

**The Use of Comparative Genomics to Investigate Mechanisms of Cadmium-Induced
Transcription**

by

Brooke Erin Tvermoes

Nicholas School of the Environment and Earth Sciences
Duke University

Date: _____

Approved:

Dr. Jonathan Freedman, Supervisor

Dr. Scott Alper

Dr. Gary Bird

Dr. Richard Di Giulio

Dr. Dennis Thiele

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
in the Nicholas School of the Environment
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ABSTRACT

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Abstract

Cadmium is a human carcinogen and a persistent environmental pollutant of increasing concern. Yet, the exact molecular targets of cadmium toxicity and the molecular mechanisms by which cadmium influences gene expression have not been fully elucidated. Therefore, the characterization of cadmium-inducible genes will provide a better understanding of the underlying mechanism involved in sensing cadmium-stress and the subsequent signaling pathways important for cellular defense against cadmium toxicity. To this end, we characterized two cadmium-responsive genes of no known biological function from the nematode *Caenorhabditis elegans* (*C. elegans*), *numr-1* and *numr-2* (Nuclear Localized Metal-Responsive gene).

Expression analysis of *numr-1* and *numr-2* revealed the same temporal and spatial expression patterns of both genes in the absence and presence of metal treatment. In the absence of metal, constitutive expression of *numr-1/-2* was developmentally regulated. When adult animals were exposed to metal, *numr-1/-2* expression dramatically increased. We show that worms overexpressing *numr-1/-2* were more resistant to metal stress and longer lived than control animals; whereas reducing *numr-1/-2* activity resulted in increased sensitivity to metal exposure. Furthermore, in the absence of metal, the two *numr-1* mutant alleles, *tm2775* and *ok2239*, exhibited decreased muscular functions. The molecular characterization of *numr-1* and *numr-2* also revealed

that the expression of these two genes, at least in part, was regulated by changes in intracellular calcium concentrations ($[Ca^{2+}]_i$). This finding lead us to reevaluate the role of calcium mobilization in cadmium-induced transcription.

While several studies have indicated that exposure to cadmium resulted in increased $[Ca^{2+}]_i$, the mechanism by which cadmium can effect $[Ca^{2+}]_i$ and concurrent effects on gene expression remain poorly understood. Therefore, we investigated the effects of low-level cadmium exposure, sufficient to induce transcription of cadmium-responsive genes, on the regulation of $[Ca^{2+}]_i$. In these studies, we utilized the protein-based calcium sensor YC 3.60 stably expressed in a HEK293 cell line. YC 3.60 is insensitive to cadmium ions, and thus is useful to monitor changes in $[Ca^{2+}]_i$ following cadmium treatment. Exposing HEK293 cells to 1-30 μ M cadmium was sufficient to induce transcription of cadmium-responsive genes such as metallothionein. Cadmium exposure from 1-10 μ M had no effect on cell viability, $[Ca^{2+}]_i$ mobilization, or increased transcriptional activity of calcium-responsive genes. In contrast, exposure to 30 μ M cadmium significantly decreased cell viability, reduced intracellular calcium stores, and significantly altered the transcriptional activity of calcium-responsive genes. Taken together, these data indicate that low-level cadmium exposures (1-10 μ M) can induce transcription of cadmium-responsive genes such as metallothionein independent of $[Ca^{2+}]_i$ mobilization.

To gain further insight into the mechanistic relationship between cadmium and calcium we investigated the effects of cadmium exposure on the defecation cycle of *C. elegans*. Defecation is a highly rhythmic behavior that is regulated by calcium oscillations. We found that low-level cadmium exposures, sufficient to induce expression of cadmium-responsive genes such as *numr-1/-2*, significantly shortened the defecation cycle but did not alter the rhythm of the cycle or the magnitude of the intestinal calcium oscillations. Modulation of lipid metabolism in *C. elegans* results in a similar shortened defecation cycle, whereas modulation of $[Ca^{2+}]_i$ results in lengthened and arrhythmic defecation cycles, suggesting that the mechanism by which cadmium alters defecation is independent of $[Ca^{2+}]_i$ mobilization.

In summary, the data in this work demonstrates that low-level cadmium exposure induces expression of cadmium-responsive genes independent of calcium mobilization. Thus, modulation of intracellular calcium is unlikely the primary mechanism by which cadmium regulates transcription at low-levels of exposure.

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List of Abbreviations

AW	amphid wing neurons	MMR	mismatch repair
BER	base excision repair	MRE	metal-responsive elements
[Ca ²⁺] _i	intracellular calcium concentrations	MT	metallothionein
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>	MTF-1	metal-responsive transcription factor
CaMKII	calcium/ calmodulin-dependent kinase	NER	nucleotide excision repair
CFP	cyan fluorescent protein	NGM	nematode growth medium
CKII	casein kinase II	NLS	nuclear localization signal
CV	coefficient of variance	NUMR-1/-2	nuclear localized metal responsive gene
DAF-16	abnormal dauer formation protein	pBoc	posterior body wall muscle contraction
DMP	stereotype motor program	PKC	protein kinase C
Egl	egg-laying defective phenotype	qRT-PCR	quantitative real-time PCR
ER	endoplasmic reticulum	RLU	relative light units
ERK	extracellular signal-regulated kinase	ROI	region of interest
Fpg	formamidopyrimidine glycosylase	ROS	reactive oxygen species
FRET	fluorescence resonance energy transfer	<i>sca-1</i>	<i>C. elegans</i> SERCA homolog
GFP	green fluorescent protein	SE	standard error
HEK293	human embryonic kidney cells	SERCA	sarcoplasmic ER Ca ²⁺ ATPase pumps
HO-1	heme oxygenase	SOC	store operated calcium entry
HSF-1	heat shock factor	STIM1	stromal interaction molecule 1
HSP	heat shock protein	TG	thapsigargin
JNK	jun kinase	XPA	xeroderma pigmentosum A
MAPK	mitogen-activated protein kinase	YFP	yellow fluorescent protein

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1. Introduction

1.1 Environmental exposure to cadmium and human health

Cadmium is a ubiquitous environmental pollutant that is very stable in the environment [1]. Cadmium is found in at least 1,014 of the 1,669 National Priority List sites and thus is ranked numbered 7 on the CERCLA Hazardous Substance Priority List [2]. Cadmium is also a natural element of the earth's crust and is commonly found associated with zinc, lead, and copper ores. The natural release of cadmium into the environment is minimal but includes emission from volcanic activity, forest fires, and the weathering of rock [3]. The main source of cadmium contamination in the environment is from anthropogenic sources such as its wide industrial use in the manufacturing of nickel-cadmium batteries, anti-corrosive products, and copper and nickel smelting. Most cadmium used in the United States today is for the manufacturing of nickel-cadmium batteries [2].

Unlike most metals, cadmium is a relatively modern toxic metal. The widespread industrial use of cadmium began around 1940 [3]. A study performed comparing metal accumulation in lung tissue autopsy samples taken from Mexico City residents from the 1950s and the 1980s observed a sharp increase in the amount of cadmium and other metals found in human lung tissue sampled from the 1980s when compared to samples from the 1950s (Table 1.1) [4]. While the use of cadmium has greatly diminished over the

past few years, cadmium production worldwide still averaged about 17,800 tons in 2004 [5, 6].

Table 1.1: Levels of several transition metals measured in lung autopsy tissues taken from residents living in Mexico City from the 1950s and the 1980s [4].

Element	Mean \pm SD (mg/g dry weight)	
	1980s (<i>n</i> = 84)	1950s (<i>n</i> = 69)
Cadmium	25.6 \pm 6.5	1.2 \pm 0.37
Cobalt	37.2 \pm 8.67	3 \pm 0.97
Copper	44.8 \pm 15.7	10 \pm 2.97
Nickel	57.6 \pm 9.3	3 \pm 0.96
Lead	134.3 \pm 26.7	12 \pm 4.97

Exposure to cadmium is implicated in a broad spectrum of adverse human health conditions, including respiratory ailments, osteoporosis, nephrotoxicity, neurotoxicity, and birth defects [7]. Furthermore, the International Agency for Research on Cancer has classified cadmium as a type 1 human carcinogen based on data that has indicated that occupational exposure to cadmium lead to an increased risk of lung cancer [8-10]. Additional studies suggest that the prevalence of cadmium-associated diseases, such as kidney dysfunction and osteoporosis, are escalating and appearing at cadmium levels below OSHA standards [11-14].

For the general population, smoking constitutes a primary source of cadmium exposure. The average cigarette contains about 1 – 2 μg of cadmium and it has been estimated that up to 60% of cadmium inhaled smoke enters the body [2, 15]. For the non-smoking population, the main routes of cadmium exposure are via occupational exposure or through the consumption of contaminated food or drinking water [16, 17]. In occupational settings, such as pigment and battery production, workers are exposed to cadmium dust and fumes, which has been reported to result in renal cadmium levels of 180-200 $\mu\text{g/g}$ kidney, which is associated with renal dysfunction [16].

The toxicity of cadmium is exacerbated by its very long biological half-life of 15-20 years in humans [18]. The main reason for this long biological half-life is the inability to excrete cadmium once it enters the body. It has been shown that cadmium accumulates mainly in the kidneys and liver, and to a lesser degree cadmium has also been found to accumulate in tissues such as the bone, breast, prostate, pancreas, and colon [12, 14, 19-22].

Epidemiological studies have found that environmental exposure to cadmium correlates with decreased bone density [14]. It was hypothesized that the toxic effects of cadmium exposure on bone were secondary effects of renal dysfunction because the kidneys play an important role in calcium reabsorption, which is essential for maintaining proper bone density [23]. However, researchers found that low-levels of cadmium exposure, which did not impair renal function, caused early bone loss [23]. A

study by Regunathan et al. (2003) further demonstrated that early bone demineralization in response to cadmium exposure resulted from increased osteoclast-mediated bone resorption, rather than decreased osteoblast-mediated bone formation and that the p38 MAP kinase pathway appeared to play a role in the imbalance between osteoclast and osteoblast activity [24].

The most well documented toxic effect of cadmium is its role as a pulmonary carcinogen. Multiple studies have indicated an association between occupational exposure to cadmium and the development of lung cancer [8-10]. The evidence for an increased risk of developing prostate and testicular cancer following exposure to cadmium has also grown [25-27]. Furthermore, evidence for adverse effects of cadmium exposure on reproduction and mental development of children have begun to emerge [28] and recent studies have linked cadmium exposure to type II diabetes in humans [29]. The effects of cadmium exposure on several organ systems are summarized in Figure 1.1. While there is a strong correlation between cadmium exposure and the development of several types of cancers and human pathologies, a detailed understanding of the molecular mechanisms by which cadmium contributes to the etiology of human disease is lacking.

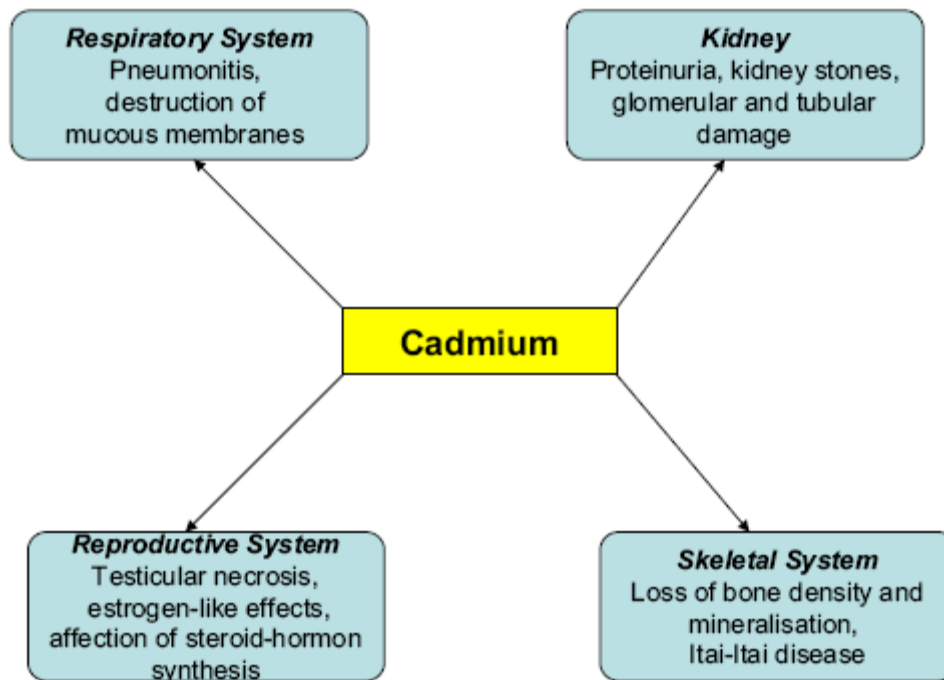


Figure 1.1: Summary of the effects of cadmium exposure on several organ systems [30].

1.2 Cadmium uptake

Ingestion or inhalation are the two main routes by which humans generally uptake cadmium. Only a very small amount of ingested cadmium (5-10%) is taken up into the body, although dietary deficiency can elevate the amount of absorbed cadmium. In contrast, it has been estimated that as much as 60% of inhaled cadmium is absorbed by the body [1, 2, 15]. Absorbed cadmium is transported throughout the body by binding to erythrocytes and high-molecular-weight plasma proteins (albumin, globulin, etc.) [31]. Cadmium is primarily distributed to the liver and kidney and cadmium

accumulation in these tissues usually accounts for 50% of the total body burden of cadmium [1, 32].

Cell culture studies indicate that cadmium uptake is rapid and biphasic. The first phase of cadmium uptake is thought to be mediated by a simple diffusion component and the second phase is thought to be mediated by an active transport mechanism [31, 33]. DelRaso et al. (2003) showed that the first phase of cadmium uptake in rat hepatocytes was via a passive diffusion mechanism that was very rapid, and reached a steady state level in less than one minute [31]. The second phase of cadmium accumulation was found to be slower than the first phase and was dependent upon the active transport of cadmium across the cellular membrane [31]. Additional studies found that excess zinc suppressed cadmium uptake, which suggested that the second phase, at least in part, involved mechanisms whereby cadmium interacts with and competes for binding sites on membrane proteins involved in the transport of essential elements such as zinc [34]. There is also evidence that cadmium enters cells through calcium channels, divalent metal transporter 1 (DMT1), and organic cation transporters (OCT) [35]. However, there is a large variability observed in the amount of cadmium uptake by cells and it appears to be largely dependent upon the experimental protocol implemented during the study.

1.3 Molecular mechanisms of cadmium toxicity

The toxicity of cadmium has various mechanisms of action and the relative contributions of each of these mechanisms has not been completely resolved [3, 20, 36, 37]. The following molecular mechanisms are believed to be involved in cadmium toxicity:

1.3.1 Oxidative Stress

Data suggests that cadmium exposure gives rise to the generation of reactive oxygen species (ROS) and that this is a major mechanism contributing to the cytotoxic effects of cadmium [3]. Oxidative stress can occur when there is an imbalance in redox homeostasis, changing the oxidative state of the cell. It has been shown in both whole animal and cell culture models that cadmium exposure results in increased lipid peroxidation and increased production of hydroxyl radicals, superoxide anions, and hydrogen peroxide [38-41]. However, cadmium is not capable of catalyzing Fenton reactions, and thus it has been proposed that cadmium must generate ROS through indirect mechanisms.

One possible mechanism is through cadmium's ability to deplete antioxidants such as glutathione, superoxide dismutase, catalase, and glutathione peroxidase [42]. Cadmium also binds with high affinity to protein-sulphydryl groups on antioxidant enzymes and interferes with glutathione synthesis [43, 44]. Furthermore, it has been shown that cadmium is able to displace iron and copper from intracellular proteins.

Cadmium has been found to bind to both ferritin and apoferritin, displacing iron from its binding sites and cadmium has been found to displace copper from metallothioneins (MT) [3]. Since both of these elements are capable of generating ROS through Fenton-type chemistry this would offer another mechanism by which cadmium exposure could induce oxidative stress [35]. However, the effects of low, non-toxic doses of cadmium on gene expression indicate that oxidative stress may play only a minimal role in mediating gene expression, further evaluation is warranted [45-47].

1.3.2 Genotoxicity

Genome stability is essential for proper cell function and survival. It is well-known that ROS can cause DNA lesions, and thus, it is no surprise that the increase in ROS following cadmium exposure has been shown to generate DNA lesions [48]. In addition to increasing ROS, numerous studies have indicated that low, non-toxic concentrations of cadmium inhibit the functional activity of DNA repair systems such as, nucleotide excision repair (NER), mismatch repair (MMR), and base excision repair (BER).

Evidence suggests that cadmium interferes with NER by displacing zinc from the zinc finger domain of xeroderma pigmentosum A (XPA) protein, which is required for recognition of DNA lesions and is critical for the recruitment of other NER proteins to repair the damaged site [6]. Jin et al. (2003) found that cadmium inhibits DNA MMR [49] and there is data indicating that inhibition of MMR by cadmium can be reversed by

incubation with zinc prior to cadmium exposure [6]. Low concentrations of cadmium also inhibit BER by targeting the zinc finger motif of the formamidopyrimidine glycosylase (Fpg) protein, which is necessary for the recognition and repair of oxidative DNA base modifications [6, 50]. Thus, inhibition of DNA repair enzymes is considered another mechanism contributing to the toxic effects of cadmium.

1.3.3 Zinc substitution

One significant mechanism underlying cadmium's inhibition of DNA repair enzymes is the displacement of zinc from zinc finger structures of DNA repair proteins. Cadmium's ability to compete with zinc for binding sites interferes with various zinc-mediated processes, such as reactions essential for DNA repair [6, 51, 52]. Another piece of evidence for cadmium's influence on zinc proteins comes from studies showing reduced cadmium toxicity in cells pretreated with zinc. For example, Souza et al. (2004) found that zinc pretreatment prevented cadmium-induced oxidative damage in hepatic stellate cells by recovering antioxidant enzyme levels to that of control cells and not by increasing heat shock protein or metallothionein production prior to cadmium treatment [53]. These data suggest that zinc pretreatment helped to maintain normal redox balance by recovering glutathione, catalase, and glutathione peroxidase levels and thus, decreased cadmium toxicity.

