** P < 0.0005, Mann-Whitney U-test with continuity correction and Bonferroni correction for multiple comparisons.
pattern consistent with both cnd-1 and ceh-13 displaying recessive phenotypes (Figure 5F, H). However, 12/20 cnd-1(gk718) ceh-13(sw1) homozygous double mutants showed no DD neuron fate induction, with the remaining animals showing variable induction of one or more DD neurons (Figure 5G, H, n = 20, P < 0.001). One animal with body morphology defects had six DD neurons. We speculate that this may have been a cnd-1 ceh-13/qC1 heterozygous or qC1 homozygous animal that happened to have a body morphology defect. Overall, this suggests that cnd-1 is primarily required for fate induction of DD3 through DD6, with ceh-13 playing only a minor role in this process.
In contrast, cnd-1 and ceh-13 have redundant roles in DD1 and probably DD2 fate induction, with loss of either transcription factor still allowing robust fate specification. Note that distinguishing between DD2 and DD3 neurons was difficult in some animals, so DD2 fate is likely to be under-counted in ceh-13 mutants.

**DISCUSSION**

The role of cnd-1 as a proneural transcription factor

Our comparative transcriptome data expand on cnd-1’s role as a proneural transcription factor, identifying the homeobox gene ceh-5 as a novel downstream target of cnd-1 (Figure 1). We find that cnd-1 controls ceh-5 gene expression in the RME neurons, suggesting that ceh-5 may function as a terminal selector transcription factor in this cell type. Analysis of publicly available single-cell RNA-seq data allows us to contextualize the relationship between cnd-1 and ceh-5 (Packet et al. 2019). Figure 7 shows sub-lineages of single-cell RNA-seq data visualized using the Viscello package. Onset of cnd-1 expression in the RME parent cell lineages (Figure 7A) occurs at the same time as onset of ceh-5 expression (Figure 7B). However, ceh-5p::GFP reporter gene data suggests that cnd-1 controls ceh-5 expression in the RME neurons. It may be that the single-cell RNA-seq data lacks the temporal resolution to define when one transcription factor is transcribed relative to another. Alternatively, cnd-1 and ceh-5 may function collaboratively to maintain ceh-5 expression, for instance in other head neuron and muscle cell types, where we see ceh-5p::GFP expression drop but is not eliminated in cnd-1 mutants. It should be noted that some aspects of RME neuron fate appear to be preserved in cnd-1 mutants as they continue to express unc-25p::GFP (a known RME marker gene), even when unc-25p::GFP is lost in posterior DD neurons (Figure 5 and 6). This suggests that ceh-5 may control a sub-module of RME terminal fate but not the actual fate of the neuron itself. The co-expression of cnd-1 and ceh-13 in terminal
fate cells such as the RMEs may be predictive of cnd-1’s ability to control ceh-5 expression in other neurons. Viscello data shows that these two transcription factors are co-expressed in RIVL/R, FLPL/R and PVQL/R neurons (Packer et al. 2019). Our data shows that cnd-1(gk718) mutants lose ceh-5p::GFP expression in PVQ neurons, supporting this hypothesis. Overall, our data adds to previously published work, placing cnd-1 as a proneural transcription factor upstream of ceh-5, unc-3, unc-4, and unc-47, to control aspects of RME, PVQ, DA, DB, and DD neuron fate respectively (Miller et al. 1992; Jin et al. 1994; Prasad et al. 1998; Kratsios et al. 2011).
CND-1 regulation of cnd-1-expressing cells

Our RNA-seq data shows that *cnd-1* transcription appears to be up-regulated in *cnd-1(ju29)* animals (Table 1B, Figure 2B and C). While there is evidence of intron inclusion, it does not appear to be sufficient to explain the almost twofold increase in *cnd-1(ju29)* transcript levels. We postulate that the intron inclusion may positively affect transcript stability, leading to higher levels of transcript for longer. We used two separate reporter gene assays to further explore the role of *cnd-1* in nervous system development. First, quantitative imaging of *unc-25p::his24::mCherry* in comma-stage embryos shows reduced *cnd-1* expression in the two *cnd-1* mutant alleles examined. Second, *cnd-1p::his24::mCherry* and *unc-25p::GFP* assays in ventral cord motorneurons reveals significantly lower cell counts in *cnd-1* mutants, with *cnd-1(ju29)* mutants displaying strong DD
neuron induction defects, although not as strong as those seen in cnd-1(gk718) mutants. This indicates that cnd-1 is required for the fate specification of cells that normally express cnd-1. Whether this is via a self-regulatory mechanism is not known.

