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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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A novel cadmium-inducible gene, *cdr-1*, was previously identified and characterized in the nematode *Caenorhabditis 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 noninducible. 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 postembryonic 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.

Keywords: *C. elegans*; cadmium; transition metal; RNA interference; transcription

Introduction

The transition metal cadmium is a pervasive and persistent environmental contaminant that is ranked in the top 10 on the Comprehensive Environmental Response, Compensation, and Liability Act Hazardous Substance Priority List.[1] Exposure to cadmium is correlated with a variety of cytotoxic effects and human pathologies.[2–4] This metal can be detoxified by chelation or exported from the cell or into lysosomes to attenuate the cytotoxic effects.[5–8] In addition, toxic by-products associated with cadmium exposure can be removed. Furthermore, metal-induced cellular damage can be repaired.[9–14]

Several genomic screens have been performed to identify cadmium-responsive genes and cognate metal-responsive regulatory pathways.[15–19] A cadmium-responsive gene, designated *cdr-1*, was identified and characterized from the nematode *Caenorhabditis 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 postembryonic development. Northern blot, DNA microarray, and RNase protection assay (RPA) 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*

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Abbreviations used: *cdr*, cadmium-responsive gene; eGFP, enhanced green fluorescent protein; RITC, rhodamine B isothiocyanate; RNAi, RNA interference; RPA, RNase protection assay; URE, upstream regulatory element; dsRNA, double-stranded RNA.

expression, using RNA interference (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, that *cdr-6* is constitutively expressed at a high level compared to the other homologs and is not cadmium inducible, and that *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, we examined *cdr-4* (cadmium inducible) and *cdr-6* (constitutive). 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 were 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 postembryonic developmental stages. However, *cdr-4* is also expressed in developing embryos and its transcription can be induced by arsenic, cadmium, mercury, and zinc and by 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 CDRs are required for optimal viability but that they do not function in osmoregulation.

```
GGTTTAATTACCCAAGTTTGAGCTCTACAATTTCAATTTTCAAAATGGTTTGTTGTTGCCCA
        SL1                                    M  V  C  C  C  P

  GTGACAACCACACTAGTGGTTGGTGCTATTGCATATTTCATCTACAAGAAATTCTTCACA
   V  T  T  T  L  V  V  G  A  I  A  Y  F  I  Y  K  K  F  F  T

  CCTCCAACAATTAAGCCAAAGCCGGCGATTCACAAAACCGACTACAAAAAAGATACAGTT
   P  P  T  I  K  P  K  P  A  I  H  K  T  D  Y  K  K  D  T  V

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

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

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

  GATCTGATCGAGATTCGTTTGAGACAACATTTCAAAATTCCATCCCTGCCCGACGAGCAA
   D  L  I  E  I  R  L  R  Q  H  F  K  I  P  S  L  P  D  E  Q

  GAAGCTCAATCAGTTGCTTTGAGCAGAATGGCTGACAATCATTTGTTCTACATTCTCATC
   E  A  Q  S  V  A  L  S  R  M  A  D  N  H  L  F  Y  I  L  I

  CGCTACAAGAGCGCTTCTGACTCGCTTTACAACATTTTCGGTGATCTCTTCAACTTGCCA
   R  Y  K  S  A  S  D  S  L  Y  N  I  F  G  D  L  F  N  L  P

  TCATTTTTAGTTCCAGTGGTTATTCCAGTGGTCCGAGCATTTTTCAAACGAAAGATTTAC
   S  F  L  V  P  V  V  I  P  V  V  R  A  F  F  K  R  K  I  Y

  TACCGATGTGTTGGAGCAATCGGAGATTTTGAACCACAAGAACTTGATGAGCTTCTTCAT
   Y  R  C  V  G  A  I  G  D  F  E  P  Q  E  L  D  E  L  L  H

  AGAGATCTCAAAGTTATTCAAGATTCAATCAAAGGAAATTTCCTGTTCGGAGACAAGATC
   R  D  L  K  V  I  Q  D  S  I  K  G  N  F  L  F  G  D  K  I

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

  CATCTGACCGATGTTCTTGACAAAGATTTCCCCAAGGTGCTTGAATATTTGGAAAGAGTA
   H  L  T  D  V  L  D  K  D  F  P  K  V  L  E  Y  L  E  R  V

  CGCAAAGAAATCTATCCTAACGATTTTACAATTTGAATTATTTTTGAAACGTTGCTCCGT
   R  K  E  I  Y  P  N  D  F  T  I  -

  TCGTTTTTCCGTGGTTTCAACAGTTTGAATAAATTTGAATTAAACTTTGAAAAAAAAAAA
```

Fig. 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 boldface. The polyadenylation signal is identified with a double underline.