1.3.4 Disruption of E-cadherin

Another possible indirect mechanism of cadmium toxicity is inhibition of E-cadherin function [3]. E-cadherin is a transmembrane glycoprotein that becomes rigid upon calcium binding, forming cell-cell adhesions. The way cadmium is thought to disrupt cell-cell adhesion is by displacing calcium from the extracellular calcium-binding region of E-cadherin [54]. Recently, Edwards et al. (2009) showed that cell-cell adhesion was disrupted in pancreatic cells of rats exposed to sub-chronic levels of cadmium (0.6 mg/ kg/ days, 5 days a week) for 12 weeks. The data suggest that E-cadherin function may have been compromised in the pancreas of cadmium exposed rats [55]. Interestingly, cadmium exposure has been associated with Type II diabetes, and thus disruption of E-cadherin in the pancreas might be one mechanism by which cadmium affects glucose levels [56]. Disruption of E-cadherin could also contribute to tumor cell metastasis and invasion.

1.4 Mechanism of cadmium detoxification

All living organisms respond to unfavorable conditions at the cellular level and this response is essential for survival, growth, and reproduction. At low-levels of exposure, cadmium activates multiple stress-responsive genes and pathways that counteract the toxic effects of cadmium. The primary mechanism for cadmium detoxification is chelation by MTs [57]. MTs are small, cysteine-rich, metal-binding proteins that sequester cadmium and other metals such as zinc and copper with high

affinity. Sequestration of cadmium by MT decreases the availability of cadmium to interact with cellular targets, protecting the cell from further injury. Although, the exact physiological functions of MTs are still unclear, there is strong evidence for the importance of MT in heavy metal detoxification [57].

Cadmium exposure has also been shown to result in a significant induction of heat shock proteins and glutathione-S-transferases [58]. The induction of these proteins proceeds cadmium toxicity and is important for cellular protection and repair. Heat shock proteins help fold misfolded or denatured proteins and prevent the accumulation of protein aggregates during times of stress. Glutathione production plays an important role in scavenging ROS and thus reduces the effects of ROS induced by cadmium exposure. Continuous exposure to cadmium or exposure to very high doses of cadmium is thought to overwhelm these cellular defense systems resulting in cadmium toxicity.

While these proteins do play an important role in cellular defense, microarray data have indicated that there are many more genes involved in response to cadmium exposure than originally anticipated [45, 58-60]. Hsiao et al. (2009) found that exposure of primary rat hepatocytes to low, non-toxic doses of cadmium up-regulated several genes that were not previously known to be cadmium-responsive. These genes included an arginine/serine-rich splicing factor (Sfrs10) and a nuclear receptor (Nr4a1) [58]. Furthermore, a recent study screening the *Saccharomyces cerevisiae* haploid and homozygous diploid deletion collection for mutants sensitive to cadmium exposure,

found a novel mechanism of cadmium resistance that involved the phosphorylation of the Snf1p kinase, which is part of the glucose sensing pathway in yeast [47].

1.5 Molecular mechanisms of cadmium-induced transcription

While several transcription factors, signaling cascades, and kinases can be activated following cadmium exposure, the exact molecular mechanisms by which cadmium influences gene expression have not been fully elucidated.

MTF-1 (metal-responsive transcription factor 1) is an evolutionarily conserved transcription factor that is a key regulator of cadmium-induced MT transcription [61]. When cells are exposed to cadmium, MTF-1 is signaled to translocate from the cytoplasm to the nucleus. Once MTF-1 reaches the nucleus, nuclear MTF-1 binds to metal responsive elements (MREs) within the MT promoter, and increases MT transcription [61]. The molecular mechanism by which cadmium activates MTF-1 remains an intense topic of research. One proposal is that activation of MTF-1 following cadmium exposure results from alterations in MTF-1 phosphorylation status [62].

A key component of the stress response is the mitogen-activated protein kinase (MAPK) family. Three major members of the MAPK pathways, p38, extracellular signal-regulated kinase (ERK), and Jun kinase (JNK), can all be activated in response to cadmium exposure [63, 64]. The activation of these MAPK pathways is thought to regulate the expression of several cadmium-inducible genes. For instance, in mesangial cells the activation of two parallel MAPK cascades following cadmium exposure results

in increased expression of the cadmium-inducible, proto-oncogene, *c-fos* [65]. Another example is HSP70 induction following cadmium treatment in NIH3T3 cells, which was found to be dependent upon the p38 and JNK MAPK pathways [64]. Finally, activation of the adaptive stress response gene, heme oxygenase-1 (HO-1), in mammary epithelial cells following cadmium treatment is dependent upon p38 signaling [66]. Interestingly, induction of HO-1 by cadmium in HeLa cells was found to utilize the ERK pathway as opposed to the p38 pathway [67]. However, activation of the p38, ERK, and JNK MAPK pathways does not account for all the genes differentially expressed following cadmium exposure. This suggests that there are additional signaling mechanisms involved in the regulation of cadmium-induced gene expression.

Cadmium can also influence the activity of numerous protein kinases both in cell culture and in animal models. Kinases activated by cadmium exposure include: protein Kinase C (PKC), casein kinase II (CKII), tyrosine kinase, stress activated protein kinase, and calcium/ calmodulin-dependent kinase II (CaMK-II) [3, 68]. It has been shown that cadmium can substitute for calcium in the activation of CAMK-II and PKC [69, 70]. In cell culture and in a nude mouse model, the induction of proto-oncogenes *c-fos*, *c-myc*, and *c-jun* were inhibited by the calcium-chelator BAPTA and PKC inhibitor, RO-31-8220 [71]. This suggested that the induction of these proto-oncogenes was dependent upon the mobilization of intracellular calcium and the activation of PKC [71]. However, these

studies are controversial and we believe there may be alternative interpretations of this data since calcium chelators such as BAPTA bind cadmium with a high affinity.

It has been hypothesized that alterations of second messengers such as ROS and calcium following cadmium exposure may play important roles in modulating cadmium-induced gene expression. It has recently been suggested that activation of ERK, p38, and JNK following cadmium exposure could result from an elevation of intracellular calcium levels ($[Ca^{2+}]_i$) [72, 73]. While several studies have indicated that exposure to cadmium results in increased $[Ca^{2+}]_i$, the mechanism by which cadmium can effect $[Ca^{2+}]_i$ and the potential induction of signaling pathways resulting from mobilization of intracellular calcium remains poorly understood. Part of this uncertainty is due in part from the use of BAPTA-based fluorescent calcium indicators which are able to bind cadmium with high affinity and elicit a fluorescence change that may be interpreted as changes in $[Ca^{2+}]_i$ (Figure 1.2) [74]. Thus the inability to distinguish between cadmium and calcium ions using BAPTA-based fluorescent calcium indicators complicates the interpretation of cadmium's effect on $[Ca^{2+}]_i$ mobilization [74]. In addition, many studies indicating an effect of cadmium on $[Ca^{2+}]_i$ have used cadmium concentrations that are toxic [75-77]. In contrast, activation of many cadmium responsive genes, such as MTs and HSPs, occurs prior to cellular toxicity [78-80]. In summary, there is a need to clarify the relationship between cadmium-exposure and its effects on transcription and the potential role of $[Ca^{2+}]_i$.

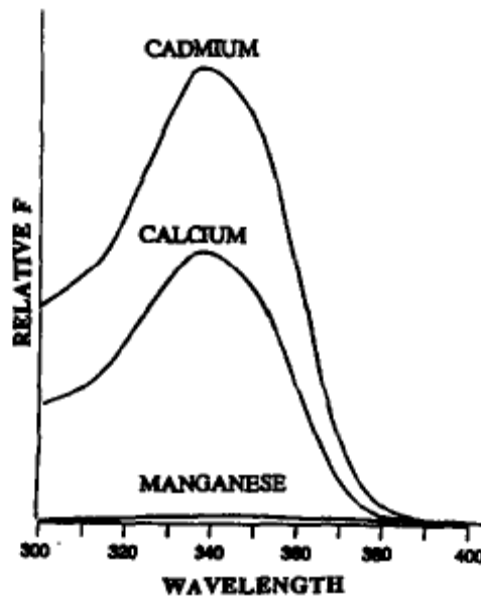


Figure 1.2: Properties of the Cd^{2+} complex of Fura 2. 1 mM calcium, 10 μM Cd^{2+} [74]

1.6 *C. elegans* as a Model System

Non-mammalian model systems such as *C. elegans* have emerged as powerful system for defining the molecular basis of complex processes, such as stress responses. *C. elegans* are non-parasitic soil dwelling nematodes about 1 mm in length. They are easily grown and maintained in a laboratory setting and have a quick life cycle. Through self-fertilization *C. elegans* produce a large number of genetically identical offspring that develop through four distinct larval stages, designated L1 – L4, and can go from an embryo to a fully mature adult with in about 72 h [81]. Despite their quick life cycle, *C. elegans* undergo a complex process of development that shares many of the essential

biological characteristics with higher eukaryotes, such as embryogenesis, morphogenesis, and ageing [81].

The adult hermaphrodite consists of 959 somatic cells and is essentially a tube, containing two smaller tubes; one consists of the pharynx and gut, and the other the reproductive system [81]. *C. elegans* are transparent throughout their life cycle, making it possible to track each cell in the living organism through all developmental stages, thus cell lineage is well defined and has relatively little variability. *C. elegans* also have highly differentiated nervous, muscular, reproductive, and digestive systems. Furthermore, the genetic tractability of *C. elegans* makes them a well-suited model for studies in genetics, developmental biology, molecular biology, and toxicology.

The molecular genetics of this nematode are very well-established, with a fully sequenced genome that has been estimated to be 40% to 60% homologous to that of humans [82]. In addition, there are a numerous helpful tools available to work with *C. elegans* when trying to establish a relationship between genotype and phenotype. For instance, DNA microarrays allow for whole genome analysis. Additionally, there are two readily available RNAi libraries, that between the two, contain nearly every gene in the genome [83, 84]. There are also many mutant *C. elegans* strains available and the construction of transgenic nematodes is relatively easy.

Numerous genes in *C. elegans* have human orthologs and the underlying mechanisms that modulate cellular response to metal exposure are highly conserved

between *C. elegans* and higher eukaryotes. This makes *C. elegans* an advantageous model for dissecting apart the molecular signaling pathways involved in regulating cadmium-induced gene expression. The MAPK signal transduction pathways, which play an important role in stress response, are one example of these evolutionarily conserved pathways in *C. elegans* (Figure 1.3) [85]. Mutations in the *C. elegans* JNK-like pathway and p38-like pathway result in animals that are hypersensitive to heavy metals, including cadmium [86-89]. Many other signal transduction pathways such as Toll, TGF, and Wnt signaling are also conserved in *C. elegans* [90].

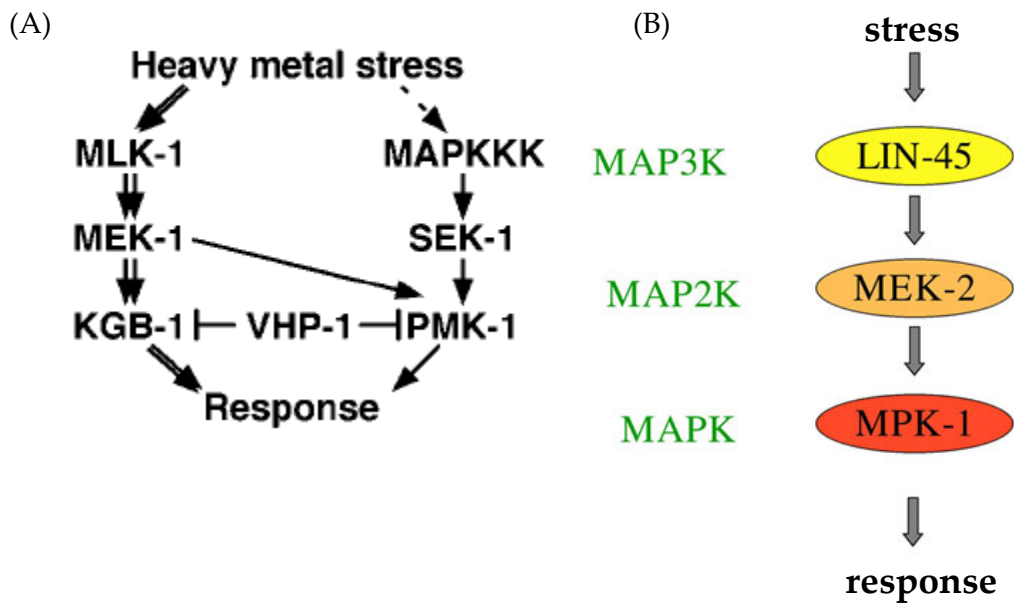


Figure 1.3: Signaling cascades for three members of the *C. elegans* MAPK family. (A) Two MAPK pathways in *C. elegans* that are involved in heavy metal response. The KGB-1 pathway is a JNK-like MAPK pathway in *C. elegans* and the PMK-1 pathway is a p38-type MAPK pathway in *C. elegans* [88]. (B) Components of the *C. elegans* ERK-like MAPK pathway involved in stress response (adapted from Ewbank 2006 [91]).

C. elegans are also a useful model system to study mechanisms of calcium homeostasis and signaling [92]. The use of *C. elegans* has been instrumental in defining fundamental aspects of calcium signaling and characterizing several plasma membrane calcium channels and proteins important for calcium signaling, such as STIM1 and Ora1 [93]. The timing of a number of biological processes in *C. elegans*, such as ovulation and defecation, are regulated by calcium signaling [93]. In *C. elegans* defecation is a highly rhythmic behavior that is the result of a stereotyped motor program, which is initiated roughly once a minute in the presence of food. The initiation of the defecation cycle and posterior body wall muscle contractions (pBoc) are thought to be, at least in part, regulated by calcium oscillations that propagate through the intestinal cells [94]. Nehrke et al. (2008) generated transgenic nematodes expressing yellow cameleon (YC) throughout the intestine, allowing for the dynamics of calcium oscillations to be investigated under physiological conditions [95]. Monitoring the defecation cycle and calcium oscillations during cadmium exposure in *C. elegans* expressing the YC transgene will provide valuable insight into the possible effects of cadmium on calcium oscillations and physiological processes regulated by calcium oscillations in a whole organism.

In brief, *C. elegans* is a highly-studied, well-established model system that possesses great simplicity. This makes it an attractive system for studying mechanisms of stress-response. Furthermore, the highly conserved nature of the genetic pathways involved in stress-responses in *C. elegans*, allows for inferences to be made on the

molecular mechanisms important for response to cadmium exposure in higher eukaryotes.

1.7 Dissertation Objectives

Cadmium is a ubiquitous environmental pollutant that constitutes a continuing threat to human health. Yet, the underlying mechanisms of toxicity and cellular defense associated with cadmium exposure are not fully understood. While several signaling cascades are activated in response to cadmium exposure, the underlying molecular mechanisms by which cadmium influences gene expression have not been fully elucidated. Therefore, unveiling new genes and shedding light on regulatory processes important for mediating cadmium-induced transcription will help further our understanding of early cellular events important for preventing and repairing adverse effects that may result from cadmium exposure. Thus, the overall goal of this dissertation project is to facilitate a better understanding of the molecular mechanisms involved in cadmium-induced gene expression. To achieve these goals three specific aims were proposed:

1.7.1 Specific Aim 1: Cloning and characterization of two cadmium-responsive genes in *C. elegans*, *numr-1* and *numr-2*

The identification and characterization of cadmium-responsive genes will facilitate an understanding of how cadmium-activated pathways can elicit a specific cellular response to prevent cadmium toxicity. Previous work in our laboratory using whole genome microarray analysis identified 290 genes that were differentially

expressed in *C. elegans* following cadmium treatment. Many genes were of no known biological function. One such gene, F08F8.5, was ranked as one of the top ten genes up-regulated following a 24 h exposure to 100 μ M CdCl₂. The first aim of this project was to clone the cDNA and characterize this gene, which we designated *numr-1* (Nuclear Localized Metal-Responsive gene). Based on sequence homology we identified another *C. elegans* gene of high similarity to *numr-1*, *C. elegans* gene F08F8.1, which we designated as *numr-2*. To determine the expression pattern of *numr-1* and *numr-2* transgenic nematodes were generated. In order to decipher the biological relevance of the transcriptional change of *numr-1/-2* both during development and in response to cadmium exposure, the effects of *numr-1/-2* overexpression and the effects of *numr-1/-2* knockdown were investigated.

1.7.2 Specific Aim 2: Describe molecular events underlying *numr-1* and *numr-2* induction in response to cadmium exposure

The goal of this aim was to understand the molecular events underlying *numr-1* and *numr-2* induction following cadmium exposure to identify signaling events pertinent in regulating cadmium-induced transcription. To this end, we compared *numr-1/-2* expression induced by cadmium exposure to other stress conditions such as oxidative stress, starvation, endoplasmic reticulum stress, heat shock, intracellular calcium mobilization, and pathogenic infection. In addition, we performed RNAi on a specific set of candidate genes to gain insight into the signaling events that influence *numr-1/-2* transcription.

