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Molecular Characterization of Two Homologs of the *Caenorhabditis elegans* Cadmium Responsive Gene *cdr-1*: *cdr-4* and *cdr-6*.

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Summary

A novel cadmium-inducible gene, *cdr-1*, was previously identified and characterized in the nematode *C. elegans* and found to mediate resistance to cadmium toxicity. Subsequently, six homologs of *cdr-1* were identified in *C. elegans*. Here we describe two homologs: *cdr-4*, which is metal-inducible, and *cdr-6*, which is non-inducible. Both *cdr-4* and *cdr-6* mRNAs contain open reading frames of 831 nt and encode predicted 32-kDa integral membrane proteins, which are similar to CDR-1. *cdr-4* expression is induced by arsenic, cadmium, mercury, and zinc exposure as well as by hypotonic stress. In contrast, *cdr-6* is constitutively expressed at a high level in *C. elegans*, and expression is not affected by these stressors. Both *cdr-4* and *cdr-6* are transcribed in post-embryonic pharyngeal and intestinal cells in *C. elegans*. In addition, *cdr-4* is transcribed in developing embryos. Like CDR-1, CDR-4 is targeted to intestinal cell lysosomes *in vivo*. Inhibition of CDR-4 and/or CDR-6 expression does not render *C. elegans* more susceptible to cadmium toxicity; however, there is a significant decrease in their lifespan in the absence of metal. Although nematodes in which CDR-4 and/or CDR-6 expression is knocked-down accumulate fluid in the pseudocoelomic space, exposure to hypertonic conditions did not significantly affect growth or reproduction in these nematodes. These results suggest that CDR expression is required for optimal viability but does not function in osmoregulation.

Introduction

The transition metal cadmium is a pervasive and persistent environmental contaminant that is ranked in the top ten on the CERCLA Hazardous Substance Priority List [1]. Exposure to cadmium is correlated with a variety of cytotoxic effects and human pathologies [2; 3; 4]. To attenuate the cytotoxic effects, this metal can be detoxified by chelation, or exported from the cell or into lysosomes [5; 6; 7; 8]. In addition, toxic by-products associated with cadmium exposure can be removed. Furthermore, metal-induced cellular damage can be repaired [9; 10; 11; 12; 13; 14].

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[1]The abbreviations used are: *cdr*, cadmium-responsive gene; eGFP, enhanced green fluorescent protein; RITC, rhodamine B isothiocyanate; RNAi, RNA interference; RPA, RNase protection assay; and URE, upstream regulatory element.

Several genomic screens have been performed to identify cadmium responsive genes and cognate metal-responsive regulatory pathways [15; 16; 17; 18; 19]. A cadmium-responsive gene, designated *cdr-1*, was identified and characterized from the nematode *Caenorhabditis elegans* (*C. elegans*) [20]. CDR-1 is a hydrophobic, 32-kDa, lysosomal, integral membrane protein. The expression of CDR-1 is limited to the intestinal cells of the nematode throughout post-embryonic development. Northern blot, DNA microarray and RPA[1] confirmed that *cdr-1* transcription is induced >50-fold in response to cadmium exposure, but the steady state level of expression is not affected following exposure to other metals or stressors [19; 20]. Inhibition of *cdr-1* expression, using RNAi or a strain in which *cdr-1* has been deleted (strain RB966; *cdr-1* (ok863)), results in increased sensitivity to cadmium exposure, clearly demonstrating a role for this gene in the defense against cadmium-induced toxicity.

BLAST [21; 22] analysis of *cdr-1* identified six homologs in *C. elegans*, designated *cdr-2*, *cdr-3*, *cdr-4*, *cdr-5*, *cdr-6* and *cdr-7* [23]. The seven homologs share a high level of both nucleotide and amino acid sequence identity. Gene expression analysis shows that *cdr-1* and *cdr-4* transcription is induced by cadmium, *cdr-6* is constitutively expressed at a high level compared to the other homologs and it is not cadmium-inducible; and *cdr-2*, *cdr-3*, *cdr-5* and *cdr-7* are expressed at low levels in both control and cadmium-treated nematodes [23]. To further characterize the *cdr* genes, *cdr-4* (cadmium-inducible) and *cdr-6* (constitutive) were examined. These genes were selected for further investigation because they had the highest levels of amino acid and DNA sequence identity with *cdr-1* (~66%), and they where the two most similar members of the CDR family. Phylogenetic analysis shows that CDR-4 and CDR-6 are most closely related, compared to the other members in the CDR family [23]. Similar to *cdr-1*, the expression of *cdr-4* and *cdr-6* predominately occurs in the intestinal cells at all post-embryonic developmental stages. However, *cdr-4* is also expressed in developing embryos and its transcription can be induced by arsenic, cadmium, mercury, zinc and hypotonic stress. In contrast, *cdr-6* transcription is not induced by any of these stressors. Based on the phenotype of fluid accumulation observed when CDR-1 expression was attenuated, it was hypothesized that members of the CDR family may function in osmoregulation [20]. Inhibition of *cdr-4* and *cdr-6* expression under hypertonic conditions did not significantly affect growth, reproduction or lifespan of *C. elegans*. These results indicate that CDR's are required for optimal viability but that they do not function in osmoregulation.

Experimental Procedures

Growth of C. elegans

The N2 Bristol strain of *C. elegans* was maintained at 20 °C on NGM agar plates seeded with *E. coli* strain OP50 as a food source [24]. The *cdr-4* knockout strain RB966 (*cdr-4(ok863)*) was obtained from the Caenorhabditis Genetics Center (University of Minnesota). To obtain large quantities of *C. elegans*, nematodes were grown in liquid S medium, collected, and stored as previously described [25]. In the experiments in which nematodes were exposed to metals, they were incubated for 24 h in media supplemented with 100 μM $NaAsO_2$, 100 μM $CdCl_2$, 18 μM $HgCl_2$, or 124 μM $ZnCl_2$ [26; 27].

RNA Isolation

Total RNA was isolated from mixed-stage populations of *C. elegans* exposed to metal, or control, non-exposed nematodes as previously described [20; 25]. In the experiment in which nematodes were exposed to osmotic stress, nematodes were grown in S medium, and then transferred to S medium in which the sodium chloride concentration was reduced from 100 mM to 10 mM [28]. Where indicated poly(A^+) RNA was isolated using Oligotex mRNA Midi Kits following the manufacturer's instructions (Qiagen).

RNase Protection Assay

cDNAs for CDR-1, CDR-4, CDR-6, and myosin light chain loading control were prepared by reverse transcriptase-PCR and then inserted into pGEM-T as previously described [23]. Sequences of the primers used in the PCR's, and characteristics of the products are described in Table 1.

To synthesize antisense RNA probes, plasmid DNA templates were linearized following digestion with either *Nco*I or *Not*I restriction enzymes. Biotin-labeled RNA probes were generated from the linearized plasmids using a MAXIscript™ *in vitro* Transcription Kit following the manufacturer's protocol (Ambion). *In vitro* transcription reactions contained SP6 RNA Polymerase when *Nco*I was used to linearize the template DNA or T7 RNA Polymerase when *Not*I was used to linearize the template DNA. Gel analysis and purification of probes were performed following the manufacturer's instructions, and the final concentration of each probe was determined from its absorbance at 260 nm.

RNase Protection Assays were performed using RPAIII™ Ribonuclease Protection Assay Kits according to the manufacturer's protocol (Ambion). In each reaction, 10 μg of total RNA from treated or control *C. elegans* was combined with 800 pg of gene specific probe and 400 pg of loading control probe. Hybridization reactions were incubated at 56 °C for 16 h, after which mixtures were incubated with RNases to degrade unhybridized RNAs. The protected fragments were resolved by 5% acrylamide/8 M urea denaturing polyacrylamide gel electrophoresis. Nucleic acids were transferred to BrightStar-Plus™ (Ambion) by electroblotting. Probes were visualized using BrightStar™ BioDetect™ Nonisotopic Detection Kit (Ambion). Steady-state levels of mRNA expression were normalized to those of the constitutively expressed myosin light chain mRNAs [29].