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 (nucleotides 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 (nucleotides 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 (nucleotides 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 (nucleotides 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[24] 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 1).

```
                  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
```

Fig. 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 boldface. The polyadenylation signal is identified with a double underline.

Table 1. 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) SIK (215–217)	TIK (29–31) SIK (215–217) SVR (265–267)
Casein kinase II phosphorylation	SLPD (121–124) SASD (150–153) TPAD (227–230)	TEQE (124–127) SSVD (150–153) 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)

Sequence analysis of the proximal 1.0 kb of the 5′-flanking DNA of *cdr-4* and *cdr-6* identified several putative upstream regulatory elements (UREs) that have been shown to affect the transcription of mammalian metal/stress-responsive genes: heat shock element, activation protein-1 binding site, and cAMP regulatory element.[25–27] These UREs have also been shown to have similar functions in *C. elegans*.[28–30] In addition, multiple GATA elements (see below) are present in the promoters of *cdr-4* and *cdr-6* (Table 2).[31,32]

Effect of metals on *cdr-4* and *cdr-6* transcription

C. elegans were exposed to arsenic, cadmium, mercury, or zinc to determine the effects of various metals on the transcription of *cdr-4* and *cdr-6*. 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, respectively, 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. *cdr-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 the *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 following 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 postembryonic 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-

Table 2. Putative UREs

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) TAACTCA (−378 to −372)	TGGCTCA (−340 to −334) TTACTCA (−865 to −859)
GATA	(A/T)GATA(A/G)	TGATAT (−288 to −283) CGATAG (−903 to −898)	AGATAG (−268 to −263) TGATAA (−438 to −433) TGATAA (−899 to −894) TGATAT (−929 to −924)
cAMP response	TGACGTCA	TCACTTCA (−52 to −45) TGTAGTCA (−192 to −185) TTCCGTCA (−539 to −532)	TAACGTCT (−83 to −76) TTTCGTCA (−115 to −108) TGAAGTGA (−472 to −465) TGTCATCA (−886 to −879)