1.7.3 Specific Aim 3: Investigate the role of calcium mobilization in cadmium-induced transcription

Alterations of intracellular calcium levels were found to induce the transcription of two cadmium-responsive genes in *C. elegans*, *numr-1* and *numr-2*. Modulation of $[Ca^{2+}]_i$ following cadmium exposure has been proposed to play an important role in cadmium-induced gene expression but the exact mechanistic relationship between cadmium and $[Ca^{2+}]_i$ remains unclear. Thus, the goal of this aim was to use a comparative genomics approach to further understand the effects of cadmium on $[Ca^{2+}]_i$. To assess the effects of low-level cadmium exposure on calcium mobilization, we used a protein-based calcium ion sensor, the yellowameleon (YC) 3.60 stably expressed in a HEK293 cell line. In addition, we assessed the effects of cadmium exposure on the transcriptional activity of cAMP/ calcium responsive genes in the HEK293 cell line. To gain further insight into the mechanistic relationship between cadmium and calcium, we investigated the effects of cadmium on calcium oscillations and on a physiological process regulated by calcium oscillations in a whole organism, the nematode *C. elegans*.

In this project, two cadmium-responsive genes of no known biological function were characterized and assessed for a functional role in the defense against cadmium toxicity in *C. elegans*. The evaluation of these two cadmium-responsive genes in *C. elegans* leads us to reevaluate the role of calcium in regulating cadmium-induced transcription in a mammalian cell culture system. There is much literature indicating

that cadmium can alter $[Ca^{2+}]_i$, yet the precise assessment of the effects of cadmium on $[Ca^{2+}]_i$ are unresolved. Our data indicate that modulation of $[Ca^{2+}]_i$ is unlikely the primary mechanism by which cadmium regulates transcription at low-levels of exposure. The results from these studies will offer insight and help provide a more complete understanding of the mechanisms involved in the regulation of cadmium-induced transcription, which will allow for better assessment of the risk associated with cadmium exposure.

2. Methods

2.1 *C. elegans* strains

The following *C. elegans* strains used in this study were obtained from the *C. elegans* Genetic Center (Minneapolis, MN): wild-type N2 Bristol, *daf-16(tj356)*, *rol-6(he1006)*, and *numr-1(ok2239)*. The Japanese knockout consortium provided the *numr-1(tm2775)* strain. Prior to use, the two *numr-1* alleles, *ok2239* and *tm2775*, were backcrossed to N2 7 and 10 times, respectively. Transgenic nematodes expressing the yellowameleon (YC) 6.1, *rnyEx001 [P_{kt2-nhx2} (nhx-2p::YC6.1), P_{cl1} (pha-1)]*, were obtained from the Nehrke lab [95]. The following transgenic strains used in this study were generated in our laboratory: *P_{numr-1}::mCherry/P_{numr-2}::GFP*, *P_{numr-1}::GFP*, *P_{numr-1}::NUMR-1-GFP*, and *P_{numr-1}::NUMR-1-GFP / P_{hsf-1}::HSF-1-mCherry*. *C. elegans* strains were maintained according to Brenner [96] and grown at 20°C.

2.2 Rapid amplification of cDNA ends

To obtain full-length cDNA corresponding to *numr-1* and *numr-2*, 3'-RACE and 5'-RACE protocols were performed (Invitrogen Life Technologies). A 3'-RACE oligo, which had an adapter region annealed ahead of oligo dT, was used to reverse transcribe 2 µg of total RNA. A set of gene specific primers (GSP) were then combined with 3'-RACE primers UAP and AUAP, respectively, and were used to amplify the possible 3' end of the poly (A)⁺ mRNA transcripts. Gene-specific primers for 3'-RACE (Table 2.1) were designed according to the predicted mRNA sequence of *numr-1* and *numr-2*

obtained from Wormbase (www.wormbase.org). For 5'-RACE, 2 µg of total RNA was reverse transcribed using gene specific primers (Table 2.1) located near the 5' end of *numr-1* and *numr-2*. PCR was then carried out to amplify dC-tailed cDNA using the 5'-RACE Abridged Anchor Primer in combination with a GSP 2 primer and a nested GSP, respectively. All the PCR fragments of 3'- and 5'-RACE were cloned into a pCR2.1 TA vector (Invitrogen, Carlsbad, CA, USA) and subjected to sequencing. DNASTar 5.05 sequence analysis software (DNASTar, Madison WI) was used to assemble continuous cDNA sequences obtained from the 3'- and 5'- RACE assays.

Table 2.1 Gene specific primer sequences used for RACE

numr-1 3' RACE

Primer	Sequence (5' to 3')
GSP 1	GAAAACTACAACCTGCAACCAATTCAATTC
GSP 2	GCTCCTGGAGCAACTGAACAA ATTTC
Nested GSP	GGGAGAACTCGGACTCAATTTCAATGTTG

numr-2 3' RACE

Primer	Sequence (5' to 3')
GSP 1 (Exon 1)	CAGAGATTCTGCTTCTATTTTTGTGATTGC
GSP 2 (Exon 2)	CCTTCTGCATATTCACCTTCCAACAACATTGCTAGACG
GSP 3 (Exon 3)	GCAATGCCGTGGAATACAAATAATCG
GSP 4 (Exon 4)	GTCCAACCATTCAATGCTTCTGAAACTTCTG

numr-1/-2 5' RACE

Primer	Sequence (5' to 3')
GSP 1	GCTTCTTCATTTGATGTCTCTTGTGATGATC
GSP 2	GATCTTGAACGATCAAATCCACGACTTCTG
Nested GSP	CACGTCTTCCACAATGACGACGATGT

2.3 The expression pattern and phenotypic analysis of *C. elegans* genes, *numr-1* and *numr-2*

2.3.1 GFP and mCherry reporter constructs

Promoter and protein fusion reporter constructs were generated using overlap extension PCR [97, 98]. Fusions of the *numr-1* or *numr-2* promoters to either mCherry or GFP or were made by amplifying 5' upstream sequences from N2 genomic DNA and fusing them to PCR fragments containing mCherry or GFP plus the *unc-54* 3' untranslated region. The 77 base pair unique intergenic region between *numr-1* and *numr-2* was used as a 5'-reference stop point. The *numr-1* and *numr-2* promoter constructs each contain 502 base pairs of upstream sequence, including the 77 base pair unique intergenic region, and the respective gene's ATG start codon. A fusion of the NUMR-1 protein to GFP was generated by amplifying the 502 bp *numr-1* promoter and the entire *numr-1* coding sequence (not including the translation termination codon) and fusing it to a PCR fragment containing GFP. A fusion of the HSF-1 protein to mCherry was generated in a similar manner using 2 kb of upstream promoter. All fusion constructs were verified by DNA sequencing. For primer sequences refer to Table 2.2 – 2.4.

To generate transgenic nematodes carrying these reporter fusions, constructs were co-injected with 50 ng/μl of the pRF4 *rol-6* plasmid as previously described [99]. Rolling F1 progeny were cloned onto individual plates and were screened for fluorescence. Three independent lines carrying extra-chromosomal arrays were obtained

for each construct. Promoter fusion constructs were injected at 50 ng/ μ l. Protein fusion constructs were injected at 10 ng/ μ l. In co-localization experiments, 100 ng/ μ l of $P_{numr-1}::mCherry$ was co-injected with 100 ng/ μ l $P_{numr-2}::GFP$ or 5 ng/ μ l of $P_{numr-1}::NUMR-1-GFP$ was co-injected with 5 ng/ μ l $P_{hsf-1}::HSF-1-mCherry$. To visualize transgenic animals, nematodes were mounted on a 3% agar pad and anesthetized using 150 mM sodium azide. Images were captured using a Zeiss LSM 510 confocal microscope.

Table 2.2: Primer sequences used for promoter fusion constructs

Primer sequences used for $P_{numr-1}::mCherry$ reporter gene

Primer	Sequence (5' to 3')
Primer A	CCGTAACCCGGACTGAGTGCCTGCCTATGTTTTCAAT
Primer B	GATGATGGCCATGTTATCCTCCTCGCCCTTGCTCACCATG-GTTGCAGTTGTAGTTTTTCATTTTTTCAGAATGATTTTC
Primer C	ATGGTGAGCAAGGGCGAGGAGGA
Primer D	CCACTGAGCCTCAAACCCAAACCTTCTTCCG
Primer D'	ATCTTTCTTGCATCGTGCTCATC

Primer sequences used for $P_{numr-1}::GFP$ reporter gene

Primer	Sequence (5' to 3')
Primer A	GGACTGAGTGCCTGCCTATGTTTTCAATCTGATTAG
Primer B	GGGACAACCTCCAGTGAAAAGTTCTTCTCCTTTACTCATGGT-TGCAGTTGTAGTTTTTCATTTTTTCAGAATGATTTTC
Primer A'	GGTCTGAAATCTGGAGAGCG
Primer C	ATGAGTAAAGGAGAAGAAGCTTTTCACTGGAGTTGTCCC
Primer D	CCACTGAGCCTCAAACCCAAACCTTCTTCCG
Primer D'	ATCTTTCTTGCATCGTGCT

Table 2.2: Continued. Primer sequences used for promoter fusions

Primer sequences used for $P_{numr-1}::GFP$ reporter gene

Primer	Sequence (5' to 3')
Primer A	CTGTAAAAATTTGCTCTCCAGATTCAGACCCTAATC
Primer B	GGGACAACCTCCAGTGAAAAGTTCTTCTCCTTTACTC- ATGGTTGCAGTTGTAGTTTTTCATTTTTCAGAATGATTTTC
Primer C	ATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCC
Primer D	CCACTGAGCCTCAAACCCAAACCTTCTTCCG
Primer D'	ATCTTTCTTGCATCGTGCT

Table 2.3: Primer sequences used for $P_{numr-1}::NUMR-1-GFP$ reporter gene

Primer	Sequence (5' to 3')
Primer A	GGACTGAGTGCCTGCCTATGTTTTCAATCTGATTAG
Primer B	GGGACAACCTCCAGTGAAAAGTTCTTCTCCTTTACTCATTCGACCAAATCTGCG- TTCAAATGGATGTTGTCTC
Primer A'	GGTCTGAAATCTGGAGAGCG
Primer C	ATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCC
Primer D	CCACTGAGCCTCAAACCCAAACCTTCTTCCG
Primer D'	ATCTTTCTTGCATCGTGCT

Table 2.4: Primer sequences used for $P_{hsf-1}::$ HSF-1-mCherry fusion

Primer sequences used for $P_{hsf-1}::$ HSF-1 cDNA Fusion

Primer	Sequence (5' to 3')
Primer A (hsf-1 promoter forward)	CGGTAAAAATGGGAAACCCTAAAATTAATGGCAAAG
Primer B (hsf-1 promoter reverse with cDNA fusion overhang)	GTATTTGATTCCCTGTTGGCTGCATTTTACGAACTAGC- ACGCGGTATCTGAAATTATTATATATAAAATATTAG
Primer C (HSF-1 cDNA forward)	ATGCAGCCAACAGGGAATCAAATACAACAAAACCAG
Primer D (HSF-1 cDNA reverse)	AACCAAATTAGGATCCGATGGACTTGGAGTACGAAGA

Primer sequences used for $P_{hsf-1}::$ HSF-1-mCherry fusion

Primer	Sequence (5' to 3')
Primer A	CGGTAAAAATGGGAAACCCTAAAATTAATGGCAAAG
Primer B	CATGTTATCCTCCTCGCCCTTGCTACCA- TAACCAAATTAGGATCCGATGGACTTGGAGTAC
Primer C	ATGGTGAGCAAGGGCGAGGAGGA
Primer D	CCACTGAGCCTCAAACCCAAACCTTCTTCCG
Primer D'	ATCTTTCTTGCATCGTGCTCATC

2.3.2 Feeding assay

Feeding assays were modified from Boyd et al. (2007) [100]. Nematodes were transferred to the sample cup of the COPAS Biosort [101] (Union Biometrica Inc., Somerville, MA, USA) and diluted to approximately 1 nematode/ μL . The COPAS Biosort was used to sort nematodes based on two size characteristics, length or “time of flight” (TOF) and optical density or “extinction” (EXT). Twenty-five adults were then dispensed into each well of a 96-well plate, containing a mixture of K-medium [102] and OP50 *E. coli* at a final volume of 50 μL . After 4 h, 5 μL aliquots of Fluoresbrite® polychromatic red microspheres (0.5 μm diameter; diluted 1:20 in K-medium) (Polysciences, Inc., Warrington, PA) were pipetted into each well and rotated on a nutator mixer for 5 min. The nematodes were allowed to ingest the microspheres for 10 additional min (15 min total) and were then anesthetized using 5 μL of sodium azide (10 mM, final concentration), inhibiting further bead ingestion. Nematodes were aspirated from each well with the COPAS Biosort ReFLx; and the TOF, EXT, and level of red fluorescence for individual *C. elegans* were measured. Red fluorescence as measured by the COPAS Biosort quantified feeding over the 15 min microsphere exposure.

2.3.3 Dye-Filling Assay

To determine which neurons expressed *numr-1/-2*, nematodes expressing a *P_{numr-1}::GFP* transgene were stained by dye filling using DiI (Molecular Probes), which labels some chemosensory neurons [103, 104]. Nematodes were suspended in 1 ml of K-

medium, to which 10 μ l of DiI solution was added. The tube was shielded from light and incubated for 2 to 3 h at room temperature. After incubation, nematodes were washed with K-medium two times and put onto a K-agar plate for 1 h before being analyzed. Assignment of the cells was based on the location of the GFP expression in comparison with neurons stained with DiI (<http://www.wormatlas.org/>).

2.3.4 Stress tolerance assays

To determine the importance of *numr-1/-2* in resistance to cadmium toxicity, we carried out a stress tolerance assay comparing the lifespan of nematodes over-expressing *numr-1/-2* to nematodes carrying the selectable marker alone. Lifespan assays were performed essentially as described by McColl et al. [105]. Assays were carried out at 20°C, on nematode-growth medium (NGM) plates containing 0 or 100 μ M cadmium chloride [96]. Embryos were synchronized by treatment of adult hermaphrodites with a mild bleach solution and allowed to develop for two days. At the L4/ young adult stage, nematodes were transferred to control plates or plates containing 100 μ M cadmium chloride and scored daily. Nematodes were scored as dead when they no longer responded to gentle prodding with a platinum wire. Lifespan was defined as the time elapsed from the time L4/ young adult nematodes were picked to NGM plates (lifespan = 0) to when they were scored dead. Nematodes that crawled off the plates during the assay were excluded from calculations. The data was analyzed using GraphPadPrism

5.0. Survival curves were compared using the log-rank (Mantel-Cox) test and a Bonferroni post-hoc test ($p < 0.05$) was used to adjust for multiple comparisons [106].

Quantitative RT-PCR indicated that *numr-2* was still actively transcribed in response to cadmium exposure in the *numr-1* null background (data not shown). Thus, to further reduce *numr-1/2* expression levels, wild-type N2 and the *numr-1* mutant allele, *ok2239*, were fed *E. coli* engineered to express *numr-1/2* dsRNA and the effects of reduced *numr-1/2* expression levels on lifespan and cadmium resistance were measured. Synchronized L1s were fed *E. coli* engineered to express *numr-1/2* dsRNA, allowed to become gravid adults and egg lay overnight. The progeny were then grown on RNAi-expressing bacteria until they were L4/young adults at which time they were transferred to fresh RNAi plates and lifespan was scored. At time of death, nematodes were also scored for bagging. All lifespan assays were repeated in at least one other independent experiment with similar effects.

2.3.5 Thapsigargin, tunicamycin, and A23187 exposures in *C. elegans*

To determine the effects of endoplasmic reticulum (ER) stress and alterations on intracellular calcium levels on *numr-1/2* expression, we exposed one day old adults carrying the *P_{numr-1}::NUMR-1-GFP* transgene to thapsigargin, tunicamycin, or the calcium ionophore A23187 [107-109]. Assays were carried out at 20°C, on NGM plates containing either 5 μ M thapsigargin, 5 μ g/ μ L tunicamycin, 20 μ M A23187, 1% DMSO as a vehicle control, or 100 μ M CdCl₂ as a positive control for 5 h.

2.3.6 *sca-1* RNA Interference

An initial RNAi screen consisting of 15 targeted genes was performed using the Ahringer bacterial RNAi library (MRC Gene Service, University of Cambridge, UK) and the Vidal ORFeome bacterial RNAi library (Open Biosystems, Huntsville, AL). RNA interference was induced by feeding nematodes bacteria producing dsRNA [110] of the following genes: *ncs-1*, *crt-1*, *itr-1*, *plc-3*, *unc-43*, *egl-8*, *stim-1*, *pkc-1*, *cnx-1*, C54E10.2, *grk-1*, *tax-6*, *osm-9*, *tax-2*, and *sca-1*. L3/ L4 stage *NUMR-1*-GFP nematodes were transferred to RNAi feeding plates and allowed to grow for 24 h before being transferred to fresh RNAi feeding plates containing 0 or 100 μ M CdCl₂. The effects on *numr-1/-2* transcription were assessed after an additional 4 or 24 h. Knockdown of *sca-1* increased *numr-1/-2* transcription under basal conditions. Thus, *sca-1* RNAi was chosen for a second round of RNAi. The *sca-1* RNAi-expressing bacteria were obtained from the ORF-RNAi feeding library. L2/ L3 stage *NUMR-1*-GFP nematodes were transferred to RNAi feeding plates with *sca-1* RNAi-expressing bacteria until they were one day old adults at which time the expression of the *P_{numr-1}::NUMR-1*-GFP transgene was assessed.

2.4 Growth of *C. elegans* and isolation of total RNA for quantitative real-time RT-PCR (qRT-PCR)

2.4.1 Growth of staged *C. elegans* for qRT-PCR

To quantitatively measure changes in *numr-1/-2* expression during development, qRT-PCR was used to assess the steady-state mRNA levels of *numr-1/-2* in the Bristol N2 strain during 6 distinct stages of development (embryo, L1, L2, L3, L4, adult). To yield

stage-specific nematode populations, gravid hermaphrodites were treated with a mild bleach solution [111], and isolated eggs were incubated overnight in M9 buffer without food. The resulting L1-staged nematodes were then recovered by centrifugation, transferred to seeded K-agar plates, and allowed to feed at 20°C for 3 h before being collected, washed, and rapidly frozen as pellets in liquid nitrogen, and stored at -80°C, as previously described [112]. For isolation of L2, L3, L4, and adult nematodes, L1-stage nematodes were fed *E. coli* OP50 at 20°C for an additional 16, 25, 34, or 45 h, respectively. After visual confirmation of stage, nematodes were collected as previously described [112].