Preparation and Analysis of cdr-4/lacZ and cdr-6/lacZ Transgenic C. elegans

A ~3.5-kb fragment of genomic DNA that is immediately 5'of the initiator ATG in *cdr-4* was generated by PCR using cosmid K01D12 as template; the 5'-primer $^{5'}$CACTGGGCAACAACAAACGAT$^{3'}$ and the 3'-primer $^{5'}$GGACACCTCCGTCTACATTC$^{3'}$. The PCR product was purified and then cloned into pGEM-T. The genomic fragment was excised from pGEM-T following digestion with *Sph*I/*Sal*I. This fragment was then inserted into the *C. elegans β*-galactosidase expression vector pPD95.03 (AddGene) that had been digested with identical enzymes. The resulting expression vector was designated p*cdr-4/lacZ*.

A ~3.5-kb fragment of genomic DNA that is immediately upstream from the initiator ATG in *cdr-6* was also generated by PCR using cosmid K01D12 as template, the 5'-primer $^{5'}$ACAGCAACACACGATTCTGG$^{3'}$, and the 3'-primer $^{5'}$GCCCGAATCTATCACCATTTTGC$^{3'}$. The PCR product was purified and then inserted into pGEM-T. A *cdr-6* expression vector, designated p*cdr-6/lacZ* was generated by inserting the genomic fragment into pPD95.03 as described above. The p*cdr-4/lacZ* and p*cdr-6/lacZ* expression vectors express a form of *β*-galactosidase that contains the SV40 large T antigen nuclear targeting sequence, which causes the protein to accumulate in nucleus [30].

Transgenic *C. elegans* were generated following microinjection of a mixture of p*cdr-4/lacZ* or p*cdr-6/lacZ* (100 μg/ml) and a plasmid containing the dominant selectable marker gene *rol-6* (su1006) into the gonadal syncytia of young adult *C. elegans.* Transgenic *C. elegans* were selected and maintained, as described previously [25; 30].

Cell-specific, developmentally regulated patterns of *cdr-4* and *cdr-6* expression were determined by staining for *β*-galactosidase as previously described [25]. Cells that actively transcribe *cdr-4* or *cdr-6* were identified from the level of *β*-galactosidase present in the nuclei.

In Vivo Intracellular Localization of CDR-4

To determine the intracellular location of CDR-4 *in vivo*, a *C. elegans* expression vector was constructed in which the expression of a CDR-4-eGFP fusion protein is regulated by a ~3.5-kb *cdr-4* promoter. To generate this vector, full-length *cdr-4* cDNA was prepared by PCR using the 5'-primer 5'TCCCCCGGGTTTTCAAAATGGTTTG3', which introduces a *Sma*I site (underlined) into the reaction product, and the 3'-primer 5'CCGGTACCATAAT**TGA**AATTGTAAAATCGTTAGG3', which introduces a *Kpn*I site (underlined) and converts the stop codon (boldface) to a serine (TGA → TCA) in the reaction product. Following amplification, the DNA was inserted into pGEM-T. The *cdr-4* cDNA was subsequently excised following incubation with *Sma*I and *Kpn*I and then inserted into the *C. elegans* GFP expression vector pPD117.01 (AddGene), digested with the same enzymes. This product was then digested with *Sph*I and *Sma*I to remove the *mec-7* promoter fragment that is present in pPD117.01, and then a ~3.5-kb *cdr-4* promoter fragment was inserted. The resulting expression vector was designated p*cdr-4*/CDR-4-eGFP. Transgenic *C. elegans* that expressed p*cdr-4*/CDR-4-eGFP were generated as described above. The intracellular location of CDR-4-eGFP was determined by fluorescence microscopy. In the co-localization experiment in which the intestinal cell lysosomes were labeled with rhodamine, *C. elegans* were fed rhodamine-labeled RITC-dextran as previously described [20; 31]. It has been demonstrated that *C. elegans* accumulate RITC-dextran in intestinal cell lysosomes [31].

RNA Interference

The full-length cDNA fragment of *cdr-4* was generated by RT-PCR using the 5'-primer 5'ATGGTTTGTTGTTGCCCAGTG3' and the 3'-primer 5'CGTTAGGATAGATTTCTTTGCG3' and then inserted into pGEM-T. This plasmid was then digested with *Nco*I and *Pst*I to excise the *cdr-4* cDNA, which was then inserted into the double T7 RNA Polymerase vector pPD129.36 (AddGene), that had been digested with the identical restriction enzymes. The resulting plasmid was designated pCDR-4i.

The full-length cDNA fragment of CDR-6 was generated by RT-PCR using the 5' primer 5'ATGGTGTGTTGCTGTCCAG3' and the 3' primer 5'GATGGTGAAATCATTGGGG3' and then inserted into pGEM-T. CDR-6 cDNA was excised following digestion with *Nco*I and *Pst*I. The fragment was then inserted into pPD129.36 digested with the identical restriction enzymes. The final RNAi vector was designated pCDR-6i. *E. coli* strain BL21 (DE3) was transformed with the dsRNA expression vectors pCDR-4i and pCDR-6i [32; 33].

RNAi-mediated inhibition of CDR-4 or CDR-6 expression was accomplished by incubating *C. elegans* on either NGM plates supplemented with 1 mM isopropyl-1-thio-β-D-galactopyranoside seeded with non-transformed *E. coli* BL21(DE3) (control) or NGM plates supplemented with 1 mM isopropyl-1-thio-β-D-galactopyranoside and 50 μg/ml ampicillin seeded with pCDR-4i- or pCDR-6i-transformed BL21(DE3) [34]. L4 nematodes were placed onto the three types of plates and incubated at 22 °C for 40 h. Subsequently, three of the offspring were isolated, and individually cultured and allowed to lay eggs at 22 °C for 24 h before being removed [34]. Progeny (F2) were cultured at 22 °C for another 48 h on RNAi plates before lifespan and other RNAi phenotypes were characterized.

Reproduction and Growth Assays

Nematodes were transferred to the sample cup of a COPAS *BIOSORT* [35] (Union Biometrica Inc., Somerville, MA) and diluted to a concentration of ~1 nematode/μL. For the reproduction assay, five L4 nematodes were added to each well of a 96-well plate, containing K-medium, chemical (if tested), and OP50 *E. coli* in a final volume of 50 μL. Nematodes were incubated at 20°C for 48 h, and then the total number of offspring was measured. For the growth assay,

twenty-five L1 nematodes were added to each well of a 96-well plate, containing K-medium, chemical (if tested), and OP50 *E. coli* in a final volume of 50 μL. Nematodes were incubated for 72 h and then length and optical density of individual *C. elegans* were measured using the COPAS *BIOSORT ReFLx*. To assess the effects of hypotonic stress, nematodes were placed in wells containing deionized water and *E. coli*.

Lifespan assay

Lifespan studies were conducted after RNAi exposure to non-functional control, or CDR-4, CDR-6, or CDR-4 and CDR-6 combined. Four replicates of 15 L4 animals each were transferred for each of the RNAi treatments and incubated at 20°C as previously described [19]. After 48 h, adults were transferred to fresh NGM plates supplemented with sodium chloride at 0, 100, or 200 mM. The final sodium chloride concentrations in the medium are 50, 150 and 250 mM. Nematodes were gently probed with a platinum wire to determine survival and then transferred every 24 h to fresh NGM plates with corresponding RNAi and salt treatments. Lifespan curves were plotted and median lifespans were calculated using GraphPad Prism software (GraphPad Software, San Diego, CA). Because the lifespans of nematodes not grown under hypertonic conditions were affected by RNAi treatment, proportional hazards regression was used to assess the differences in survival between different RNAi treatment groups.

Results

Sequence Analysis of cdr-4 and cdr-6

The full-length cDNA sequence of CDR-4 is 944 nt, and contains an 831 nt open reading frame. An initiator codon ATG (nt 23−25) lies within the context of the consensus *C. elegans* translation start site. The 5' end of the CDR-4 mRNA is *trans*-spliced with the 22-nt SL1 spliced leader. A 3'-untranslated region of 73 nt follows the translation stop codon (nt 856−858) and terminates with a poly(A) tail with a typical polyadenylation signal (AATAAA) (Fig. 1).