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

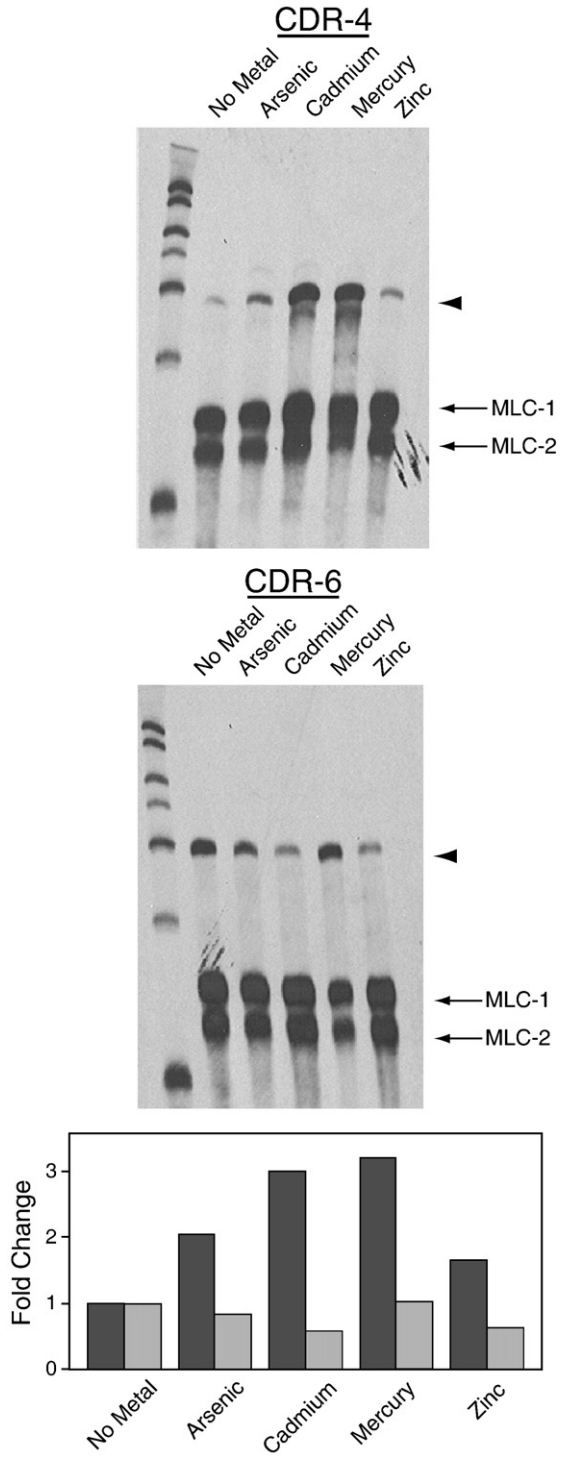

Fig. 3. Effects of metals on *cdr-4* and *cdr-6* transcription. Total RNA was prepared from control, nontreated *C. elegans* and *C. elegans* exposed to 100 μM $NaAsO_2$, 100 μM $CdCl_2$, 18 μM $HgCl_2$, or 124 μM $ZnCl_2$ for 24 h. Ten micrograms of total RNA, 800 pg of a *cdr-4* (upper panel) or *cdr-6* (middle panel) 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.

galactosidase in *cdr-4*-transcribing cells was greater in the metal-treated nematodes, compared to the nonexposed animals.

CDR-4 protein localization *in vivo*

Transgenic *C. elegans* that express a CDR-4–enhanced green fluorescent protein (eGFP) fusion protein, whose expression is regulated by the *cdr-4* promoter, were generated to determine the intracellular location of CDR-4. 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.[20,35] It has been shown that when a nonfusion form of eGFP is expressed in intestinal cells, it accumulates in the cytoplasm, not in vesicles (unpublished observation). The transgenic nematodes were fed rhodamine B isothiocyanate (RITC)-labeled dextran, which labels intestinal cell lysosomes, to confirm that CDR-4 was targeted to the lysosomes.[35] In the double-labeling experiment, both eGFP and rhodamine colocalized to the same structures, confirming that CDR-4 is targeted to intestinal cell lysosomes (Fig. 6).

Effect of hypotonic stress on *cdr-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. 7).

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. 8). This phenotype was observed in the absence of metal exposure. Concomitant exposure to cadmium or the simultaneous inhibition of CDR-4 and CDR-6 expression did not significantly affect the magnitude of the RNAi phenotype (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 or hypertonic or hypotonic stress. There were no 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).

C. elegans lifespan assays were performed to further assess the effects of the relation between

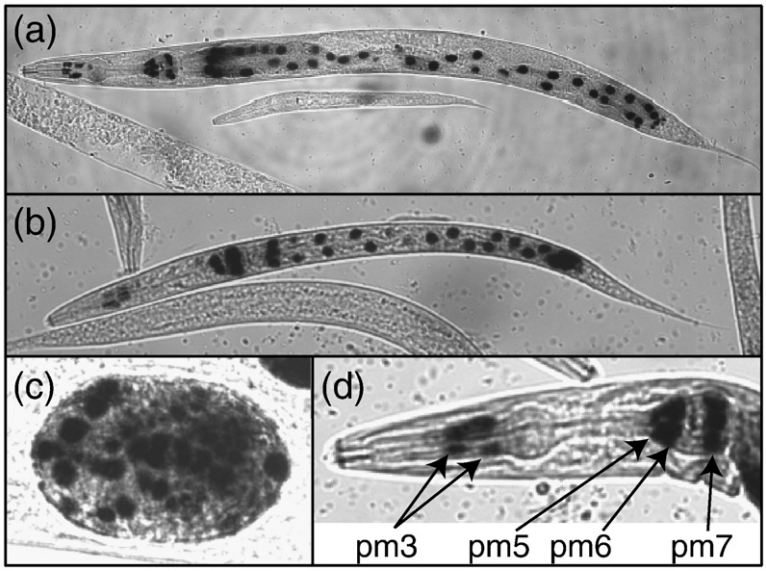

Fig. 4. Cell-specific and developmental expression of *cdr-4*. Transgenic *C. elegans* containing p*cdr-4/lacZ* were stained for β-galactosidase activity.[33] The typical expression pattern of *cdr-4* in an adult (a), L1 larva (b), and embryo (c) is shown. An enlarged view of an adult *C. elegans* pharynx with pharyngeal muscle nuclei identified (d).