2.4.2 Growth and stress treatments of mix-staged *C. elegans* for qRT-PCR

For cadmium exposure studies investigating the time course of steady-state *numr-1/-2* mRNA accumulation, the Bristol N2 strain of *C. elegans* was used. Nematodes were cultured in 150 ml of S-medium at 20°C for five days using *E. coli* OP50 as a food source [112]. Additional *E. coli* OP50 was added after three days to maintain an adequate amount of food. Nematodes were exposed to 100 µM cadmium chloride for the last 4, 12, 24 or 36 h of incubation in the S-medium. At the end of the five-day culture, nematodes were collected as previously described [112].

For studies assessing the effects of various metal treatments and heat shock on *numr-1/-2* mRNA levels, the N2 strain of *C. elegans* was cultured in 70 ml of K Plus-medium (K-medium plus 1 M CaCl₂, 1 M MgSO₄, and 5 mg/mL cholesterol) at 20°C for

five days. Metal stress was evoked by exposing the nematodes to the following metals at the indicated concentrations for the last 24 h in K Plus-medium: 100 μ M sodium arsenite, 100 μ M cadmium chloride, 100 μ M cobalt chloride hexahydrate, 100 μ M chromium oxide, 100 μ M copper sulfate, 25 μ M mercury chloride, 100 μ M nickel sulfate hexahydrate, and 100 μ M zinc sulfate heptahydrate. Metal stock solution was directly added to the culture medium. The heat shock condition was at 37°C for the last 30 minutes of the five-day liquid culture. At the end of the five-day culture, nematodes were collected as previously described [112].

2.4.3 Isolation of total RNA from nematodes and qRT-PCR

Total RNA was isolated from staged nematodes using a Qiagen RNeasy Midi Kit (Qiagen). Frozen nematode pellets were ground into a fine powder using a liquid nitrogen-chilled mortar and pestle before being homogenized in a cell lysis buffer. Isolated RNA was subsequently purified using RNase-Free DNase Set and RNeasy Midi Kit (Qiagen) to eliminate possible DNA contamination prior to qRT-PCR. RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clare, CA, USA) using RNA 6000 Nano LabChip kits prior to qRT-PCR. qRT-PCR was performed using QuantiTect SYBR Green RT-PCR kits (Qiagen) following the manufacture's instructions in an ABI Prism 7900H system (Applied Biosystems, Foster City, CA, USA). At least three biological replicates for each treatment were prepared, and each biological replicate was measured in triplicate.

To investigate the change in *numr-1/-2* steady-state mRNA levels during development, *numr-1/-2* mRNA level measurements were normalized to a non-variable control gene, *rpb-12* (RNA Polymerase II (B) subunit) [113]. The fold change in *numr-1/-2* steady-state mRNA levels during development was calculated via the comparison of *numr-1/-2* expression in adult nematodes. Final results are presented by mean $\log_2(\text{fold change}) \pm$ standard error (SE; $n=3$). Primer sequences can be found in Table 2.5.

To investigate changes in the steady-state mRNA level of *numr-1/-2* in response to various stress conditions, *numr-1/-2* expression was normalized to *mlc-2* (myosin light chain) before the fold change in gene expression was calculated. The fold change in *numr-1/-2* transcript following metal exposure or heat shock was calculated via the comparison of *numr-1/-2* expression in untreated *C. elegans* samples. Final results are presented by mean $\log_2(\text{fold change}) \pm$ standard error (SE; $n=3$). qRT-PCR was performed as described above, except an ABI Prism 7000 system was used (Applied Biosystems). Refer to Table 2.5 for primer sequences used for *C. elegans* qRT-PCR.

Table 2.5: Primer sequences used for *C. elegans* qRT-PCR

Gene	Forward (5' to 3')	Reverse (5' to 3')
<i>mlc-2</i>	TTGACAGGAACTGACCCAGAGG	ATAGCCTTGACCTCATCCTCG
<i>cdr-1</i>	TCTTCTCTCAATTGGCAACTG	TTTGGGTAAACTTCATGACGA
<i>numr-1/-2</i>	GGAAGACGAGGAATGGATCATC	CTGCGTTCAAATGGATGTTGTCTC
<i>rpb-12</i>	CGCCGAAAATGAAATCAAAC	GGGCGTCGTACACCATCA

2.5 Calcium imaging in *C. elegans*

Calcium oscillations were measured in one day old transgenic nematodes expressing the YC 6.1 regulated by the *nhx-2* promoter. Transgenic nematodes were imaged on 500 μ l NGM pads seeded with 50 μ l *E. coli* OP50. Optical recordings were performed on a Zeiss LSM 510 confocal microscope using a 10x objective. The FRET signal was monitored as described below in Section 2.8.2. Cellular calcium dynamics were determined by the fluorescent ratio of YFP and CFP emissions (F_{530}/F_{490}) [95]. The first four fluorescent ratio frames at the beginning of the acquisition were averaged and used to normalize the subsequent emission ratios.

2.6 Analysis of posterior body wall muscle contraction (pBoc) in *C. elegans*

Posterior body wall muscle contraction (pBoc) was monitored at room temperature in one day old adult nematodes expressing the YC 6.1 transgene. pBoc rhythmicity in individual nematodes was assessed by calculating the coefficient of variance (CV), which is the standard deviation of the pBoc period expressed as a percent of the mean, from 5 successive contractions.

2.7 Cell culture

2.7.1 Cell maintenance

Stably transfected human embryonic kidney HEK293 cells expressing the yellow cameleon 3.60 (YC 3.60; a generous gift from Dr. Atsushi Miyawaki) were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% fetal

bovine serum, and Geneticin (G418) at a final concentration of 250 $\mu\text{g}/\text{ml}$. The cells were maintained at 37°C in a 5% CO₂ incubator. Stably transfected cells were used for all cell culture experiments. In preparation for calcium measurements, cells were cultured to about 70% confluence and then transferred onto 30 mm round glass coverslips (#1.5 thickness). The cells allowed to attach for a period of 12 h, after which additional DMEM was added to the coverslip, and the cells maintained in culture for an additional 24-36 h before use in calcium measurements.

2.7.2 HEK293 cell viability assays

Sensitivity of HEK293 cells to increasing concentrations and exposure times to cadmium chloride was determined by the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega Corporation). This assay measures cell viability by level of ATP content [114]. HEK293 cells stably transfected with the YC 3.60 construct were plated in 96-well plates and allowed to grow for 48 h. Cells were exposed to 0, 1, 3, 10, or 30 μM cadmium chloride for the last 24 or 4 h of culture time. After 48 h, cells were treated with 0.1 ml of CellTiter-Glo™ reagent, lysised for 2 minutes, and allowed to incubate for an additional 10 minutes at room temperature to stabilize luminescent signal. Luminescence was recorded using a BMG FLUOstar luminometer. Relative light units (RLU) were corrected for background using readings of an equivalent amount of cell culture medium without cells. Tests were performed in quadruplicates and each point represents the mean \pm SEM

($n = 4$) of four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's post-test.

2.7.3 Isolation of total RNA from HEK293 cells and qRT-PCR

To assess the effects of cadmium-induced transcription in a HEK293 cell line, qRT-PCR was used to quantify the steady-state mRNA levels of *mt-1*, *c-fos*, and *grp-78*. Cells were grown on plastic 100 x 20 mm cell culture plates using complete DMEM medium and allowed to grow for 48 h before being harvested. Cells were treated with cadmium chloride or thapsigargin at the indicated concentrations for the last 1, 4, or 24 h of the 48 h culture. Total RNA was collected from both treated and control HEK293 cells using an RNeasy Mini Kit (Qiagen).

Two-step qRT-PCR was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and SYBR Green (Qiagen). A total of 0.5 μg of total RNA was reverse transcribed to yield cDNA and transcription was determined by using an ABI 7900H system. Gene expression was normalized to actin before the fold change in gene expression was calculated. The fold changes in gene expression in cadmium-treated cells were calculated via the comparison of gene expression of that gene in untreated cells. The fold changes in gene expression in thapsigargin-treated cells were calculated via the comparison of gene expression of that gene in vehicle-treated (DMSO) control cells. Three biological replicates for each treatment were prepared, and each

biological replicate was measured in triplicate. Final results are presented by mean $\log_2(\text{fold change}) \pm \text{SEM}$. Primer sequences can be found in Table 2.6.

Table 2.6: Primer sequences used for qRT-PCR in HEK293 cells. Primer sequences for *mt-1*, *c-fos*, and *grp-78* were designed using Primer3 (<http://fokker.wi.mit.edu/primer3>).

Gene	Forward (5' to 3')	Reverse (5' to 3')
<i>actin</i>	GATATCGCTGCGCTGGTCGTC	ACGCAGCTCATTGTAGAAGGTGTGG
<i>mt-1</i>	GCAAATGCAAAGAGTGCAAA	CAGCTGCACTTCTCTGATGC
<i>c-fos</i>	GATACACTCCAAGCGGAGACAGA	GTGAGCTGCCAGGATGAACT
<i>grp-78</i>	GTCTTTCACCTTCATAGACCTTGAT	GTCCCCTTACACTTGGTATTGA

2.8 Intracellular calcium ($[Ca^{2+}]_i$) measurements

2.8.1 Fura-5F-based calcium measurements

Coverslips with cells were mounted in a Teflon chamber and incubated in DMEM with 1 μM fura-5F/AM at 37°C in the dark for 25 min. Before $[Ca^{2+}]_i$ measurements were made, cells were washed 3 times and incubated for 15-30 min at room temperature (25°C) in a HEPES-buffered salt solution (HBSS, in mM: NaCl 120; KCl 5.4; Mg_2SO_4 0.8; HEPES 20; $CaCl_2$ 1.8 and glucose 10 mM, with pH 7.4 adjusted by NaOH). In these experiments, nominally calcium-free solutions were HBSS with no added $CaCl_2$.

The Teflon chamber was then mounted onto a Nikon TS-100 inverted microscope equipped with a 20x fluor objective (0.75 NA) and fluorescence images recorded and

analyzed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging Inc., Cincinnati, OH) equipped with a light sensitive CCD camera (Cooke PixelFly, ASI, Eugene, OR). Fura-5F fluorescence was monitored by alternately exciting the dye at 340 and 380 nm, and collecting the emission wavelength at 510 nm. Changes in intracellular calcium are represented as the ratio of fura-5F fluorescence due to excitation at 340 nm to that due to excitation at 380 nm (Ratio= F340/F380). The ratio changes in fields of fura-5F-loaded cells were collected from a multiple regions of interest (ROI), with each ROI representing an individual cell. Typically, 25 to 35 ROIs were monitored per experiment. In all cases, ratio values have been corrected for contributions by autofluorescence, which is measured after treating cells with 10 μ M ionomycin and 20 mM MnCl₂. Since many BAPTA-based calcium indicators can bind cadmium with very high affinity [74], we also used the fura-5F-loaded HEK293 cells to monitor the accumulation of intracellular cadmium ([Cd²⁺]_i).

2.8.2 YC 3.60-based calcium measurements

HEK293 cells stably expressing YC 3.60 were mounted in a Teflon chamber and maintained HBSS as described above, but not loaded with fura-5F. The Teflon chamber was mounted on the stage of a Zeiss LSM 510 confocal microscope equipped with a 20x objective (NA 0.8). Since YC 3.60 is a FRET-based calcium sensor, the FRET signal was monitored by exciting the cells at 458 nm (for CFP), and collecting the emission images at 475-525 for CFP and 530 nm for YFP. After correcting for background signals,

intracellular calcium levels ($[Ca^{2+}]_i$) were monitored by calculating the fluorescence ratio of YFP and CFP emissions (F_{530}/F_{490}). An increase in $[Ca^{2+}]_i$ will be observed as and increase in the (F_{530}/F_{490}) FRET ratio. Typically we monitored the fluorescence signals of 25-30 cells on a single coverslip. Data were analyzed by one-way ANOVA followed by Dunnett's post-test.

2.8.3 Effects of cadmium exposure on intracellular $[Ca^{2+}]$ pools

Briefly, the status of intracellular calcium pools was measured by exposing HEK293 cells to 10 μ M ionomycin in the presence of 3mM BAPTA [115]. The observed transient calcium response is an indication of the size of intracellular calcium pools, and can be quantified by calculating the peak YC 3.60 response (as defined by the peak response minus the resting fluorescence ratio). Data were analyzed by one-way ANOVA followed by Dunnett's post-test.

2.9 Human cAMP/ calcium Signaling RT² ProfilerTM PCR Array

To determine the effects of cadmium exposure on calcium-responsive gene expression in HEK293 cells, Human cAMP/ calcium Signaling RT² ProfilerTM PCR Arrays were used to analyze the gene expression profile of 84 genes known to be responsive to cAMP or calcium (SABiosciences, PCR array PAHS-066). Cells were grown and RNA was isolated as described above. The purity and quality of extracted RNA were further assessed using the RNA 6000 LabChip and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clare, CA, USA). Procedures for array analysis were carried out

according to manufacturer's protocol. Briefly, 0.5 µg of total RNA was used as a template for reverse-transcription of cDNA. The cDNA was then diluted and added to a SYBR Green master mix. Aliquots (25 µl) of this mix were added to each well of a 96-well plate, where each well contained a gene-specific primer set.

The array data was normalized to the average threshold cycle (C_t) value of the following three endogenous housekeeping genes on the PCR array: Beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase (HPRT1), ribosomal protein L13a (RPL13A). These housekeeping genes were chosen for PCR array normalization because the C_t value did not differ by more than one cycle across the compared samples with the exception of cells exposed to 30 µM cadmium chloride for 24 h. Under this exposure condition the average C_t value for the three housekeeping genes did differ by more than one cycle. Thus, to make a meaningful biological analysis of differentially expressed genes in cells treated with 30 µM cadmium chloride for 24 h, a normalization factor was calculated for each of the three independent experiments (0.91, 0.88, and 0.89) so that the normalized expression ratio of the average C_t value for each housekeeping gene was equal across compared samples (cadmium treated vs. control). This normalization factor was then used to appropriately scale the expression value for each of the 84 calcium specific genes within each array for cells treated with 30 µM cadmium chloride for 24 h. These normalized expression values were then used to determine fold change. Data was processed with SABiosciences web-based RT²

Profiler™ PCR Array Data Analysis to calculate the C_t value and relative gene expression values according to the standard $\Delta\Delta C_t$ method [116]. A list of differentially expressed genes was identified using a Student's t -test. Only two-fold or greater changes in gene expression with a $P < 0.05$ were considered significant and reported.

3. Results

3.1 Cloning and sequence analysis of *numr-1* and *numr-2* cDNAs

3.1.1 Cloning of *numr-1* and *numr-2*

Previous work in our laboratory identified *numr-1* (Nuclear Localized Metal-Responsive gene) as a highly inducible cadmium-responsive gene of unknown function [60]. *numr-1* was predicted to consist of only a single exon. Based on sequence homology; we identified a second *C. elegans* gene of high similarity to *numr-1*, *C. elegans* gene F08F8.1, which we designated as *numr-2*. *numr-1* and *numr-2* are located less than 1 kb apart in an inverse complement orientation on Chromosome III (Figure 3.1A). The 502 bp promoters of these two genes are 99% identical, separated by only 77 bp of unique DNA. *numr-2* was predicted (www.wormbase.org) to consist of 5 exons, in which the first exon was nearly identical to *numr-1*. *numr-2* was also predicted to initiate protein synthesis from a second ATG that was 221 bp downstream and in-frame from the first predicted translation initiation codon that was in an analogous position to that of the predicted ATG for *numr-1*.

To verify the predicted cDNA sequences of *numr-1* and *numr-2*, 3'- and 5'-RACE were used. Sequencing of the *numr-1* cDNA clones isolated from the 3' RACE assay was consistent with the predicted 3' end cDNA sequence for *numr-1*. Using the 3' RACE assay, however, we were unable to isolate cDNA corresponding to the predicted 3' end of *numr-2*, suggesting that *numr-2* was annotated incorrectly.

To confirm the 5' end of *numr-1* and *numr-2*, 5'RACE was used. Although *numr-1* and *numr-2* were predicted to start translation at two different start codons, cDNA corresponding to only one translation initiation site was isolated using 5' RACE. The 5' end identified using the RACE assay corresponded with the predicted ATG of *numr-1*. Only a single consensus TATA sequence was identified 40 base pairs upstream from the first translational start codon, but none was found in the analogous position upstream of the second predicted translational start codon. Our data therefore suggest that *numr-2* was annotated incorrectly and consists of only a single exon that is 100% identical to *numr-1* (Figure 3.1B). Therefore, *C. elegans* gene F08F8.1 was renamed *numr-2*. The full-length cDNA sequence of *numr-1* and *numr-2* are 1029 bp and contain open reading frames of 846 bp. The 5'-untranslated regions identified using 5'RACE are roughly 12 bp in length and the 3'-untranslated regions consisted of 142 bp terminating with a poly(A) tail with typical polyadenylation signals.