The full-length cDNA sequence of CDR-6 is 916 nt and contains an 831 nt open reading frame. An initiator codon ATG (nt 2−4) lies within the context of the consensus *C. elegans* translation start site. The 5' end of the CDR-6 mRNA is also *trans*-spliced with the 22-nt SL1 spliced leader. A 3'-untranslated region of 62 nt follows the translation stop codon (nt 833−835), and terminates with a poly(A) tail with the typical polyadenylation signal (Fig. 2).

The open reading frames of CDR-4 and CDR-6 are identical in length (277 amino acids) and are predicted to encode proteins with molecular masses of 32,033 and 31,650, respectively. Pairwise comparison of the deduced amino acid sequences of CDR-4 and CDR-6 shows a high level of identity, 83.39%. CDR-4 and CDR-6 amino acid sequences also have a high level of similarity with the other five members in the CDR gene family [23].

Both CDR-4 and CDR-6 are predicted to be highly hydrophobic, integral membrane proteins with two transmembrane spanning domains [23]. PROSITE analysis [36] reveals that both CDR-4 and CDR-6 contain putative cAMP- and cGMP-dependent protein kinase phosphorylation sites, protein kinase C phosphorylation sites, casein kinase II phosphorylation sites, and tyrosine kinase phosphorylation sites. In addition, both are predicted to contain N-glycosylation and N-myristoylation sites (Table 2).

Sequence analysis of the proximal 1.0 kb of the 5'-flanking DNA of *cdr-4* and *cdr-6* identified several putative URE's that have been shown to affect the transcription of mammalian metal/stress-responsive genes: heat shock element (HSE), activation protein-1 (AP-1) binding site, and cyclic AMP regulatory element (CRE) [37; 38; 39]. These URE's have also been shown to have similar functions in *C. elegans* [40; 41; 42]. In addition, multiple GATA elements (see below) are present in the promoters of *cdr-4* and *cdr-6* (Table 3) [43; 44].

Effect of Metals on cdr-4 and cdr-6 Transcription

To determine the effects of various metals on the transcription of *cdr-4* and *cdr-6*, *C. elegans* were exposed to arsenic, cadmium, mercury or zinc. Exposure to cadmium resulted in a 3.0-fold increase in the steady state level of *cdr-4* mRNA, and caused a 1.7-fold reduction in the level of *cdr-6* mRNA. In nematodes exposed to arsenic, mercury or zinc, the expression of *cdr-4* increased 2.1-fold, 3.2-fold and 1.7-fold compared to that observed in control nematodes, respectively (Fig. 3). In contrast, the expression of *cdr-6* decreased 1.3-fold, and 1.7-fold in response to arsenic and zinc exposure, and was unaffected by exposure to mercury (Fig. 3).

Cell Specific and Developmental Expression of cdr-4 and cdr-6

The expression patterns of *cdr-4* and *cdr-6* were investigated in transgenic *C. elegans* containing *cdr-4/lacZ* or *cdr-6/lacZ* transgenes. Several independent lines of transgenic *C. elegans* containing the *cdr-4/lacZ* transgene were investigated. c*dr-4* expression was observed in all of the intestinal cells of *C. elegans* in the absence of the exposure to any stressor (Fig. 4). This result is consistent with that observed by RPA in which *cdr-4* gene was constitutively expressed in control *C. elegans* (Fig. 3). Expression was also observed in the terminal bulb and procorpus of the pharynx; likely in the pharyngeal muscle cells; pm3, pm5, pm6, and pm7. The highest level of *cdr-4* transcription was observed in pm5, pm6 and pm7. *cdr-4* transcription was observed in all post embryonic developmental stages and in developing embryos (Fig. 4). Although *cdr-4* transcription is induced by cadmium (Fig. 3), the cell-specific, developmental pattern of expression of *cdr-4* was not affected by metal exposure (data not shown).

In transgenic *C. elegans* containing p*cdr-6/lacZ*, *cdr-6* promoter activity was observed in the intestinal cells, in the absence of the exposure to any stressor, similar to *cdr-4* (Fig. 5). *Cdr-6* expression was also observed throughout the pharynx: terminal bulb, isthmus, metacarpus and procorpus. Location of the stained nuclei suggests that pharyngeal muscle cells pm3, pm4, pm5 and pm6 express *cdr-6*. The highest levels of transcription were observed in pm3, pm4, and pm5. In contrast to *cdr-4*, *cdr-6* expression was not observed in the pm7 cells. A low level of *cdr-6* transcription was observed in several body wall muscle cells, located in the head and tail regions. Unlike *cdr-4*, *cdr-6* transcription was not observed in developing embryos. Exposure to cadmium, zinc, mercury or arsenic did not affect the cellular patterns of expression of *cdr-4* or *cdr-6*. Constant with the RPA results the amount of β-galactosidase in *cdr-4* transcribing cells was greater in the metal-treated nematodes, compared to the non-exposed animals.

CDR-4 Protein Localization In Vivo

To determine the intracellular location of CDR-4, transgenic *C. elegans* were generated that express a CDR-4-eGFP fusion protein whose expression is regulated by the *cdr-4* promoter. The CDR-4-eGFP fusion protein was expressed predominately in the intestinal cells of the nematodes. It was concentrated in small punctate structures within these cells (Fig. 6). The size and distribution of these structures were similar to those of lysosomes and of CDR-1-eGFP [20; 31]. It has been shown that when a non-fusion form of eGFP is expressed in intestinal cells, it accumulates in the cytoplasm, not in vesicles (unpublished observation). To confirm that CDR-4 was targeted to the lysosomes, the transgenic nematodes were fed RITC-labeled dextran, which labels intestinal cell lysosomes [31]. In the double-labeling experiment, both eGFP and rhodamine co-localized to the same structures confirming that CDR-4 is targeted to intestinal cell lysosomes (Fig. 6).

Effect of Hypotonic Stress on cd-4 and cdr-6 expression

Since CDR-4 and CDR-6 are predicted to function in osmoregulation, the effects of hypotonic stress on the transcription of *cdr-4* and *cdr-6* were assessed. Exposure to hypotonic stress resulted in a 1.54-fold increase in the steady state level of *cdr-4* mRNA. In contrast hypotonic stress did not have any effect on the steady-state level of *cdr-6* relative to control. In addition *cdr-1* mRNAs were undetectable in both control and hypotonic stressed *C. elegans* (Fig. 8).

Functional Analysis of CDR-4 and CDR-6

The biological function of CDR-4 and CDR-6 was determined using RNAi. Inhibition of CDR-4 or CDR-6 expression resulted in the accumulation of fluid-filled droplets in the pseudocoelom and the tissues (Fig. 7). This phenotype was observed in the absence of metal exposure. Concomitant exposure to cadmium did not significantly affect the magnitude of the RNAi phenotype, nor did the simultaneous inhibition of CDR-4 and CDR-6 expression (results not shown).

Two assays were used to assess potential differences in reproduction and growth between wild-type and *cdr-4(ok863)* mutants after exposure to cadmium, hypertonic or hypotonic stress. There were not significant differences between the wild type and the *cdr-4 (ok863)* nematodes, in the absence of stress. Furthermore, both strains responded similarly to all treatments (result not shown).

To further asses the effects the relation between the *cdr* genes and osmotic stress, *C. elegans* lifespan assays were performed. Nematodes grown under isotonic conditions and CDR-4 or CDR-6 RNAi alone had a decreased lifespan compared to the control vector (log rank test; $p < 0.0001$). Furthermore, the combination of both CDR RNAi's led to an even shorter lifespan than either RNAi treatment alone (Fig. 9). For these reasons, multiple comparisons were performed using proportional hazards regression. Hypertonic conditions led to decreased median lifespans regardless of RNAi treatment. However, when the differences in lifespan without salt treatment were taken into account, nematodes with knocked down *cdr-4* and/or *cdr-6* expression did not exhibit increased sensitivity in terms of decreased lifespans compared to isosmotic conditions (Fig 9). These results suggest that the CDR's are required for optimal viability but that they may not function in osmoregulation.