the *cdr* genes and osmotic stress. 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 CDRs 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 postembryonic 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]

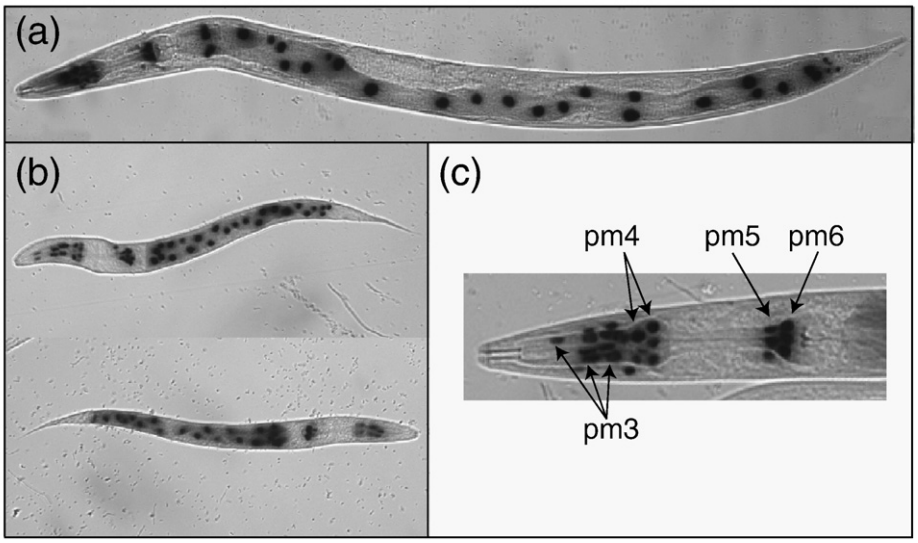

Fig. 5. Cell-specific and developmental expression of *cdr-6*. Transgenic *C. elegans* containing p*cdr-6/lacZ* were stained for β-galactosidase activity as previously described.[33] The typical expression pattern of *cdr-6* in an adult *C. elegans* (a), two L1 larvae (b), and an adult pharynx with the pharyngeal muscle cell nuclei identified (c).

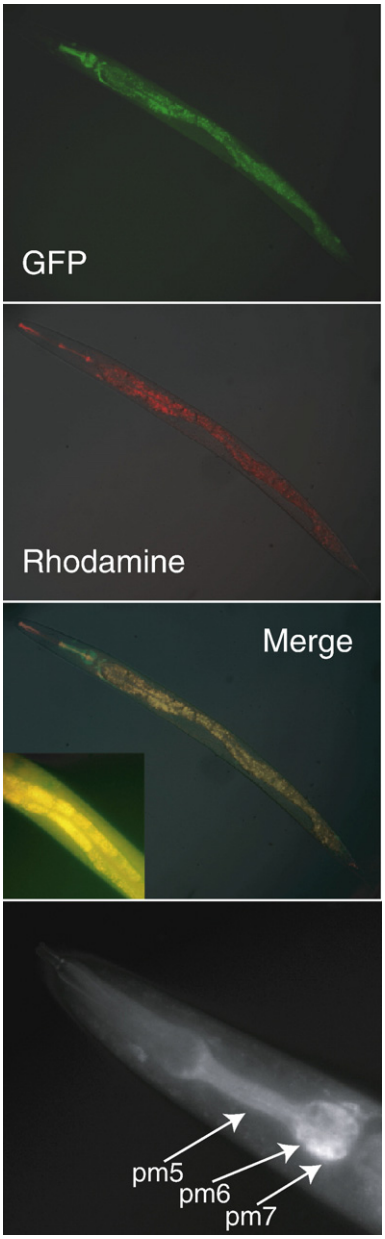

Fig. 6. Intracellular location of CDR-4 *in vivo*. Transgenic *C. elegans* that contain a *cdr-4*/CDR-4–eGFP expression vector were fed RITC-dextran. The location of CDR-4–eGFP (GFP) and RITC-labeled lysosomes (Rhodamine) was visualized using the appropriate filter sets. A false-color, composite image (Merge) that demonstrates the localization of CDR-4 in lysosomes is presented. A higher magnified (400×) image of the identical transgenic *C. elegans* is presented in the inset. Locations where the eGFP and rhodamine signals are coincident can be identified by the yellow color. The lowermost panel indicates the location of CDR-4–eGFP within the pharynx of an adult *C. elegans*. The location of pharyngeal muscle cells pm5, pm6, and pm7 is indicated. pm5 cells are columnar cells, which extend from the anterior region of the terminal bulb to the posterior of the metacorpus.[34]