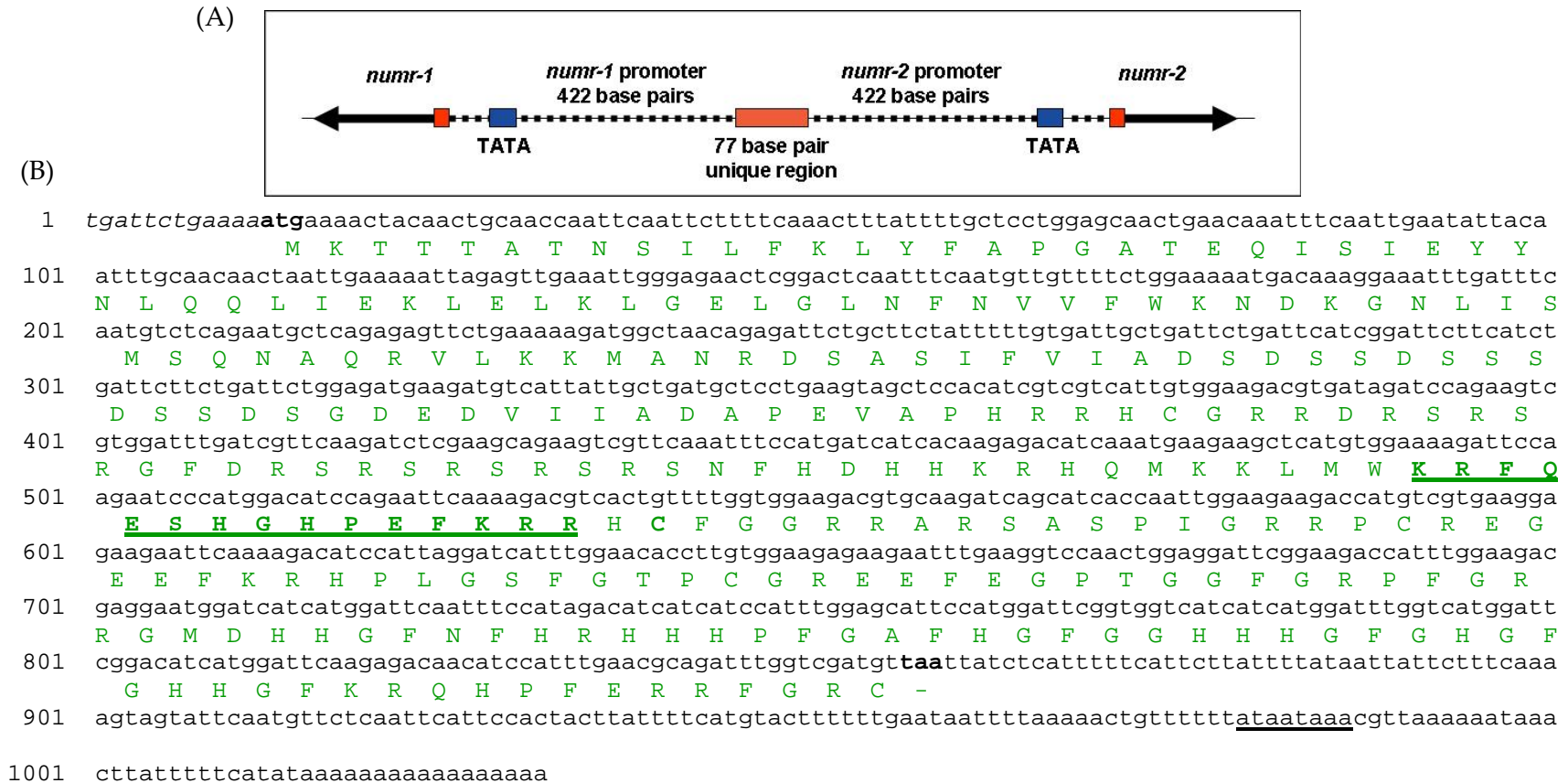


Figure 3.1: Sequences of NUMR-1/-2 cDNA and protein. (A) Gene organization of *numr-1* and *numr-2* (B) The nucleotide sequence of the NUMR-1/-2 cDNA is shown with the derived amino acid sequence presented below the corresponding codons. The sequence of the 5'-untranslated region is shown in italics. The translation start and stop codons are presented in boldface. The polyadenylation signal is underlined. The amino acid sequence for a predicted bipartite nuclear targeting sequence is shown in bold and underline.

3.1.2 Protein motif analysis of NUMR-1 and NUMR-2

The proteins encoded by *numr-1* and *numr-2* are 281 amino acids in length. ProSite analysis was used to identify potential functional protein domains within NUMR-1/-2¹ (Table 3.1) [117]. The NUMR-1/-2 proteins contain an arginine rich region near a predicted nuclear localization signal, which could imply that *numr-1/-2* plays a role in RNA processing because this region is similar to that of other RNA-binding proteins with arginine rich domains [118, 119]. ProSite analysis also identified two potential Casein Kinase II phosphorylation sites. Casein Kinase II has been shown to be activated in cells exposed to cadmium suggesting that *numr-1/-2*² may be part of a signaling cascade that is affected by cadmium exposure [62, 120]. A histidine rich region was also identified in NUMR-1/-2, which could suggest metal-binding capabilities. A group of copper-zinc superoxide dismutases contain a high affinity histidine-rich metal-binding domain that can directly bind copper [121, 122].

¹ NUMR-1 refers to just the NUMR-1 protein, whereas NUMR-1/-2 refers to both the NUMR-1 and NUMR-2 proteins

² *numr-1* refers to just the expression of *numr-1*, whereas *numr-1/-2* refers to both *numr-1* and *numr-2*

Table 3.1: Predicted protein motifs within NUMR-1/-2

Motif	Residues
Arginine-rich region	118-194
Bipartite nuclear targeting sequence	160-176
Histidine-rich region	234-272
N-myristoylation sites	GLNFNV (46-51) GNLISM (59-64) GGRRAR (178-183) GTPCGR (208-213) GAFHGF (247-252)
Casein Kinase II phosphorylation sites	SSSD (94-97) SGDE (101-104)
Amidation sites	CGRR (121-124) GGRR (178-181) IGRR (188-191) FGRR (227-230)

3.1.3 Putative upstream regulatory elements within the *numr-1* and *numr-2* promoters

In order to identify potential motifs important for regulation of *numr-1/-2* expression, we searched for putative upstream regulatory elements within the proximal promoters of *numr-1* and *numr-2* (Table 3.2). Highly conserved Sp1 consensus sites were identified upstream of the TATA boxes. Sp1 belongs to the Sp family of zinc-finger transcription factors, which are ubiquitously expressed. This family of transcription

factors have been found to be essential for the proper expression of a large variety of cellular genes that take part in a diverse array of cellular functions, including cell proliferation, apoptosis, and differentiation [123, 124]. In addition, GATA-1 motifs were found in the *numr-1/-2* promoters. The transcription factor ELT-2 is known to bind to GATA motifs and regulates the expression of many intestinal genes in *C. elegans* [125]. Furthermore, binding motifs for two well-studied stress responsive transcription factors, DAF-16 and SKN-1, were identified in the promoter regions of *numr-1/-2*, which is consistent with a possible role for *numr-1/-2* in stress response [86, 126, 127]. A binding site for the *C. elegans* UNC-86 transcription factor was also identified. A study by Sze et al. (2003), demonstrated the importance for proper UNC-86 activity in modulating olfactory sensitivity in *C. elegans* [128]. There is also data indicating that UNC-86 is required for proper cell determination and differentiation for several different neuronal lineages, including those of egg-laying neurons [128]. Finally, a binding site for a highly responsive calcium transcription factor found in mammals, NFAT, was also identified [129]. However, a *C. elegans* homolog for the mammalian NFAT has yet to be identified.

Table 3.2: Putative upstream regulatory elements of the *numr-1* and *numr-2* promoters.

^a Positions are relative to *numr-1* translation start site. ^b Inverse complement of the consensus sequence.

<i>C. elegans</i> homolog	Element	Sequence and location in <i>numr-1</i> ^a
	TATA box	TATAAAA (-43 to -37)
	GATA	GATA (-169 to -176)
	GAGAFACOR	GGAGAGCGAAA (-443 to -432)
	NFAT	TAAAAATTGGAAATTTAG (-42 to -24)
<i>mef-2</i>	MEF-2	CTGTAAAAATTCGCT ^b (-440 to -424) AAGTGATCGAAAAATAGTTTTT ^b (-422 to -400)
<i>mef-2</i>	RSRFC4 (MEF-2A)	CTGTAAAAATTCGCT ^b (-440 to -424) AAGTGATCGAAAAATAGTTTTT ^b (-422 to -400)
<i>sptf-3/ sptf-2</i>	SP1	GGTAGGCGGAGCT (-66 to -56)
<i>nhr-64/ nhr-69</i>	TCF (NHF4alpha)	GAATATCAAAAGATCA (-184 to -168)
<i>C. elegans</i> specific	SKN-1	TACTGTCATCAC ^b (-147 to -135)
<i>C. elegans</i> specific	UNC-86	CATTAGAAAT ^b (-333 to -323)
<i>C. elegans</i> specific	CES-2	ATTTCGAAAT ^b (-95 to -85)
<i>C. elegans</i> specific	DAF-16	AAATTGTTTAATGT ^b (-105 to -91)
<i>C. elegans</i> specific	TRA-1	ATGGAGGT (-51 to -43)

3.2 Expression analysis of *numr-1* and *numr-2*

To determine the expression pattern of *numr-1* and *numr-2*, we generated transgenic nematodes harboring both the *numr-1* promoter fused to *mCherry* and the *numr-2* promoter fused to *gfp* (Figure 3.2A). Additionally, to determine the subcellular localization of NUMR-1, we fused the *numr-1* promoter and coding sequence to *gfp*, generating a $P_{numr-1}::NUMR-1$ -GFP translational fusion.

3.2.1 Expression of *numr-1* and *numr-2* is developmentally regulated

We found that $P_{numr-1}::mCherry$ and $P_{numr-2}::GFP$ were expressed similarly both temporally and spatially. In the absence of metal, the *numr-1* and *numr-2* promoters were expressed maximally in the intestine of L1 larvae. The expression decreased as nematodes continued to develop into adults (Figure 3.2B-F). In accordance with the expression pattern of *numr-1* and *numr-2*, qRT-PCR analysis showed that *numr-1/-2* steady-state mRNA levels varied significantly throughout development with the highest level of *numr-1/-2* steady-state mRNA accumulation being observed at the L1 stage (Figure 3.3). As the animal continued to develop past the L1 stage, *numr-1/-2* steady-state mRNA abundance gradually decreased.

In the absence of metal, adult nematodes expressed both *numr-1* and *numr-2* in the same pair of sensory neurons in the head, the egg-laying muscles of the vulva, and a few cells in the posterior end of the nematode (Figure 3.2F). To determine which sensory neurons *numr-1* and *numr-2* were expressed, we exposed transgenic nematodes

harboring only the $P_{numr-1}::GFP$ construct to the fluorescent dye DiI. DiI preferentially fills six amphid head neurons (ASI, ADL, ASK, AWS, ASH and ASJ) and the two phasmid tail neurons (PHA and PHB) [130]. Expression of *numr-1* (green fluorescence) was detected in a pair of sensory neurons with processes extending towards the external environment, but this expression did not overlay with cells stained by DiI (red fluorescence), suggesting that the *numr-1/-2* was constitutively expressed in one of the amphid wing neuron pairs (Figure 3.4) [131].

(A)

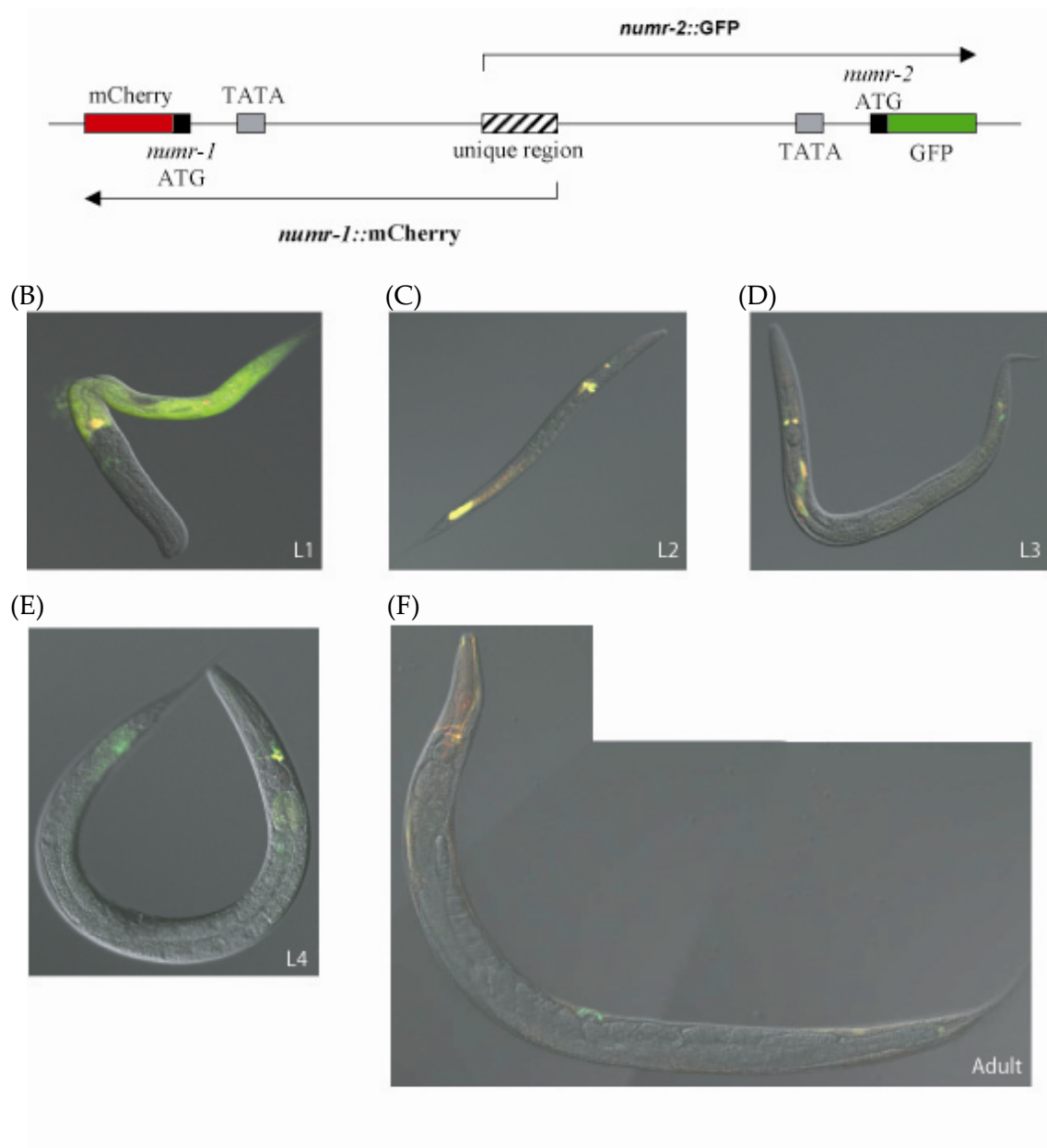


Figure 3.2 Promoter activity of *numr-1* and *numr-2*. (A) Construction of the $P_{numr-1}::mCherry$ and $P_{numr-2}::GFP$ promoter transgenes. (B - F) Differential expression of *numr-1* and *numr-2* during development in *C. elegans*.

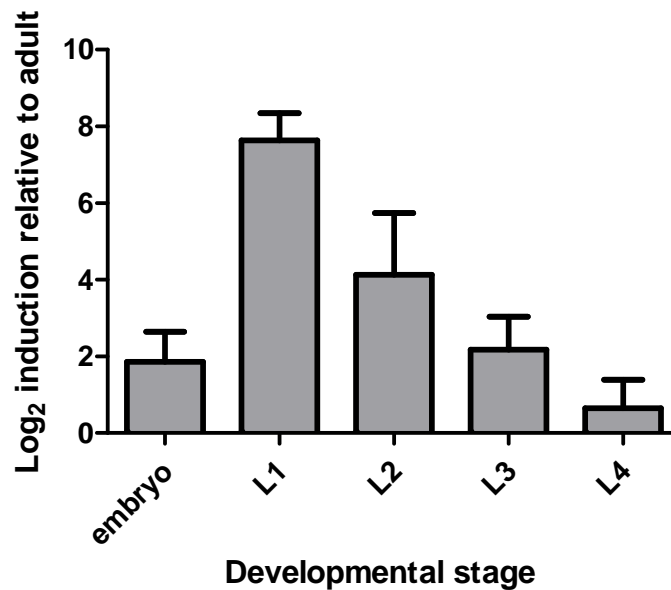


Figure 3.3: Constitutive expression of *numr-1/-2* is developmentally regulated. Quantitative RT-PCR analysis of changes in *numr-1/-2* steady-state mRNA levels during *C. elegans* development. Total RNA was isolated from different developmental stages of *C. elegans*, and steady state mRNA levels of *numr-1/-2* were analyzed in Egg, L1, L2, L3, L4, and adult. All measurements were normalized to the mRNA level of *rbp-12* and the fold change was normalized to the mRNA levels in adult nematodes. Results are displayed in mean $\log_2 \pm SE$ (n=3).

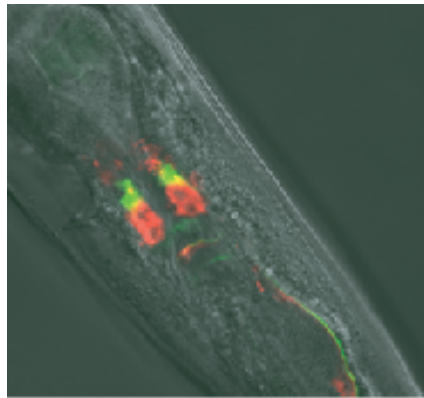


Figure 3.4: Expression of *numr-1/-2* in the AWA neurons. Overlay of *numr-1/-2* expression (green fluorescence) in the hermaphrodite head and the DiI staining of the ciliated amphid neurons (red fluorescence). The cells that are stained by DiI are not expressing GFP indicating these are the AW sensory neurons.