DISCUSSION

A novel cadmium-inducible gene, *cdr-1*, was identified during a genomic screen for cadmium-responsive genes in *C. elegans*. *cdr-1* encodes a 32-kDa, integral membrane protein that is expressed exclusively in intestinal cells of post-embryonic nematodes following cadmium exposure. The CDR-1 protein is targeted to the intestinal cell lysosomes. The role of *cdr-1* in cadmium detoxification was demonstrated when inhibition of its expression using RNAi, or with a *cdr-1* null strain, made *C. elegans* more sensitive to cadmium toxicity. In addition, inhibition of CDR-1 expression causes the accumulation of fluid within the nematode. Therefore, CDR-1 is predicted to function in osmoregulation, possibly as a pump that can transfer ionic material across lysosomal membranes [20].

Six homologs of CDR-1 designated CDR-2, −3, −4, −5, −6 and −7 were identified and characterized [23]. Among the seven genes, *cdr-1* and *cdr-4* transcription significantly increases following exposure to cadmium; however *cdr-4* is also constitutively expressed. In contrast, *cdr-6* is constitutively expressed in control nematodes, and it is not metal-inducible. Both *cdr-4* and *cdr-6* mRNAs contain open reading frames of 831 nt, and are *trans*-spliced with the 22-nt SL1 spliced leader RNA (Figs. 1 and 2). They provide predicted ~32-kDa, integral membrane proteins with two transmembrane domains. In contrast, CDR-1 is predicted to

contain four transmembrane spanning domains and is not *trans*-spliced. Like CDR-1, both CDR-4 and CDR-6 have multiple, potential post-translational modification sites (Table 3 and [20]).

Although the primary and predicted structures of CDR-1, CDR-4, and CDR-6 are similar, there is considerable variation in the ability of different stressors to activate their transcription. Exposure to arsenic, cadmium, mercury and zinc increases the steady state level of *cdr-4* mRNA (Fig. 3). This confirms that metals are strong activators of *cdr-4* transcription. In contrast, *cdr-6* expression is not affected by metal exposure (Fig. 3). Similarly, *cdr-1* transcription increases in response to cadmium exposure, however, it is not responsive to other metals [20]. The mechanism by which metal exposures induces *cdr-1* and *cdr-4* transcription remains to be resolved. Analysis of the 1.0-kb promoter regions of *cdr-1*, *cdr-4* and *cdr-6* identified consensus sequences for several stress-responsive URE's (Table 2 and [20]). Although these genes contain similar URE's, their responsiveness to different environmental stressors is unique. Sequence comparisons among *cdr-1*, *cdr-4*, and the metal responsive genes *mtl-1* and *mtl-2* did not identify conserved DNA sequences, other that GATA elements. This suggests that multiple metal-regulatory processes may control the metal-inducible transcription in *C. elegans*. This is further supported by the observation that cadmium is the only metal capable of activating *cdr-1* transcription, whereas multiple metals are able to affect *cdr-4* transcription; similar to that observed for *mtl-1* and *mtl-2* [27]. The presence of multiple metal-responsive regulatory pathways has been observed in higher eukaryotes. For example, cadmium can induce transcription via MTF-1, NFκB, Heat shock factor, Nrf2 and by affecting MAPK signaling cascades [45; 46; 47; 48; 49]. Detailed analysis of the regulatory regions of the *cdr* genes and comparisons with other stress responsive genes will reveal the mechanisms for constitutive, metal-inducible and cell-specific transcription.

CDR-1, CDR-4 and CDR-6 have similar primary and predicted structures, cellular expression patterns, and loss-of-function phenotypes; suggesting that they have comparable or redundant biological functions. Variations in their activity may be controlled by the ability of the cognate genes to be expressed in response to environmental stimuli or in specific cell types. For example *cdr-1* transcription is induced only by cadmium, whereas *cdr-4* transcription is affected by multiple environmental stressors, and *cdr-6* is constitutively expressed at a level greater than any other member of the CDR family. Thus, the biological activity of CDR proteins may be controlled at the transcriptional level (i.e., their ability to be transcribed in response to specific biological stimuli or in unique patterns of cellular expression).

Studies using transgenic *C. elegans* confirm that both *cdr-4* and *cdr-6* are expressed in intestinal cells (Figs. 4, 5, and 6). This pattern is similar to that of *cdr-1*; other metal-responsive genes, *mtl-1* and *mtl-2* [25]; and several other non-stress responsive genes including the six vitellogenins, *vit-1* through *vit-6*, the cysteine protease *cpr-1* and the gut esterase *ges-1* [42; 50; 51]. Intestinal cell-specific transcription in *C. elegans* is regulated by the binding of the transcription factor ELT-2 to GATA elements in the promoters of these genes [42; 43; 50; 51; 52; 53; 54; 55; 56]. GATA-binding transcription factors constitute a family of structurally related proteins that are expressed in distinct developmental and tissue-specific patterns. Their involvement in the regulation of cell-specific transcription has been well established [57]. Sequence analysis of the *cdr-4* and *cdr-6* promoters identified two and four GATA elements, respectively (Table 2). Further analysis of the GATA elements present in *cdr-1*, *cdr-4* and *cdr-6* will provide new insights into the mechanisms that govern the constitutive and inducible gene expression through GATA elements and factors in *C. elegans*.

Unlike *cdr-1*, *cdr-4* and *cdr-6* are also expressed in non-intestinal cells; specifically pharyngeal muscles cell (Figs. 4 and 5). *cdr-4* and *cdr-6* have overlapping patterns of cellular expression within the pharynx: pm3 and pm6. The major difference in pharyngeal expression is that

cdr-4 is actively transcribed in the posterior region of the pharynx, in pharyngeal muscle cells pm6 and pm7, while *cdr-6* is actively transcribed in the anterior region of the pharynx in cells pm3, pm 4 and pm5. Both *cdr-4* and *cdr-6* are expressed in the terminal bulb of the pharynx. In addition to muscle, the terminal bulb contains the dorsal and ventral gland cells. These cells contain vesicles and secrete digestive enzymes into the grinder of the pumping pharynx. In addition, the cells have processes that extend into the procorpus and isthmus [58; 59]. The expression of *cdr-4* and *cdr-6* in the pharynx may be related to a biological activity, where these proteins transport material into the gland cells. The functional consequence of the distribution in of *cdr-4* and *cdr-6* expression remains to be resolved.

CDR-1 is targeted specifically to the intestinal cell lysosomes [20]. Similarly, CDR-4 is also targeted to the intestinal cell lysosomes (Fig. 6). A transgene was generated in which the expression of a CDR-6-eGFP fusion protein is regulated by ~3.5 kb of the *cdr-6* promoter. Strains of *C. elegans* containing this transgene were not viable, which suggests that over-expression of CDR-6 may severely disrupt *C. elegans* metabolism. This response may not have been observed in CDR-1-eGFP and CDR-4-eGFP expressing transgenic nematodes because the basal levels of the endogenous proteins are significantly lower than that of CDR-6 [23] (Fig. 3 and 8).

A proposed biological role for CDR-1 was determined using RNAi [20; 23]. Using identical protocols, the functions of CDR-4 and CDR-6 were investigated. Nematodes fed CDR-4 dsRNA, CDR-6 dsRNA, or a combination of CDR-4 and CDR-6 dsRNA proliferated and appeared to develop normally. This response was similar to that observed when CDR-1 expression was inhibited in *C. elegans* grown in the absence of cadmium [20; 23]. In the absence of stress, there was a significant decrease in the lifespan of *C. elegans* fed CDR-4 and/or CDR-6 dsRNA. The addition of cadmium did not significantly enhance the decrease in lifespan. This is in contrast to the effect of inhibiting *cdr-1* expression, where blocking *cdr-1* expression made the nematodes more susceptible to cadmium toxicity.