Six homologs of CDR-1, designated CDR-2, CDR-3, CDR-4, CDR-5, CDR-6, and CDR-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 posttranslational modification sites (Table 1 and Ref. 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 induce *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 UREs (Table 2 and Ref. 20). Although these genes contain similar UREs, 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 than 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*.[36] 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, and Nrf2 and by affecting mitogen-activated protein kinase signaling cascades.[37–41] 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

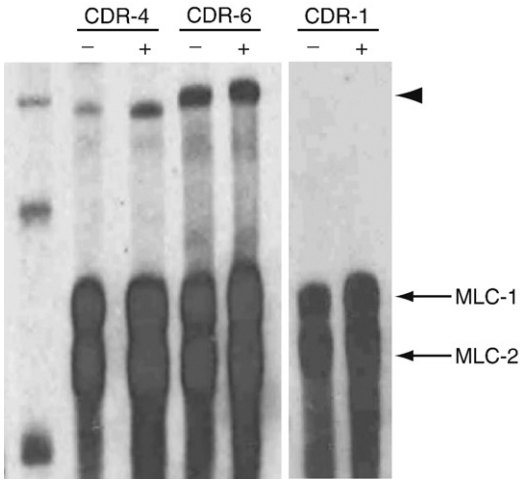

Fig. 7. Effects of hypotonic stress on *cdr* transcription. Total RNA was prepared from nontreated *C. elegans* (−) or *C. elegans* exposed to a hypotonic stress for 24 h (+). Ten micrograms of total RNA; 800 pg of *cdr-1*, *cdr-4*, or *cdr-6* mRNA-specific probe; and 400 pg of myosin light chains 1 and 2 (MLC-1 and MLC-2) probe were hybridized in each reaction. The arrowhead indicates the position of the protected product.

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*[33]; 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*.[30,42,43] 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.[30,31,42–48] 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.[49] 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 nonintestinal cells, specifically pharyngeal muscle cells (Figs. 4 and 5, respectively). *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 pharyngeal muscle cells pm3, pm4, 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.[50,51] 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 *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 in which the expression of a CDR-6–eGFP fusion protein is regulated by ~3.5 kb of the *cdr-6* promoter was generated. Strains of *C. elegans* containing this transgene were not viable, which suggests that overexpression 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] (Figs. 3 and 7).

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* double-stranded RNA (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. 8). This phenotype is similar to that observed when the cells of the *C. elegans* secretory–excretory system were ablated.[52] In addition, similar phenotypes have been observed in hyperactive *egl-15* signaling and in *clr-1* mutants.[53] In all cases, it is proposed that disruption of the biological activity of these genes, or cognate signaling pathways, causes fluid imbalances or disruption in *C. elegans* osmoregulation. *C. elegans* in which the expression of these two genes was attenuated were grown under hypertonic conditions to assess the roles of *cdr-4* and *cdr-6* in osmoregulation. The lack of response to hypertonic stress suggests that these genes may not be involved in the osmoregulation.

The CDR family may be part of a new class of transmembrane proteins. Orthologs of the *C. elegans*