3.2.2 Cadmium and other metals induce the expression of *numr-1* and *numr-2*

The $P_{numr-1}::mCherry$ and $P_{numr-2}::GFP$ transgenic nematodes were used to determine the expression pattern of *numr-1/-2* in response to metal treatment. Exposure of nematodes to cadmium resulted in increased *numr-1* and *numr-2* expression throughout the intestine and two bulbs of the pharynx (Figure 3.5A). In contrast, exposure to copper resulted in increased expression of *numr-1/-2* only within the pharynx (Figure 3.5B). We also used transgenic nematodes expressing the transcriptional reporter, $P_{numr-1}::GFP$, to test the effects of additional non-metal stress conditions on *numr-1* activation. Oxidative stress (200 μ M jugalone and hydrogen peroxide), starvation, heat shock (37°C 15 minutes and 35°C 1 hr) and pathogenic infection (*Serratia marcesans* and *Pseudomonas aeruginosa*) failed to activate $P_{numr-1}::GFP$ transcription (Table 3.3).

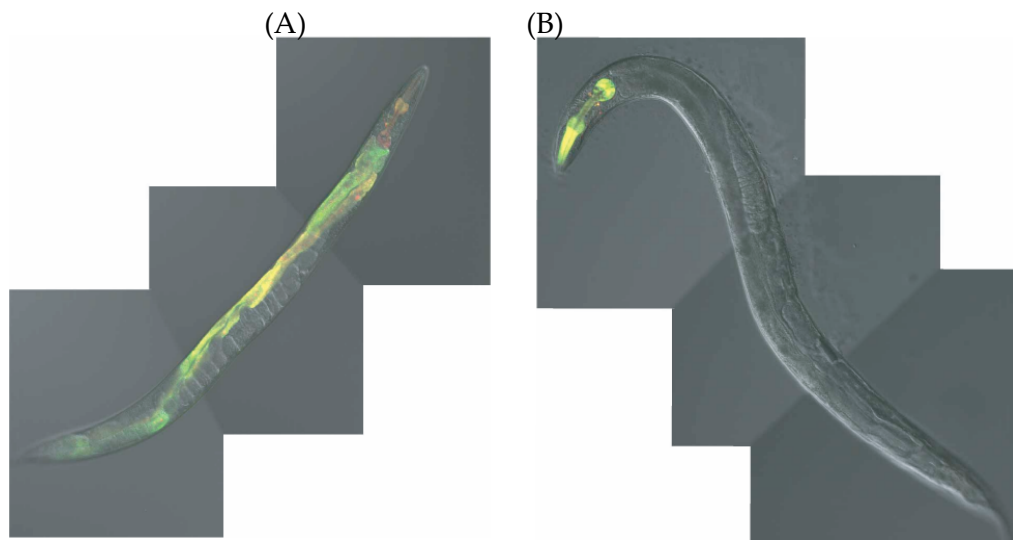


Figure 3.5: The effects of (A) cadmium and (B) copper on *numr-1* and *numr-2* expression in adult nematodes carrying both *P_{numr-1}::mCherry* and *P_{numr-2}::GFP*.

Table 3.3: The effects of various non-metal stress conditions on *numr-1/-2* expression

Treatment	Increase in GFP Expression
100 uM cadmium	YES
Heat shock	NO
Hydrogen peroxide	NO
Juglone	NO
Starvation	NO
<i>serratia marcesans</i>	NO
<i>pseudomonas aeruginosa</i>	NO

The time course of *numr-1/-2* induction following exposure to 100 μ M cadmium chloride was investigated using qRT-PCR (Figure 3.6). The *C. elegans* gene *cdr-1* was used as a positive control because *cdr-1* is known to be highly induced by cadmium exposure [132]. In mixed stage nematodes, expression of *numr-1/-2* increased with time in response to cadmium. Increase in *numr-1/-2* steady-state mRNA levels were detected after only a 4 h exposure to cadmium, and transcript levels continued to increase until 24 h, at which point maximal *numr-1/-2* transcript levels were reached. The time course of *cdr-1* steady-state mRNA accumulation was consistent with previous data from our laboratory [60].

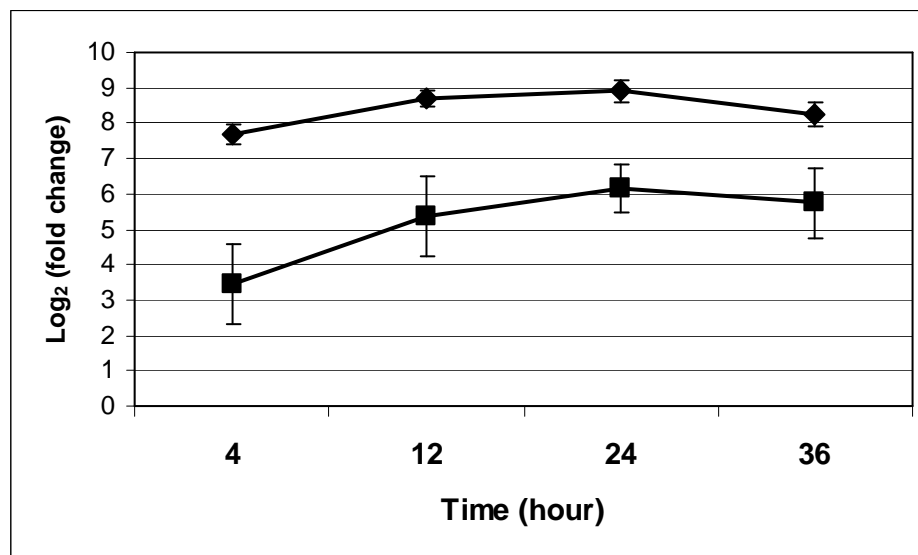


Figure 3.6: Time course of *numr-1/-2* transcript induction after cadmium treatment
 Total RNA was isolated from non-treated or cadmium-treated *C. elegans*, and mRNA levels of *cdr-1* (triangle) and *numr-1/-2* (square) genes were measured using qRT-PCR. All measurements were normalized to the mRNA level of *mlc-2* and the fold change was normalized to the mRNA levels in non-treated nematodes. Results are displayed in mean $\log_2 \pm$ SE (n=4).

To further quantify the response of *numr-1/-2* to additional metal treatments and to assess the effects of a more general stress condition on *numr-1/-2* transcript levels, qRT-PCR was used. Mixed stage wild type nematodes were treated with eight different metals: CdCl₂, CuSO₄, CoCl₂, CrO₃, NiSO₄, AsNaO₂, ZnSO₄, HgCl₂, and the effect of heat shock was also examined (Table 3.4). The qRT-PCR data along with the promoter data, indicated a range of metals can increase *numr-1/-2* transcript levels following a 24 h exposure. According to the qRT-PCR data, cadmium was the strongest inducer followed by copper. Heat shock did not result increased *numr-1/-2* mRNA abundance, suggesting *numr-1/-2* is a metal-specific responsive gene and not a general stress response gene.

Table 3.4: The effects of various metal treatments and heat shock on *numr-1/-2* steady-state mRNA levels. Total RNA was isolated from untreated or treated *C. elegans*, and mRNA levels of the *numr-1/-2* gene was measured using qRT-PCR. All measurements were normalized to the mRNA level of *mlc-2* and the fold change was normalized to the mRNA levels in non-treated nematodes. Results are displayed in mean $\log_2 \pm$ SE (n=3).

Stress Condition	$\log_2(\text{fold change}) \pm \text{standard error}$
100 μ M arsenite	1.97 \pm 0.06
100 μ M cadmium	1104.49 \pm 24.35
100 μ M cobalt	5.77 \pm 2.09
100 μ M chromium	7.67 \pm 2.70
100 μ M copper sulfate	16.12 \pm 6.73
25 μ M mercury	5.20 \pm 2.17
100 μ M nickel	3.37 \pm 1.80
100 μ M zinc	2.65 \pm 2.47
Heat shock	0.80 \pm 0.12

3.2.3 NUMR-1 protein localizes to discrete punctate structures within intestinal nuclei following exposure to cadmium

The *NUMR-1*-GFP fusion protein localized to the nuclei of all cells in which it was expressed. This suggests that the predicted NLS of NUMR-1/-2 is functional. In accordance with expression of *numr-1* and *numr-2*, and qRT-PCR data, the expression of NUMR-1 in the absence of metal was observed throughout the intestinal nuclei during the L1 larval stage and expression decreased as the animals developed into young adults. In the absence of metals, constitutive NUMR-1 expression in adult *P_{numr-1}::NUMR-1*-GFP animals was minimal. NUMR-1 was observed in the nuclei of chemosensory neurons in the head (most likely the amphid wing neurons), the nuclei of cells comprising the egg-laying muscles of the vulva, and a few cells in the tail (Figure 3.7A-C). These are the same cell types in which we observed expression of *numr-1* and *numr-2* (Figure 3.2B-F).

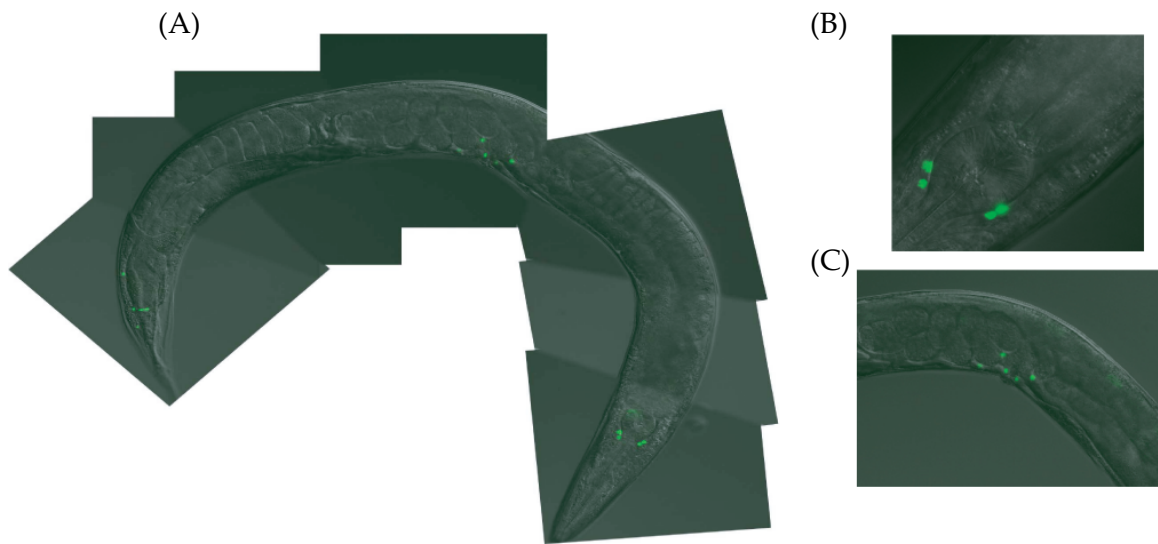


Figure 3.7: Expression of *NUMR-1-GFP* in adult nematodes (A) Constitutive expression of *NUMR-1* in adult *NUMR-1-GFP* animals in the absence of metal is observed in a pair of sensory neurons in the head, a few cells around the vulva and tail (B) Enlargement of *NUMR-1* expression in neuronal head cells (C) Enlargement of *NUMR-1* expression in cells around the vulva.

Following copper exposure, *NUMR-1* expression in adult nematodes increased only within the pharyngeal nuclei (Figure 3.8A), where as, cadmium stress induced *NUMR-1* expression throughout intestinal nuclei and multiple nuclei within the two bulbs of the pharynx (Figure 3.8B). After cadmium treatment, *NUMR-1* localized to discrete foci within the intestinal nuclei (Figure 3.9A). We proposed that these punctate nuclear structures might be nuclear stress granules. In cell culture, Heat Shock Factor-1 (HSF-1) has been shown to localize to nuclear stress granules under various types of stress conditions, including metal stress [133, 134].

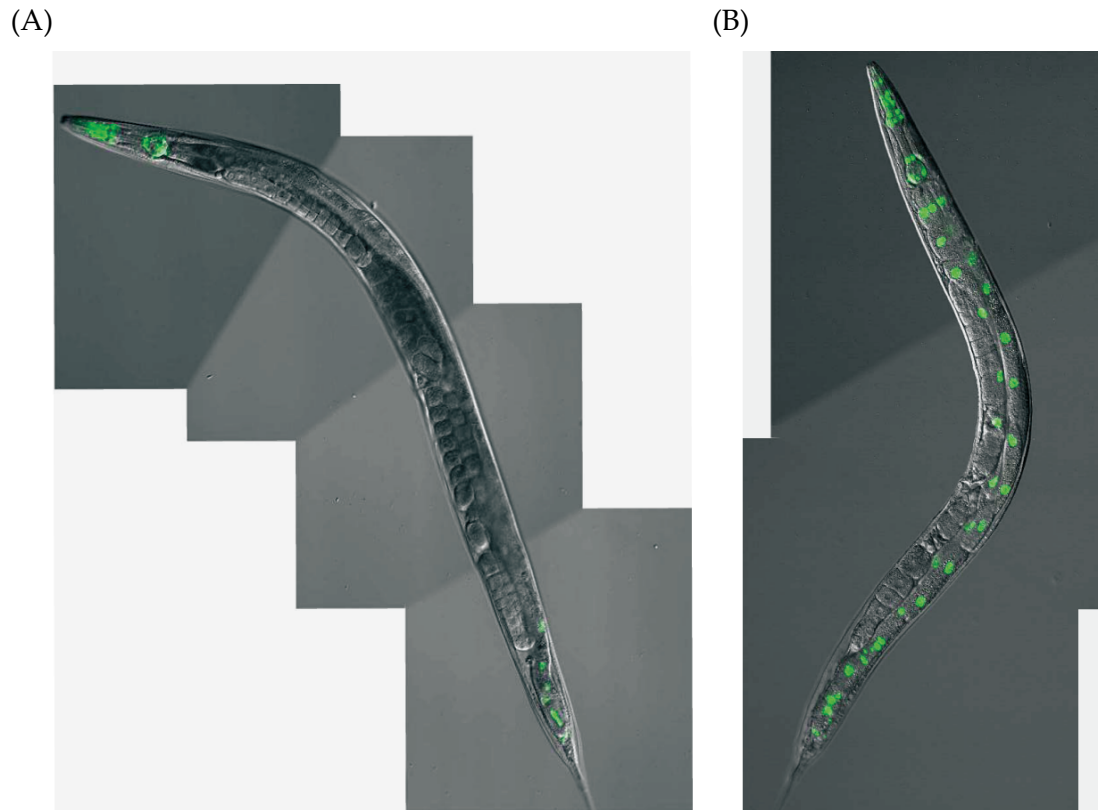


Figure 3.8: Expression of *numr-1* in adult NUMR-1-GFP animals after metal exposure. (A) exposure to 100 μM CuSO₄ for 24 h (B) exposure to 100 μM CdCl₂ for 24 h.

To test whether NUMR-1 localized to nuclear stress granules, we analyzed the location of *P_{numr-1}::NUMR-1-GFP* in animals also expressing a *P_{hsf-1}::HSF-1-mCherry* translational fusion. We found that NUMR-1 did colocalize with HSF-1 after cadmium treatment, suggesting that these punctate foci in the intestinal nuclei may be nuclear stress granules (Figure 3.9B and C). These punctate nuclear structures are not common among all nuclear localized stress response genes. When DAF-16 localizes to intestinal

nuclei in response to stress, its expression pattern is diffuse throughout the entire nucleus and not within discrete nuclear foci (Figure 3.9D). This result demonstrated that the punctate foci within which NUMR-1 and HSF-1 localized in response to stress, were not artifacts caused by the fluorescent protein fusion.

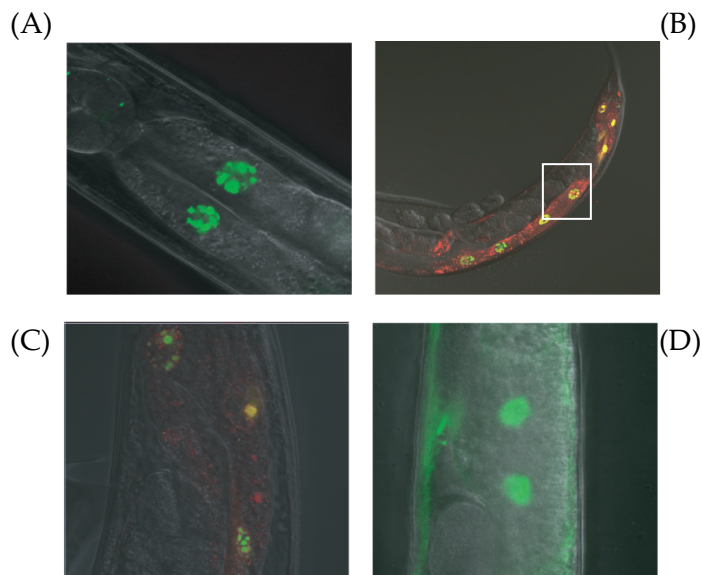


Figure 3.9: After cadmium exposure NUMR-1 localizes to discrete foci within the intestinal nuclei of adult NUMR-1-GFP animals. (A) *NUMR-1*-GFP expression in intestinal nuclei after exposure to 100 μ M CdCl₂ for 24 h (B) Nematodes co-expressing *NUMR-1*-GFP and *HSF-1*-mCherry after exposure to 100 μ M CdCl₂ for 24 h (C) Enlargement of the boxed portion of B. (D) Expression of *DAF-16*-GFP(*tj356*) in intestinal nuclei of animals exposed to 35°C for 1 h.