Inhibiting *cdr-4* or *cdr-6* expression causes the nematodes to accumulate fluid-filled droplets in the pseudocoelom and tissues throughout the organism (Fig. 7). This phenotype is similar to that observed when the cells of the *C. elegans* secretory-excretory system were ablated [28]. In addition, similar phenotypes have been observed in hyperactive *egl-15* signaling and in *clr-1* mutants [60]. In all cases, it is proposed that disruption of the biological activity of these genes, or cognate signaling pathways, cause fluid imbalances or disruption in *C. elegans* osmoregulation. To assess the roles of *cdr-4* and *cdr-4* in osmoregulation, *C. elegans* in which the expression of these two genes was attenuated were grown under hypertonic conditions. The lack of response to hypertonic stress suggests that these genes may not be involved in the osmoregulation (Fig. 8),

The CDR family may be part of a new class of transmembrane proteins. Orthologs of the *C. elegans cdr* genes have been identified in other *Caenorhabditi* species: *C. briggsae* and *C. remanei*. Partial orthologs have also been tentatively identified in other invertebrates [61]. To date, orthologs have not been identified in mammals. Further analysis will determine if CDR proteins are unique to lower organisms, or if they have evolved into more complex proteins, which are present in higher organisms.

Footnotes

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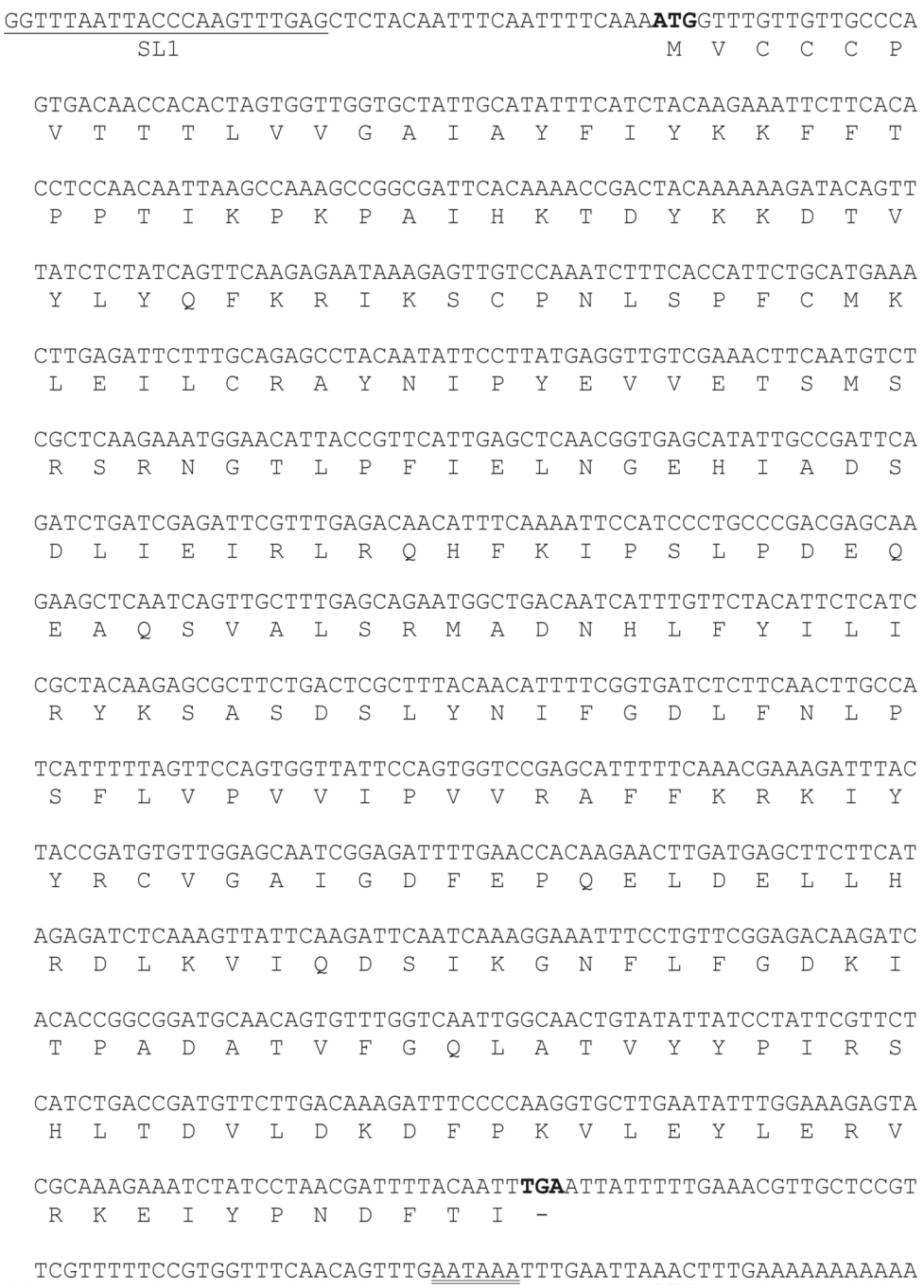

Figure 1. Sequences of *cdr-4* cDNA and protein

The nucleotide sequence of the *cdr-4* cDNA is shown with the deduced amino acid sequence presented below the corresponding codons. The SL1 sequence is *underlined*, and the translation start and stop codons are presented in **bold**. The polyadenylation signal is identified with a *double-underline*.

```
                    GGTTTAATTACCCAAGTTTGAGAATGGTGTGTTGCTGTCCAGTGACA
                              SL1            M  V  C  C  C  P  V  T

ACAACCCTAGTGGTTGGTGCAATCGCTTTTTTCATTTACAAGAAATTCTTCACGCCACCA
 T  T  L  V  V  G  A  I  A  F  F  I  Y  K  K  F  F  T  P  P

ACAATTAAACCAAAGCCGGCGATTCACAAAACCGACTACAAGAAAGATACTGTTTATCTT
 T  I  K  P  K  P  A  I  H  K  T  D  Y  K  K  D  T  V  Y  L

TACCAATTCAAAAGACTGAAAAACTGCCCAAATCTTTCACCATTCTGCATGAAAATTGAG
 Y  Q  F  K  R  L  K  N  C  P  N  L  S  P  F  C  M  K  I  E

ATTCTTTGCAGAGTCTACAATATTCCTTATGAGATTGTGGAGAGTTCAATGGCTCGATCG
 I  L  C  R  V  Y  N  I  P  Y  E  I  V  E  S  S  M  A  R  S

AGAAATGGAACTTTACCTTTTATCGAGCTCAATGGGGAACACATTGCAGACTCTGATCTC
 R  N  G  T  L  P  F  I  E  L  N  G  E  H  I  A  D  S  D  L

ATTGAGATTCGCCTGAGACAACATTTTAAGATTCCAAGCCTCCCAACCGAACAAGAAGCG
 I  E  I  R  L  R  Q  H  F  K  I  P  S  L  P  T  E  Q  E  A

CAGTCAGTTGCTTTGAGCAGAATGGCAGATAATCATCTTTTCTATGTACTTCTCCGCTAC
 Q  S  V  A  L  S  R  M  A  D  N  H  L  F  Y  V  L  L  R  Y

AAGAGCTCAGTTGACATGTTTTACGAGATCATTGTCGGGCTTCTCGGTTTACCATCAGCA
 K  S  S  V  D  M  F  Y  E  I  I  V  G  L  L  G  L  P  S  A

TTCAACGCCGTATTGGTTCCTCTGGTCAAAGCTGTGTTTGGAAGCAAAGTTTATAGTCGT
 F  N  A  V  L  V  P  L  V  K  A  V  F  G  S  K  V  Y  S  R

TGTGTTGGGGCAATTGGAGATTTTGAGCCACATGAGCTTGATGAGCTTCTTCATAGAGAT
 C  V  G  A  I  G  D  F  E  P  H  E  L  D  E  L  L  H  R  D

CTCAAAGTAATTCAAGACTCTATCAAAGGGAAATTCCTTTTCGGAGACAAGATCACACCG
 L  K  V  I  Q  D  S  I  K  G  K  F  L  F  G  D  K  I  T  P

ACTGATGCAACCGTATTTGGACAATTGGCATCCGTGTATTACCCACTTCGTTCGCATATC
 T  D  A  T  V  F  G  Q  L  A  S  V  Y  Y  P  L  R  S  H  I

AACGACGTGCTGGAAAAGGATTTCCCAAAAATTCTTGAGTACTGCGAAAGTGTTCGCAAG
 N  D  V  L  E  K  D  F  P  K  I  L  E  Y  C  E  S  V  R  K

GAAGTGTACCCCAATGATTTCACCATCTAACTTCAATTTTTAGTTAATTTTTCTTGTCTT
 E  V  Y  P  N  D  F  T  I  -

ACCTTTTCTTTAGCCAAATAAAAGTGTCTATAAAAAAAAAAAAAAAAAAAA
```

Figure 2. Sequences of *cdr-6* cDNA and protein

The nucleotide sequence of the *cdr-6* cDNA is shown with the deduced amino acid sequence presented below the corresponding codons. The SL1 sequence is *underlined*, and the translation start and stop codons are presented in **bold**. The polyadenylation signal is identified with a *double-underline*.