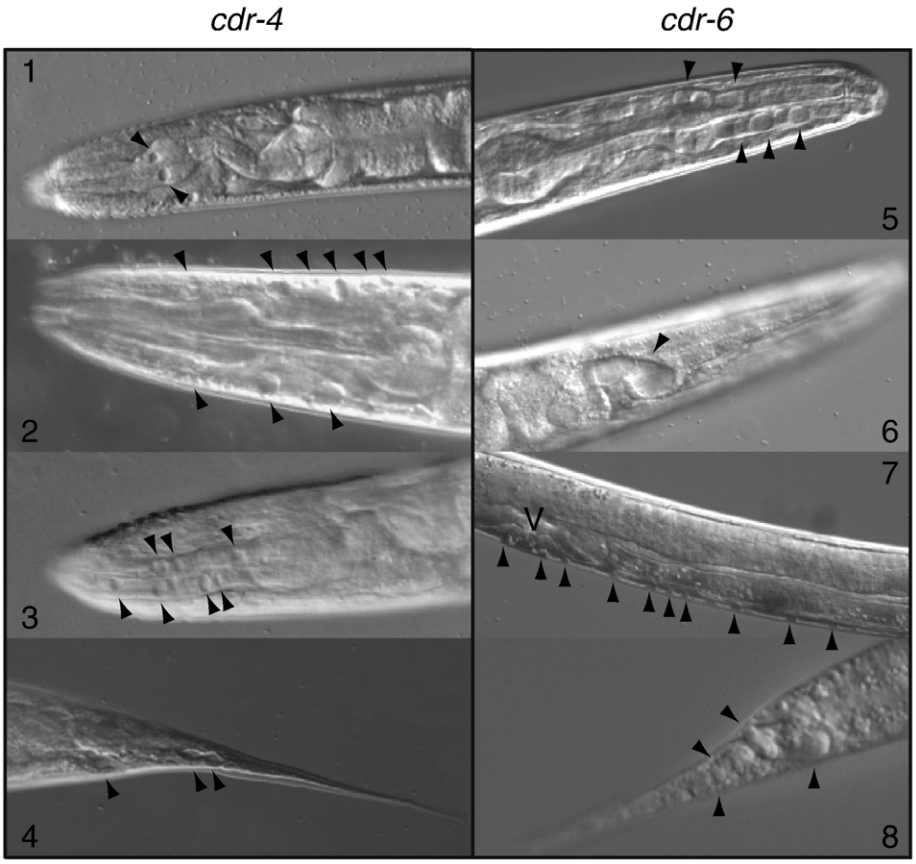

Fig. 8. Phenotype associated with RNAi of *cdr-4* and *cdr-6*. Wild-type *C. elegans* were grown on NGM plates containing 1 mM isopropyl-1-thio-β-D-galactopyranoside and 50 μg/ml ampicillin, seeded with BL21(DE3) containing pCDR-4i (left) or pCDR-6i (right). Adult pharynx with vesicles in the pharyngeal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

cdr genes have been identified in other *Caenorhabditis* species: *C. briggsae* and *C. remanei*. Partial orthologs have also been tentatively identified in other invertebrates.[54] 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.

Experimental Procedures

Growth of *C. elegans*

The N2 Bristol strain of *C. elegans* was maintained at 20 °C on NGM agar plates seeded with *Escherichia coli* strain OP50 as a food source.[55] The *cdr-4* knockout strain RB966 [*cdr-4(ok863)*] was obtained from the Caenorhabditis Genetics Center (University of Minnesota). Nematodes were grown in liquid S medium, collected, and stored as previously described to obtain large quantities of *C. elegans*.[33] In the experiments in which nematodes were exposed to metals, they were incubated for 24 h in S media supplemented with 100 μM $NaAsO_2$, 100 μM $CdCl_2$, 18 μM $HgCl_2$, or 124 μM $ZnCl_2$.[36,56]

RNA isolation

Total RNA was isolated from mixed-stage populations of *C. elegans* exposed to metal or control, nonexposed nematodes as previously described.[20,33] 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 to 10 mM.[52] Where indicated, poly(A^+) RNA was isolated using Oligotex mRNA Midi Kits following the manufacturer's instructions (Qiagen).

RPA

cDNAs for CDR-1, CDR-4, and 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 PCRs and characteristics of the products are described in Table 3.

Plasmid DNA templates were linearized following digestion with either NcoI or NotI restriction enzymes to synthesize antisense RNA probes. Biotin-labeled RNA probes were generated from the linearized plasmids using a MAXIscript™ *in vitro* Transcription Kit following the