3.3 *NUMR-1* expression is required for certain muscular functions

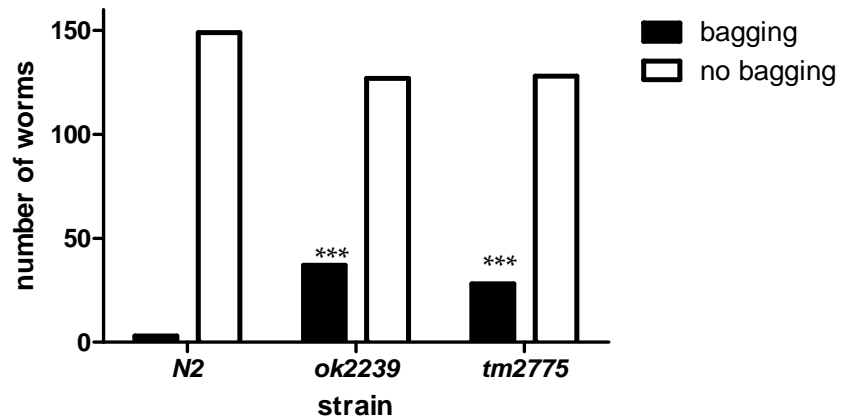
To gain a better understanding of the biological role of *numr-1/-2* during normal development, we investigated the effects of decreased *numr-1* expression in two different *numr-1* deletion alleles, *ok2239* and *tm2775*. The *numr-1* deletion allele, *ok2239*,

has a deletion that spans most of the coding region for *numr-1* plus the *numr-1* 3'UTR. Whereas, the *tm2775* mutant harbors a deletion that spans ~59 base pairs of the *numr-1* promoter, including the TATA box, and an additional 147 base pairs of the *numr-1* coding region. The coding region for *numr-2* is unaffected in either *numr-1* mutant.

First, we examined the egg-laying behavior of the *numr-1* mutants since *numr-1/2* expression was observed in the vulva muscles of adult hermaphrodites. To determine how the *numr-1* mutations might affect egg-laying, we quantified the number of adult nematodes that died of bagging. When nematodes fail to lay their eggs, embryos hatch inside the adult and this phenomena is referred to as bagging. We found that the *numr-1* deletions inhibit *C. elegans* egg-laying behavior (Egl phenotype) (Figure 3.10A). This data suggested to us that NUMR-1 function is required for proper egg-laying behavior, implying a role for *numr-1* in certain muscular functions.

We also investigated the effects of decreased *numr-1* expression on another muscular function, feeding. We found that the *numr-1* mutant alleles exhibited lower feeding levels (Figure 3.10B) when compared to wild-type nematodes [135]. Taken together, this data suggest a role for *numr-1* in certain muscular functions.

(A)



(B)

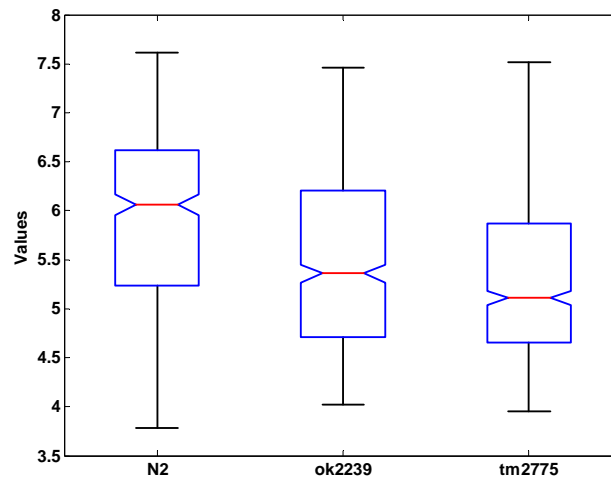


Figure 3.10: Proper *numr-1* expression is required for normal egg-laying and feeding
(A) Downregulation of *numr-1* increases the propensity of worms to bag. Fisher's exact test indicated a highly significant impact of genotype on the propensity of worms to bag *** $P < 0.001$, for comparison of wild-type to the *numr-1* mutant allele *ok2239* and *tm2775*.
(B) Comparison of wild-type and *numr-1* mutant *C. elegans* feeding. Boxplots of normalized fluorescence for adult nematodes aggregated over three independent experiments. The upper and lower limits of each box give the 25th and 75th percentiles of the data respectively, and the line inside the box gives the median.

3.4 NUMR-1/-2 is required for defense against cadmium treatment

To determine the importance of NUMR-1/-2 in protecting the nematode against metal stress; we measured the longevity of nematodes overexpressing NUMR-1/-2 raised in the presence or absence of 100 μ M cadmium chloride. We found that worms overexpressing NUMR-1/-2 from an extra chromosomal array were more resistant to cadmium stress ($p < 0.0001$) and slightly longer lived than control nematodes ($p < 0.003$) (Figure 3.11A and B). We also found that NUMR-1/-2 overexpression mediated resistance to cadmium toxicity at a level similar to that of nematodes overexpressing DAF-16. It is known that overexpression of DAF-16 results in increased longevity and stress resistance [127]. Thus, *numr-1/-2* is sufficient to increase nematode resistance to cadmium and increase longevity.

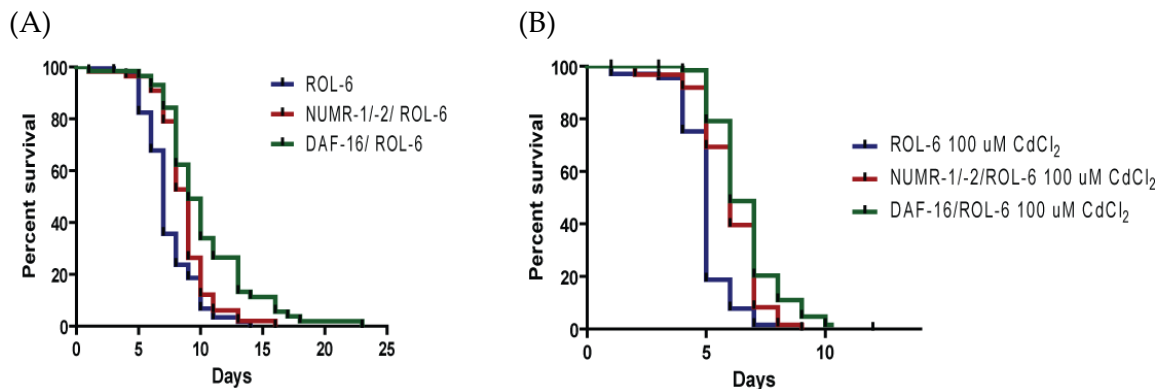


Figure 3.11: Overexpression of NUMR-1/-2 results in increased resistance to metal exposure and increased life-span. (A) No treatment - Red, animals carrying additional copies of *numr-1/-2*; green, animals carrying additional copies of *daf-16(tj356)*; blue, animals carrying the *rol-6(he1006)* selectable marker alone. *rol-6(he1006)*: $n = 60$, $m = 7$. *numr-1/-2*: $n = 50$, $m = 9$, $p = 0.0028$. *daf-16(tj356)* is shown for comparison. *daf-16(tj356)*: $n = 54$, $m = 9$ (B) 100 μ M CdCl₂ - *rol-6(he1006)*: $n = 64$, $m = 5$. *numr-1/-2*: $n = 61$, $m = 6$, $p < 0.0001$. *daf-16(tj356)* is shown for comparison. *daf-16(tj356)*: $n = 65$, $m = 6$. All experiments were repeated at least once with similar results.

To demonstrate that *numr-1/-2* is necessary for mediating resistance to cadmium in nematodes, we examined the effects of decreased *numr-1* expression in *numr-1(ok2239)*, in response to cadmium exposure. We found that *numr-1(ok2239)* alone did not increase sensitivity to cadmium and did not affect lifespan, possibly due to continued *numr-2* expression (data not shown). Thus, to try to abolish as much *numr-1/-2* expression as possible we fed *numr-1/-2(RNAi)* to *numr-1(ok2239)* and wild-type nematodes. RNAi-mediated inhibition of *numr-1/-2* expression in wild-type animals did not shorten life-span and did not increase sensitivity to metal exposure (Figure 3.12A and B). It is plausible that there is incomplete penetrance of *numr-1/-2(RNAi)*, which would explain why wild-type animals fed *numr-1/-2(RNAi)* did not exhibit a shortened life span or increased sensitivity to cadmium exposure. The median life span of *numr-1(ok2239)* animals, fed *numr-1/-2(RNAi)*, in the absence of metal was not significantly different from wild-type nematodes (Figure 3.12A). The initial difference in life span between *numr-1(ok2239)* animals, fed *numr-1/-2(RNAi)* and wild-type nematodes under control conditions is due to the increased bagging frequency observed in the *numr-1* mutants. However, RNAi-mediated inhibition of *numr-1/-2* expression in *numr-1(ok2239)* animals resulted in increased sensitivity to metal exposure when compared to down regulation of *numr-1/-2* expression in wild-type animals (p= 0.0007, Figure 3.12B). This suggests that *numr-1/-2* plays a role in resistance to cadmium toxicity.

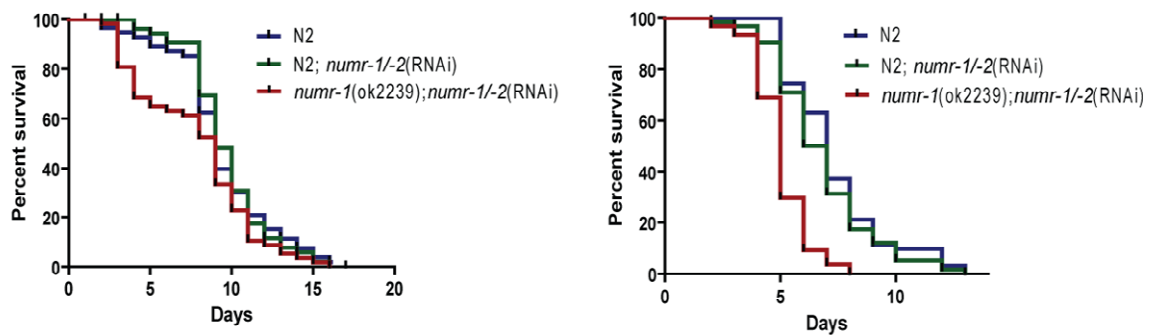


Figure 3.12: Knockdown of *numr-1/-2* results in increased sensitivity to metal exposure but does not affect life span. Worms fed RNAi control vector are represented in blue (N2). Worms fed *numr-1/-2*(RNAi) are represented in green (N2) and red (*numr-1(ok2239)*). (A) *numr-1/-2* knockdown does not affect life-span in the absence of metal treatment but (B) *numr-1/-2* knockdown increases sensitivity of animals grown on plates containing 100 μ M CdCl₂ (N2 versus *numr-1(ok2239)* fed *numr-1/-2*(RNAi) N2: $n = 62$, $m = 7$. *numr-1(ok2239); numr-1/-2*(RNAi): $n = 59$, $m = 5$, $P < 0.0001$) All experiments were repeated at least once with similar results.

3.5 NUMR-1/-2 responds to alteration in $[Ca^{2+}]_i$

To understand the signaling mechanisms by which cadmium activates *numr-1* transcription, we first asked whether ER stress might be acting as a signal to activate *numr-1/-2* transcription. This hypothesis was derived from the knowledge that in *C. elegans* the intestinal cells experience endogenous ER stress during early larval stages of development and the intestinal cells are the primary sites for metal detoxification in *C. elegans* [136, 137]. Therefore, since *numr-1/-2* was expressed throughout the intestine during these early larval stages of development and in response to cadmium exposure, it seemed plausible that ER stress might be acting as a signal to activate *numr-1/-2* expression. To test this idea we exposed adult *NUMR-1*-GFP animals to tunicamycin or

thapsigargin for 5 h. Tunicamycin induces ER stress by inhibiting glycosylation of newly synthesized proteins [138]. Whereas, thapsigargin induces ER stress by inhibiting sarcoplasmic ER Ca²⁺ ATPase (SERCA) pumps, depleting ER calcium stores [139, 140]. Interestingly, we found that thapsigargin resulted in increased *numr-1/-2* transcription throughout the intestine (Figure 3.13C). Whereas, tunicamycin did not significantly alter *numr-1/-2* expression relative to DMSO vehicle control treated animals (Figure 3.13B and B). This raised the intriguing possibility that *numr-1/-2* expression, at least in part, might be regulated by changes in [Ca²⁺]_i. Therefore, we exposed adult *NUMR-1-GFP* animals to a calcium ionophore (A23187) for 5 h and found that this resulted in increased *numr-1/-2* transcription (Figure 3.13D). These findings suggest that *numr-1/-2* does respond to changes in [Ca²⁺]_i and ER calcium store depletion.

If ER calcium store depletion resulted from SERCA pump inhibition following thapsigargin exposure, then genetic inhibition of the *C. elegans* SERCA pump should also result in increased *numr-1/-2* expression. Therefore, we inhibited the *C. elegans* SERCA homolog by feeding *NUMR-1-GFP C. elegans sca-1* dsRNA. The *sca-1* gene encodes for the only *C. elegans* SERCA homolog [109]. As seen in Figure 3.14B, *sca-1* RNAi increased *numr-1* transcription when compared to *NUMR-1-GFP* animals fed the RNAi control vector alone. Taken together, this data indicates that inhibition of

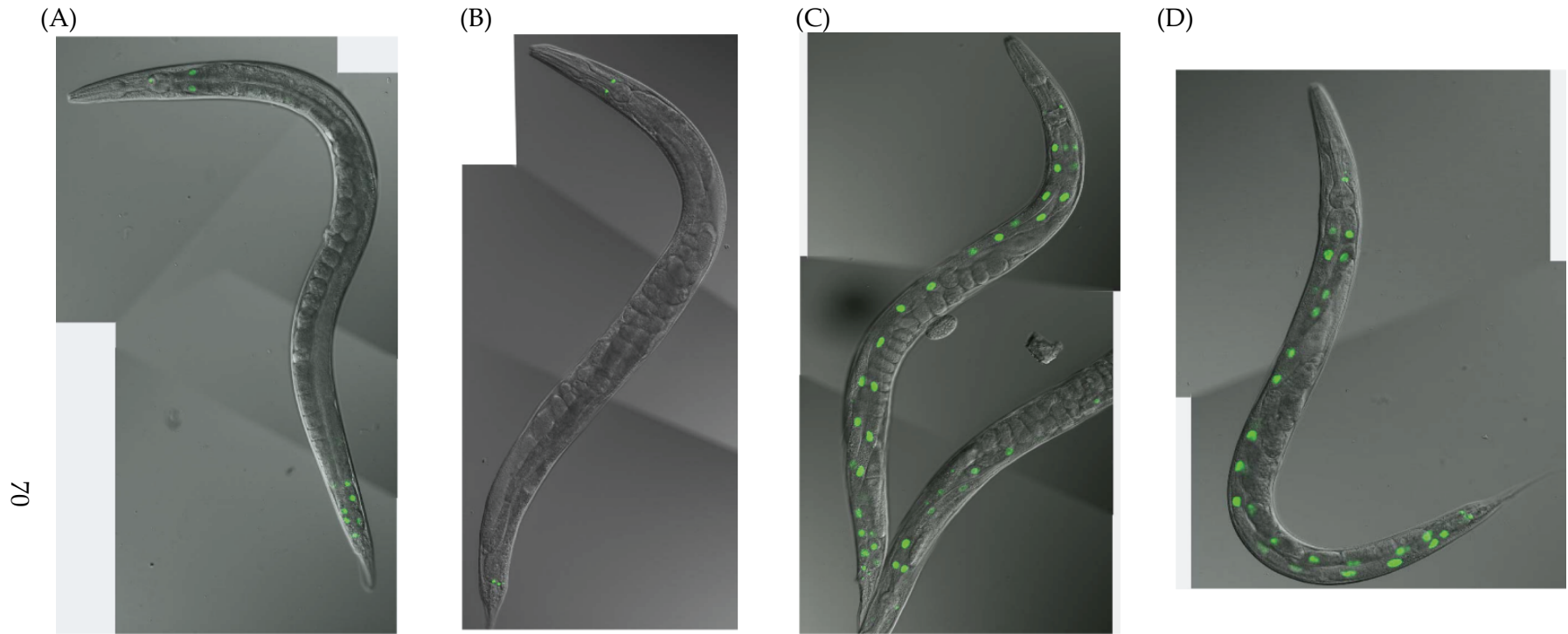


Figure 3.13: Changes in intracellular Ca^{2+} levels activates *numr-1/-2* transcription. *NUMR-1*-GFP nematodes were exposed to the following conditions for 5 h (A) 1% DMSO vehicle control worm (B) 5 $\mu\text{g}/\mu\text{l}$ tunicamycin (C) 5 μM thapsigargin (D) 20 μM A23187.

SERCA pumps and/ or alteration in intracellular calcium levels activates *numr-1/-2* transcription. Therefore, to address this mechanism and better define the relationship between cadmium and intracellular calcium we moved to a cell culture system.