**CND-1 functions redundantly with CEH-13 to specify DD1 and DD2 cell fate**

Our data corroborate previous work showing that loss-of-function in the Hox gene ceh-13/labial leads to loss of ventral cord motorneurons in a manner similar to that seen in cnd-1 mutants (Hallam et al. 2000; Stefanakis et al. 2015). While ceh-13 is not significantly different in our whole embryo RNA-seq dataset, a previous RNA-seq study, using Fluorescence-Activated Cell Sorting to enrich for our whole embryo RNA-seq dataset, a previous RNA-seq study, using the Viscello data display tool (https://cello.shinyapps.io/celegans). Co-expression of cnd-1 and ceh-5 transcripts occurs in all RME-class neurons. We find that ceh-5:p::GFP expression in RME neurons is lost in cnd-1(gk718) mutants suggesting that cnd-1 is responsible for driving ceh-5 expression in these cells. Based on the above expression overlap, we predict that cnd-1 also controls ceh-5:p::GFP expression in RIVL/R. Note that ceh-5 does not control RME neuron fate, because those cells can still be visualized using an unc-25p::GFP reporter gene. Similarly, cnd-1 and ceh-13 are co-expressed in DD3-6 motorneurons but not in DD1 and DD2. unc-25p::GFP expression in DD3-6 is primarily controlled by cnd-1, with a weak contribution from ceh-13. However, unc-25p::GFP expression in DD1 and DD2 is redundantly controlled by both cnd-1 and ceh-13. An alternative interpretation is that both cnd-1 and ceh-13 are required for successful induction of ABpl/rrpapp fates. Loss of these genes may mean that this cell division is lost, leading to a default anterior fate that permits aspects of DA2-6 to be specified normally but leads to loss of all DD neurons. (E) model summarizing cnd-1, ceh-5, and ceh-13 function in the control of RME sub-module transcription and DD neuron fate specification.

**Figure 7** Summary of cnd-1's role in controlling a ceh-5-dependent RME sub-module and DD motorneuron fate. (A - D) Single-cell RNA-seq expression lineages of (A, C) cnd-1, (B, ceh-5, and (D) ceh-13 expression. Data derived from Packer et al. (2019) and visualized using the Viscello data display tool (https://cello.shinyapps.io/celegans). Co-expression of cnd-1 and ceh-5 transcripts occurs in all RME-class neurons. We find that ceh-5:p::GFP expression in RME neurons is lost in cnd-1(gk718) mutants suggesting that cnd-1 is responsible for driving ceh-5 expression in these cells. Based on the above expression overlap, we predict that cnd-1 also controls ceh-5:p::GFP expression in RIVL/R. Note that ceh-5 does not control RME neuron fate, because those cells can still be visualized using an unc-25p::GFP reporter gene. Similarly, cnd-1 and ceh-13 are co-expressed in DD3-6 motorneurons but not in DD1 and DD2. unc-25p::GFP expression in DD3-6 is primarily controlled by cnd-1, with a weak contribution from ceh-13. However, unc-25p::GFP expression in DD1 and DD2 is redundantly controlled by both cnd-1 and ceh-13. An alternative interpretation is that both cnd-1 and ceh-13 are required for successful induction of ABpl/rrpapp fates. Loss of these genes may mean that this cell division is lost, leading to a default anterior fate that permits aspects of DA2-6 to be specified normally but leads to loss of all DD neurons. (E) model summarizing cnd-1, ceh-5, and ceh-13 function in the control of RME sub-module transcription and DD neuron fate specification.
Figure 7C and D shows the relationship between cnd-1 and ceh-13 transcript expression as identified via single-cell RNA-seq (Packer et al. 2019). Similar to the relationship between cnd-1 and ceh-5, there does not appear to be any temporal sequence in their expression. Intriguingly, this visualization shows loss of cnd-1 and ceh-13 expression in the DD1 and DD2 mother cells (ABplppappa and ABprpppappa respectively). We speculate that this renders DD1 and DD2 resistant to changes in either cnd-1 or ceh-13 expression, such that both cells are correctly specified in either single mutant background. Perhaps loss of both cnd-1 and ceh-13 promotes premature cell cycle exit in DD mother cells (as postulated by Hallam et al. 2000), preventing any DD neurons (and presumably RIGL and RIGR) from being born. While the DA2-5 mother cells express cnd-1 and ceh-13, their grandparents (ABplppappa and ABprpppappa) only express cnd-1 (and at a lower level than the posterior daughter). This raises the possibility that this anterior branch of the lineage is less sensitive to these transcription factors and may give rise to a default set of cell fates, which means that DA2-5 are born whereas DD1-6 are lost. Figure 7E summarizes our analysis on the genetic interactions between cnd-1 and ceh-13 and the sub-fate terminal selector transcript factors ceh-5 in RME and unc-30 in DD neurons respectively. unc-30 was previously reported to control the GABAergic neurotransmission module of DD and RME neurons (Eastman et al. 1999). While our RNA-seq assay does not reveal significant changes in unc-30 expression, it may have been below the threshold for statistical significance.

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