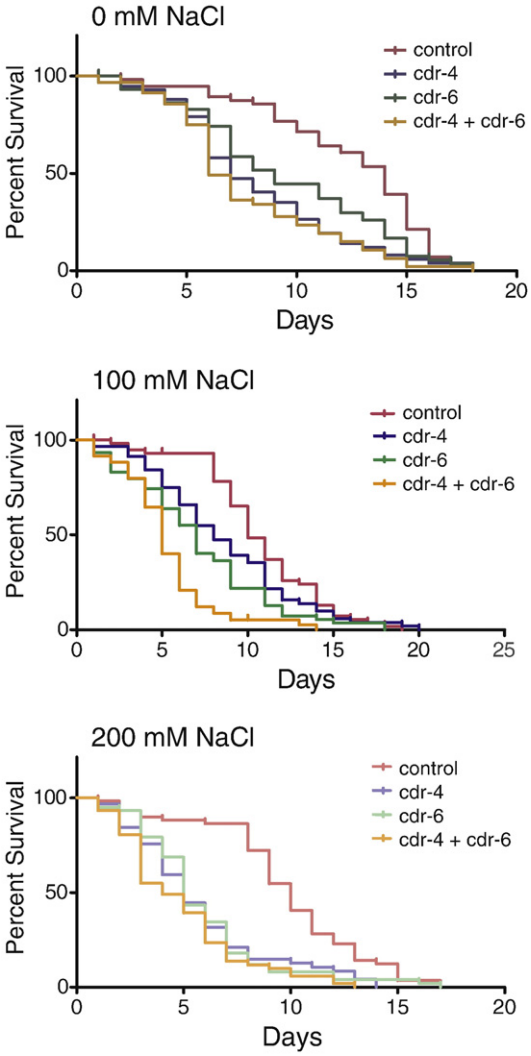

Fig. 9. Effect of osmotic stress on *C. elegans* lifespan. Wild-type *C. elegans* were grown on NGM plates containing 0, 100, or 200 mM added sodium chloride. Plates contained bacteria that expressed control, *cdr-4*, *cdr-6*, or a mixture of *cdr-4* and *cdr-6* dsRNA. Percentage of survival was calculated as indicated.

manufacturer's protocol (Ambion). *In vitro* transcription reactions contained SP6 RNA Polymerase when NcoI was used to linearize the template DNA or T7 RNA Polymerase when NotI 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.

RPAs 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.[57]

Preparation and analysis of *cdr-4/lacZ* and *cdr-6/lacZ* transgenic *C. elegans*

An ~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′CACTGGGCAACAACAAACGAT3′, and the 3′-primer 5′GGACACCTCCGTCTACATTC3′. The PCR product was purified and then cloned into pGEM-T. The genomic fragment was excised from pGEM-T following digestion with SphI/SalI. 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*.

An ~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′ACAGCAACACACGATTCTGG3′, and the 3′-primer 5′GCCCGAATCTATCACCATTTTGC3′. 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.[58]

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.[33,58]

Cell-specific, developmentally regulated patterns of *cdr-4* and *cdr-6* expression were determined by staining for β-galactosidase as previously described.[33] 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

A *C. elegans* expression vector in which the expression of a CDR-4–eGFP fusion protein is regulated by an ~3.5-kb *cdr-4* promoter was constructed to determine the intracellular location of CDR-4 *in vivo*. Full-length *cdr-4* cDNA was prepared by PCR to generate this vector, using the 5′-primer 5′TCCCCCGGGTTTTCAAAATGGTTTG3′, which introduces an SmaI site (underlined) into the reaction

Table 3. Probes used in RPA

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	

product, and the 3′-primer 5′CCGGTACCATAAT**TGA**-AATTGTAAAATCGTTAGG3′, which introduces a KpnI 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 SmaI and KpnI and then inserted into the *C. elegans* GFP expression vector pPD117.01 (AddGene), digested with the same enzymes. This product was then digested with SphI and SmaI to remove the *mec-7* promoter fragment that is present in pPD117.01, and an ~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 colocalization experiment in which the intestinal cell lysosomes were labeled with rhodamine, *C. elegans* were fed RITC-labeled dextran as previously described.[20,35] It has been demonstrated that *C. elegans* accumulate RITC-dextran in intestinal cell lysosomes.[35]

RNAi

The full-length cDNA fragment of *cdr-4* was generated by RT-PCR using the 5′-primer 5′ATGGTTTGTTGTTGCC-CAGTG3′ and the 3′-primer 5′CGTTAGGATAGATT-TCTTTGCG3′ and then inserted into pGEM-T. This plasmid was then digested with NcoI and PstI 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′ATGGTGTGTT-GCTGTCCAG3′ and the 3′-primer 5′GATGGTGAAA-TCATTGGGG3′ and then inserted into pGEM-T. CDR-6 cDNA was excised following digestion with NcoI and PstI. 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.[59,60]

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 nontransformed *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).[61] 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, individually cultured, and allowed to lay eggs at 22 °C for 24 h before being removed.[61] 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[62] (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 the total number of offspring was measured. For the growth assay, 25 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 length and optical density of individual *C. elegans* were measured using the COPAS BIOSORT ReFLx. Nematodes were placed in wells containing deionized water and *E. coli* to assess the effects of hypotonic stress.

Life span assay

Life span studies were conducted after RNAi exposure to nonfunctional control, *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. Life span 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.

Acknowledgements

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