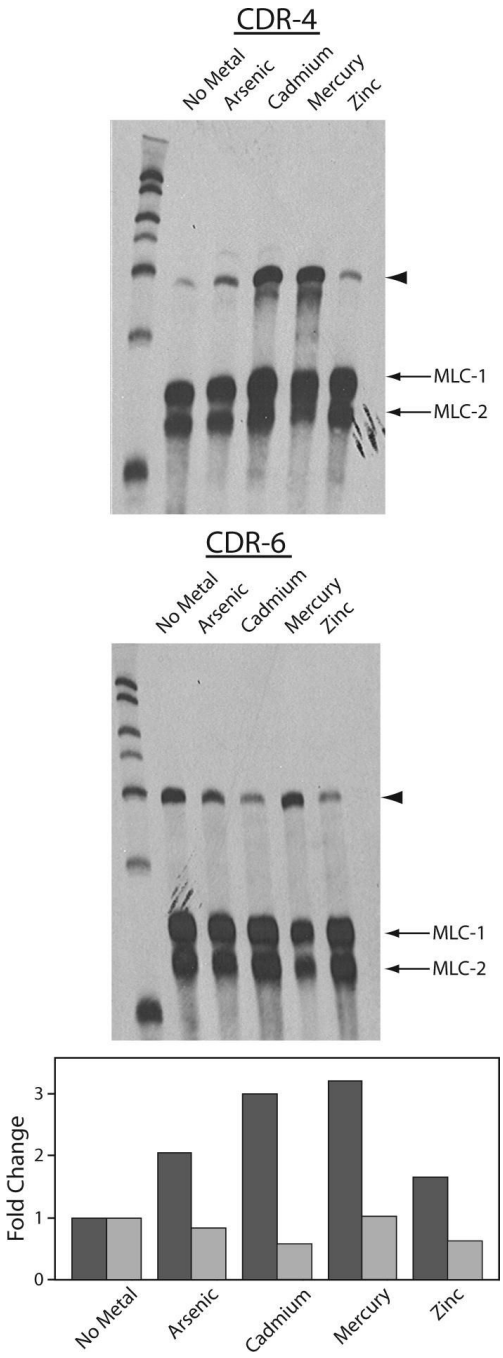

Figure 3. Effects of metals on *cdr-4* and *cdr-6* transcription

Total RNA was prepared from control, non-treated *C. elegans* and *C. elegans* exposed to 100 μM $NaAsO_2$, 100 μM $CdCl_2$, 18 μM $HgCl_2$, or 124 μM $ZnCl_2$ for 24 h. 10 μg of total RNA, 800 pg of a *cdr-4* (*upper*) or *cdr-6* (*middle*) mRNA specific probe, and 400 pg of myosin light chain 1 and 2 (MLC-1 and MLC-2), were hybridized in each reaction. The *arrowhead* indicates the position of the CDR target. The *lower panel* presents the fold change, relative to untreated controls, of *cdr-4* (■) and *cdr-6* (▨) mRNAs in response to metal treatment. The *fold change* was normalized to the level of myosin light chain mRNA.

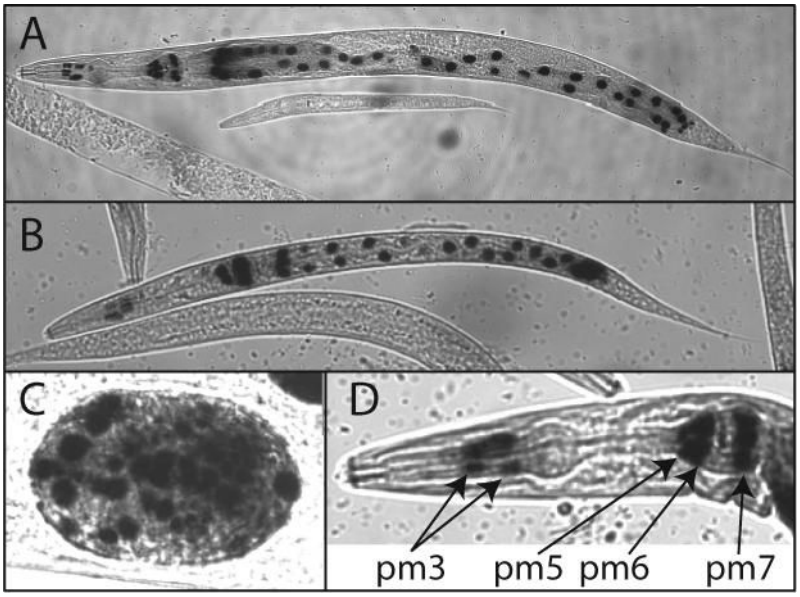

Figure 4. Cell-specific and developmental expression of *cdr-4*

Transgenic *C. elegans* containing pcdr-4/lacZ were stained for *β*-galactosidase activity [25]. The typical expression pattern of *cdr-4* in an adult (*panel A*), L1 larva (*panel B*), and embryo (*panel C*) are shown. An enlarged view of an adult *C. elegans* pharynx with pharyngeal muscle nuclei identified (*panel D*).

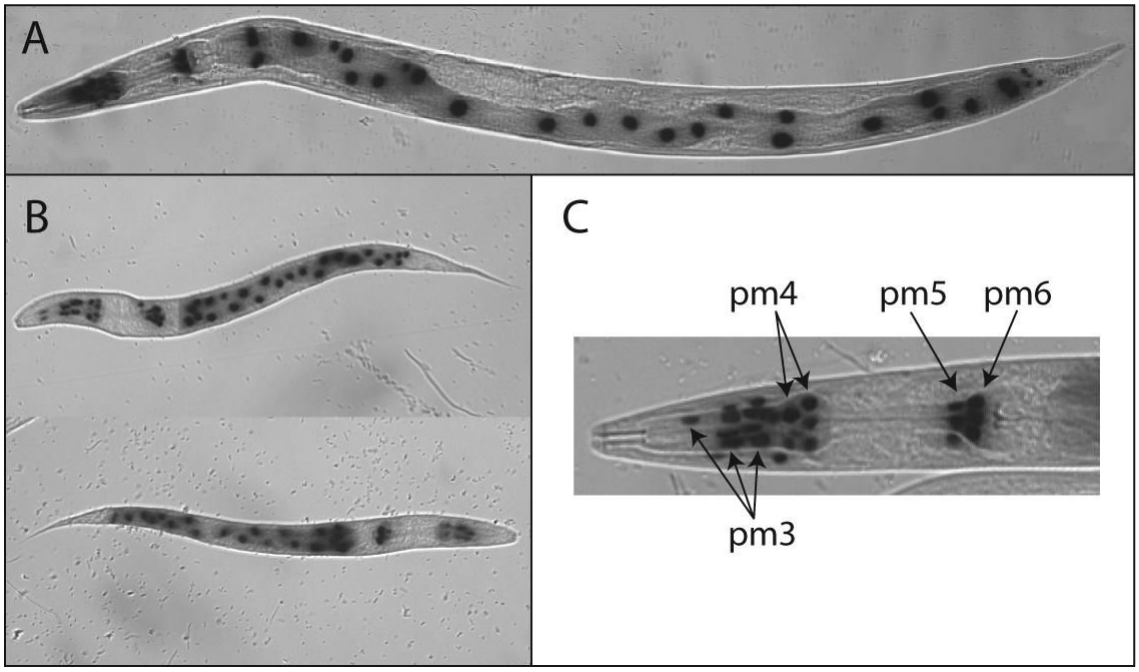

Figure 5. Cell-specific and developmental expression of *cdr-6*

Transgenic *C. elegans* containing pcdr-6/lacZ were stained for *β*-galactosidase activity as previously described [25]. The typical expression pattern of *cdr-6* in an adult *C. elegans* (*panel A*), two, L1 larva (*panel B*), and an adult pharynx with the pharyngeal muscle cell nuclei identified (*panel C*).

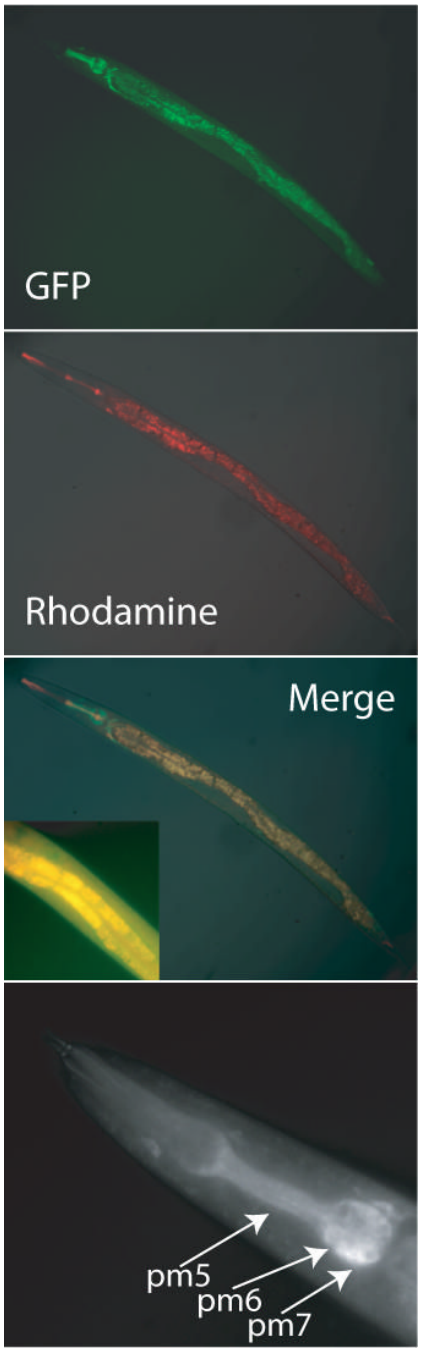

Figure 6. Intracellular location of CDR-4 *in vivo*

Transgenic *C. elegans* that contain a *cdr-4*/CDR-4-eGFP expression vector, were fed RITC-dextran. The location of CDR-4-eGFP (*GFP*) and RITC-labeled lysosomes (*rhodamine*) were visualized using the appropriate filter sets. A false-color, composite image (*merge*) is presented that demonstrates the localization of CDR-4 in lysosomes. A higher magnified (400×) image of the identical transgenic *C. elegans* is presented in the *insert*. Locations where the eGFP and rhodamine signals are coincident can be identified by the yellow color. The lower panel indicates the location of CDR-4-eGFP within the pharynx of an adult *C. elegans*. The location of pharyngeal muscles cells; pm5, pm6, and pm 7; are indicated. Pm5 cells are columnar cells,

which extend from the anterior region of the terminal bulb to the posterior of the metacorpus [62].

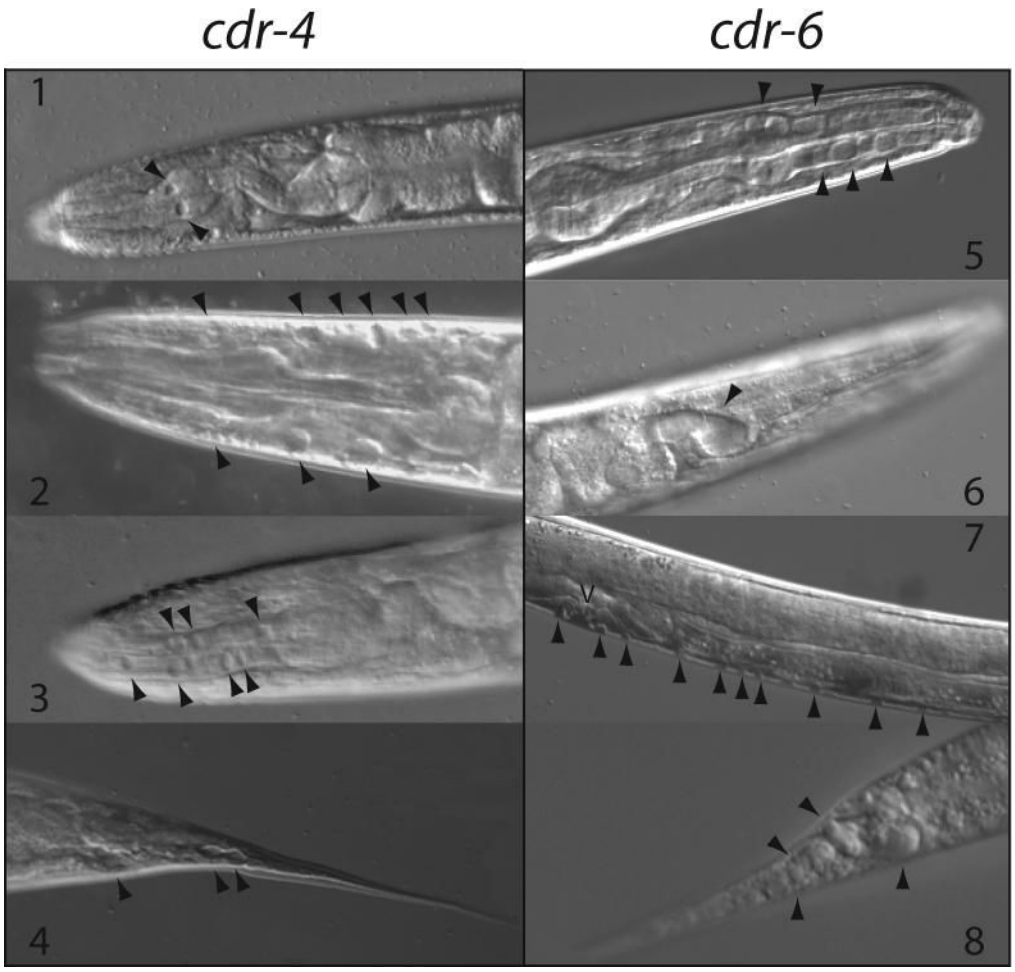

Figure 7. Phenotype associated with RNAi of CDR-4 and CDR-6

Wild type *C. elegans* were grown on NGM plates containing 1 mM isopropyl-1-thio-β-D-galactopyranoside and 50 μg/ml ampicillin, seeded with BL21(DE3) containing pCDR-4i (*left*) or pCDR-6i (*right*). *Panels 1*, *3* and *5*, adult pharynx with vesicles in the pharyngeal muscle cells; *Panels 2* and *6*, adult pharynx with vesicle in the pseudocoelomic space between the pharynx and hypodermis; *Panel 7*, *C. elegans* larva with vesicles along the body wall in the pseudocoelomic space between the hypodermis and intestine; *Panels 4* and *8*, adult tail containing fluid filled vesicles. *Arrowheads* indicate the position of vesicles.

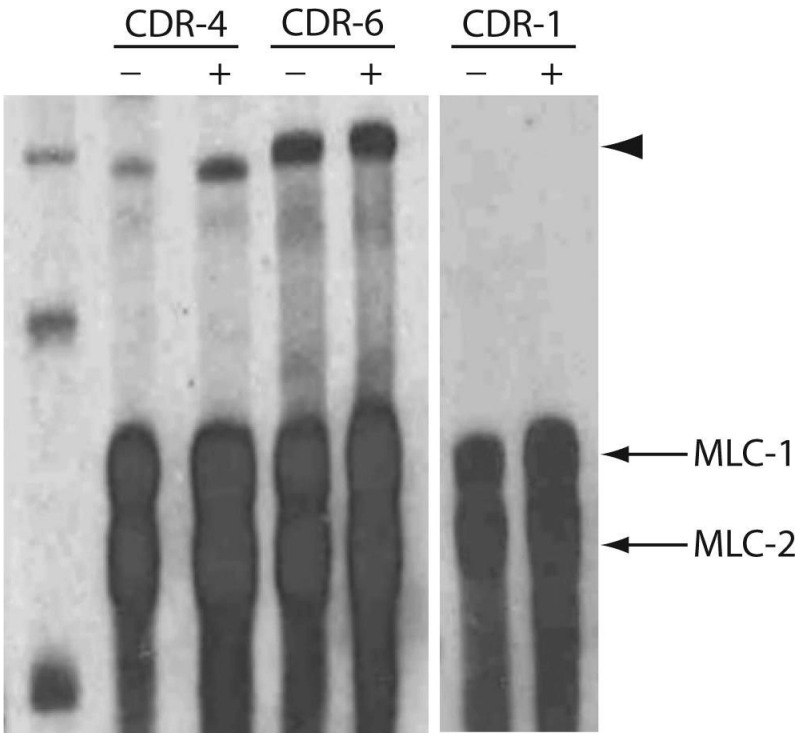

Figure 8. Effects of hypotonic stress on *cdr* transcription

Total RNA was prepared from non-treated *C. elegans* (−) or *C. elegans* exposed to a hypotonic stress for 24 h (+). 10 μg of total RNA, 800 pg of *cdr-1, cdr-4* or *cdr-6* mRNA specific probe, and 400 pg of myosin light chains 1 and 2 (MLC-1 and MLC-2) probe were hybridized in each reaction. The *arrowhead* indicates the position of the protected product.

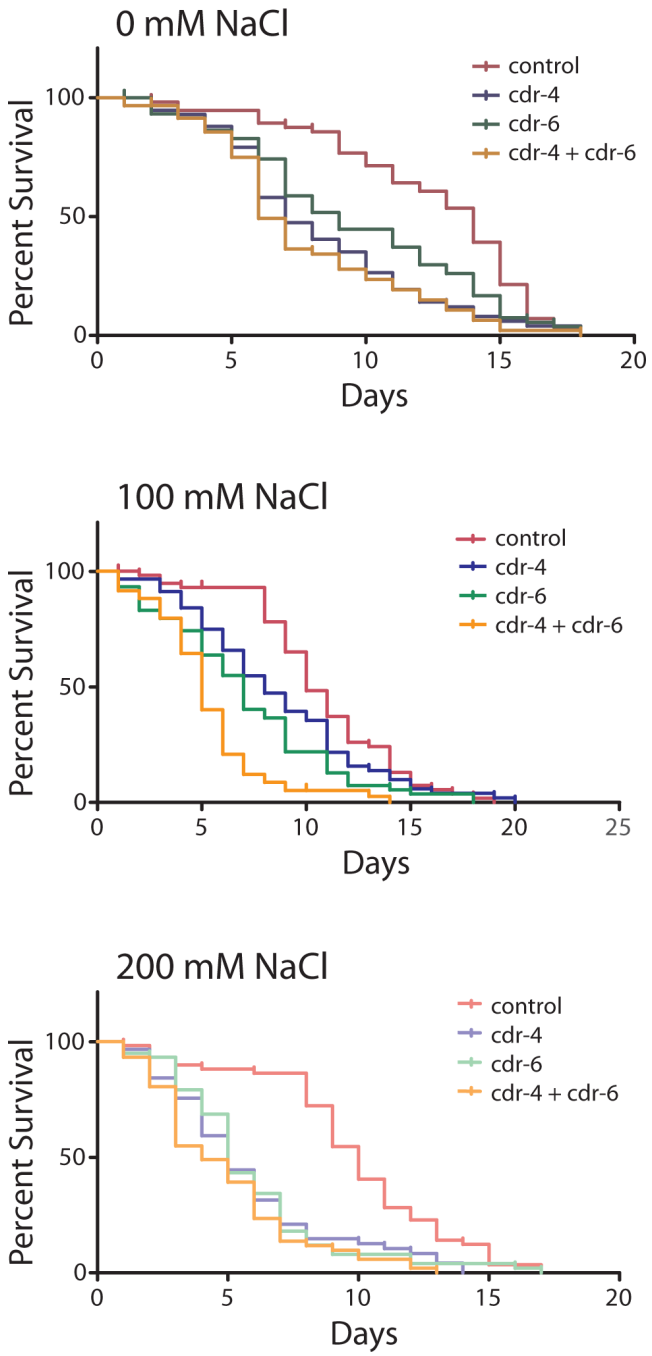

Figure 9. Effect of osmotic stress on *C. elegans* lifespan

Wild type *C. elegans* were grown on NGM plates containing 0, 100, or 200 mM added sodium chloride. Plates contained bacteria that expressed, control, CDR-4, CDR-6 or a mixture of CDR-4 and CDR-6 dsRNA. Percent survival was calculated as indicated.

TABLE 1

Probes used in RNase Protection Assay

Probe Name	PCR Primer Sequence (5′→3′)		Probe Length (bp)
rpa-cdr-1	5′	TAAAATTCCGTCGCTTCC	271
	3′	TCTGTGAAGAATCTCGTCGAGC	
rpa-cdr-4	5′	GTGATCTCTTCAACTTGC	275
	3′	AACATCGGTCAGATGAGAACG	
rpa-cdr-6	5′	TCAACGCCGTATTGGTTCCTC	278
	3′	CTCAAGAATTTTTGGGAAATCC	
rpa-mlc	5′	TTGACAGGAACTGACCCAGAGG	149
	3′	ATAGCCTTGACCTCATCCTCG	

TABLE 2

Predicted protein motifs

Motif	Residues	
	CDR-4	CDR-6
cAMP- and cGMP-dependent protein kinase phosphorylation	KKDT (42−45)	KKDT (42−45)
Protein kinase C phosphorylation	TIK (29−31)	TIK (29−31)
	SIK (215−217)	SIK (215−217)
		SVR (265−267)
Casein kinase II phosphorylation	SLPD (121−124)	TEQE (124−127)
	SASD (150−153)	SSVD (150−153)
	TPAD (227−230)	TPTD (227−230)
Tyrosine kinase phosphorylation	KSASDSLY (149−156)	KSSVDMFY (149−156)
N-glycosylation	NGTL (90−93)	NGTL (90−93)
N-myristoylation	GQLATV (235−240)	GLPSAF (164−169)
		GQLASV (235−240)

TABLE 3

Putative upstream regulatory elements

Element	Consensus sequence	Sequence (location)[a]	
		cdr-4	*cdr-6*
Heat shock	NGAANNTTCNNGAAN		TGGAAATTCTCGAAG (−726 to −712)
Antioxidant Response	TGA(C/G)TCA	TGAGTAA (−153 to −147)	TGGCTCA (−340 to −334)
		TAACTCA (−378 to −372)	TTACTCA (−865 to −859)
GATA	(A/T)GATA(A/G)	TGATAT (−288 to −283)	AGATAG (−268 to −263)
		CGATAG (−903 to −898)	TGATAA (−438 to −433)
			TGATAA (−899 to −894)
			TGATAT (−929 to −924)
cAMP Response	TGACGTCA	TCACTTCA (−52 to −45)	TAACGTCT (−83 to −76)
		TGTAGTCA (−192 to −185)	TTTCGTCA (−115 to −108)
		TTCCGTCA (−539 to −532)	TGAAGTGA (−472 to −465)
			TGTCATCA (−886 to −879)

[a] Positions are relative to the translation start site.