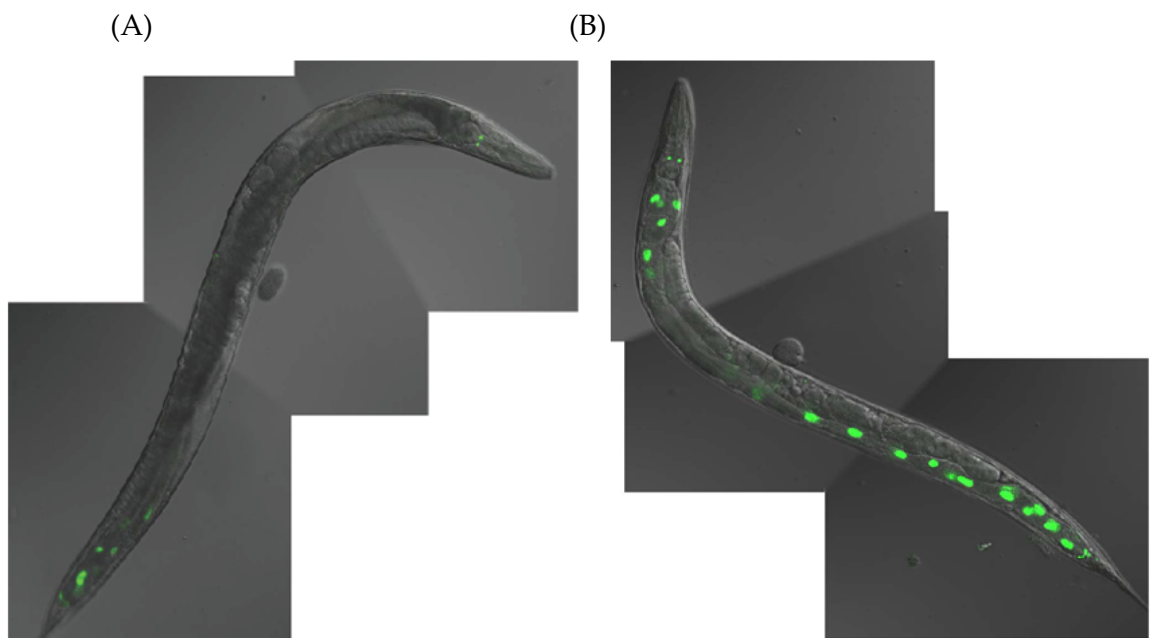


Figure 3.14: Knockdown of the *C. elegans* SERCA pump homolog, *sca-1*, activates *numr-1/-2* transcription in NUMR-1-GFP nematodes. (A) NUMR-1-GFP nematode fed RNAi control vector. (B) NUMR-1-GFP nematode fed bacteria expressing *sca-1* dsRNA.

3.6 Quantification of cadmium-response and cell viability in HEK293 cells

3.6.1 HEK293 cells are an appropriate system to study cadmium-inducible transcription

Human Embryonic Kidney (HEK) 293 cells are a well established cell line widely used for studying intracellular calcium signaling mechanisms. To ensure that HEK293 cells were also an appropriate system to investigate mechanisms of cadmium-induced transcription, qRT-PCR was used to quantify the response of three well characterized cadmium-inducible genes: metallothionein (*mt-1*), *c-fos*, and *grp-78* (78-kDa glucose-regulated protein / HSPA5) (Figure 3.15). MT-1 is one of the most widely studied cadmium-inducible genes [3, 68] and as shown in Figure 3.15A, cadmium exposure results in a rapid and significant induction of *mt-1* steady-state mRNA levels in HEK293 cells. In addition, cadmium exposure caused a significant induction of *c-fos* and *grp-78* (Figure 3.15B and C, respectively). Previous studies have also demonstrated *c-fos* and *grp-78* to be calcium- as well as cadmium-responsive genes [141-145]. To test this, we treated HEK293 cells with the SERCA pump inhibitor, thapsigargin (TG), to fully activate store-operated calcium (SOC) signaling and elevate intracellular $[Ca^{2+}]_i$ [139, 140]. Under these conditions, the steady-state mRNA levels of both *c-fos* and *grp-78* increased (Figure 3.15B and C, respectively).

In summary, HEK293 cells display a classic cadmium stress response where cadmium, over a wide dose range (1-30 μ M), increases the mRNA abundance of three

known cadmium-responsive genes. These data support the use of HEK293 cells as an appropriate system for investigating underlying mechanisms of cadmium-induced transcription.

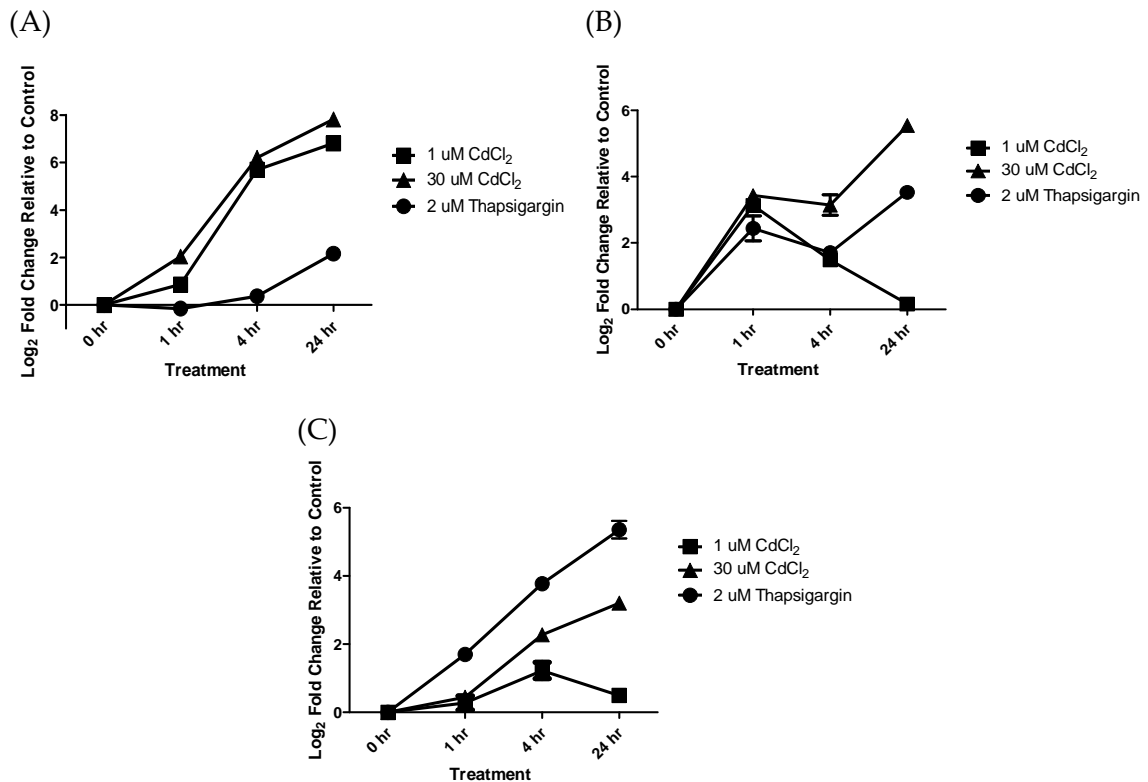


Figure 3.15: Effects of cadmium and thapsigargin on the transcription of three well characterized cadmium-responsive genes. Total RNA was extracted from HEK293 cells treated with either 1 μ M CdCl₂ (square), 30 μ M CdCl₂ (triangle), or 2 μ M thapsigargin (circle) for 1, 4, or 24 h and mRNA levels of (a) *mt-1*, (b) *c-fos*, and (c) *grp-78* were measured with qRT-PCR. All measurements were normalized to the mRNA levels of *actin*. Fold change was normalized to the mRNA levels observed in control cells. Results were displayed in mean log₂fold \pm SEM ($n = 3$).

3.6.2 Increased cadmium uptake in HEK293 correlates with decreased cell viability

Previous studies utilized BAPTA-based fluorescent calcium sensors for investigating the effects of cadmium exposure on calcium signaling. However, it has been demonstrated that cadmium can bind to BAPTA-based $[Ca^{2+}]_i$ fluorescent dyes with high affinity [74] and elicit a fluorescence change that could be misinterpreted as a change in $[Ca^{2+}]_i$. This affect of Cd^{2+} ions is demonstrated in Figure 3.16A using HEK293 cells loaded with fura-5F [115]. To ensure that changes in the fluorescence ratio represented the influx of cadmium into the cells, rather than potential changes in $[Ca^{2+}]_i$ that may result from cadmium exposure, intracellular calcium stores were first depleted by exposure to 10 μ M ionomycin for 5 minutes in nominally calcium-free medium. In Figure 3.16A, elevating extracellular Cd^{2+} ions lead to a rapid and significant increase in the fura-5F fluorescence ratio which could only result from Cd^{2+} ions entering the cells and interacting with the calcium indicator. While the interaction between Cd^{2+} ions and fura-calcium indicators raises serious concerns for interpreting cadmium effects on calcium signaling using this technical approach, it does present an opportunity for monitoring Cd^{2+} ion accumulation.

To assess Cd^{2+} ion accumulation in chronically exposed cells, HEK293 cells were exposed to various cadmium concentrations for either 4 or 24 h and then loaded with fura-5F (Figure 3.16B). The fura-5F fluorescence ratios were measured as an indication of intracellular Cd^{2+} ion accumulation. Cadmium uptake was both time- and concentration-

dependent, with the highest fura-5F fluorescence ratio occurring in cells exposed to 30 μM cadmium chloride for 24 h.

Under these cadmium exposure conditions HEK293 cell viability was assessed by use of the CellTiter-Glo™ assay (Figure 3.16C). Short-term exposure (4 h) to cadmium concentrations ranging from 1 - 30 μM cadmium chloride did not significantly alter HEK293 cell viability according to ATP content. Long-term exposure (24 h) to low-levels of cadmium chloride (1 and 3 μM) also had no significant effect on cell viability. However, exposing HEK293 cells to 10 or 30 μM cadmium chloride for 24 h, significantly reduced cell viability by 26% and 62% respectively. The 30 μM cadmium chloride exposure for 24 h resulted in the highest intracellular Cd^{2+} ion accumulation (Figure 3.16B) and the highest decrease in cell viability.

The effects of this exposure condition are significant since many studies investigating the effects of cadmium exposure on gene expression and intracellular signaling processes, including calcium signaling, use exposure conditions that are toxic. Therefore, signaling pathways and genes activated under cadmium exposure conditions that decrease cell viability ($> 10 \mu\text{M}$, 24 h) could be considered non-specific and unrelated to the key mechanisms of cadmium-induced gene expression. Our data suggest that exposing cells to cadmium in the range of 1 – 3 μM for 4 h is sufficient for studying mechanisms of cadmium-induced transcription.

Despite previous studies investigating the effects of cadmium on calcium homeostasis, we believe the exact mechanistic relationship between cadmium and calcium still remains largely unresolved. This is primarily due to the fact that (i) Cd^{2+} ions binding to BAPTA-based calcium indicators can interfere with $[\text{Ca}^{2+}]_i$ measurements potentially creating experimental artifacts, and (ii) most studies used cadmium exposure conditions that can potentially decrease cell viability and lead to non specific effects on cell signaling.

To avoid these problems we reassessed the effects of low, non-toxic cadmium exposures on $[\text{Ca}^{2+}]_i$ and utilized a protein based calcium ion sensor that is insensitive to Cd^{2+} ions, the yellow cameleon (YC) 3.60, stably expressed in HEK293 cells. The YC is a chimeric protein composed of four domains: a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) linked via a calmodulin (CaM) and CaM-binding peptide domain [146]. When calcium binds to the CaM-domain this results in a conformational change of the protein, which brings the CFP and YFP domains closer together and enhances the efficiency of fluorescence resonance energy transfer (FRET). The resulting change in fluorescence ratio is a measure of $[\text{Ca}^{2+}]_i$ changes. Using the same protocol described in Figure 3.16A, we demonstrate in Figure 3.16D that the YC 3.60 can distinguish between Ca^{2+} ions and Cd^{2+} ions, presenting a useful technique to investigate the effects of cadmium exposure on changes in intracellular calcium levels.

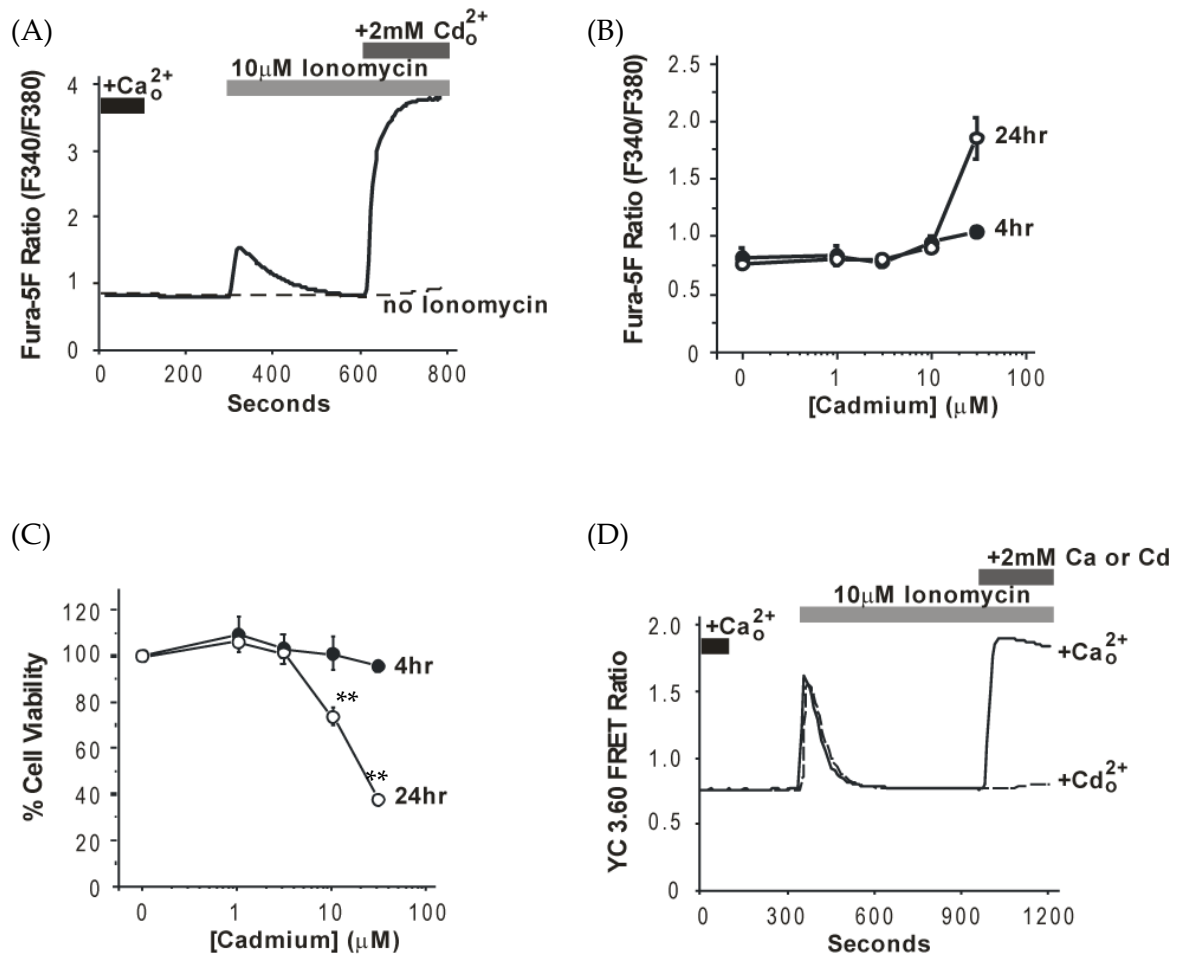


Figure 3.16: Cadmium uptake and cell viability in HEK 293 cells. (A) Trace represents the $[Cd^{2+}]_i$ measured in fura-5F loaded HEK293 cells treated with ionomycin to deplete $[Ca^{2+}]_i$ stores. At the end of the run the media was supplemented with 2 mM $CdCl_2$ in Ca^{2+} -free HBSS. (B) Detection of $[Cd^{2+}]_i$ in fura-5F loaded HEK293 cells exposed to 0, 1, 3, 10 and 30 μM $CdCl_2$ for 4 and 24 h. (C) Cell viability in HEK293 cells following a 4 and 24 h exposure to 0, 1, 3, 10 and 30 μM $CdCl_2$. Data were expressed as the mean \pm SE and were analyzed by one-way ANOVA followed by Dunnett's post-test. Cell viability is indicated as percent control. Asterisks indicate a significant difference between control and cadmium treated groups (** $P < 0.001$). (D) The YC 3.60 stably expressed in HEK293 cells, is only sensitive to changes in $[Ca^{2+}]_i$ and not $[Cd^{2+}]_i$. Trace represents the $[Ca^{2+}]_i$ measured in HEK293 cells stably expressing the YC 3.60 following ionomycin exposure in Ca^{2+} -free HBSS. At the end of the run, the media was supplemented with either 2 mM Ca^{2+} or 2mM Cd^{2+} . All traces are representative of a typical response observed in at least 3 independent experiments.

3.7 Low level cadmium exposure does not disrupt calcium homeostasis or deplete intracellular calcium stores

We investigated whether low, non-toxic concentrations of cadmium had an effect on ER calcium stores. In these experiments, we treated HEK293 cells with the SERCA pump inhibitor thapsigargin (TG, 2 μM ; Figure 3.17A). TG treatment results in a biphasic change in $[\text{Ca}^{2+}]_i$ which represents depletion of calcium from ER calcium stores and the entry of calcium across the plasma membrane by activation of store operated calcium entry (SOCE) [147, 148]. The biphasic calcium response induced by TG reveals to several components of calcium homeostasis: (i) that there is SERCA pump activity that can be inhibited by TG, (ii) plasma membrane Ca^{2+} -ATPases are operating which leads to a transient TG response in the absence of extracellular calcium, and (iii) SOCE can be activated. To test if cadmium exposure can alter the activity of all or any of these calcium homeostatic processes, we compared the TG-induced biphasic calcium response in HEK293 cells exposed to 1 μM cadmium chloride for 4 h to untreated cells. This exposure condition is sufficient to induce transcription of cadmium-responsive genes. As shown in Figure 3.17A, the TG-induced biphasic calcium response in HEK293 cells exposed to 1 μM cadmium chloride for 4 h was indistinguishable from untreated cells, suggesting that low level cadmium exposure does not disrupt calcium signaling processes.

The size of the TG-induced calcium peak (in the absence of extracellular calcium) is somewhat indicative of ER calcium store content. Since the TG-induced peak was

similar in control and cadmium treated conditions, it would suggest that cadmium does not deplete ER calcium stores. However, to get a better estimate of ER calcium stores we utilized an ionomycin depletion protocol [115]. In the absence of extracellular calcium, treating cells with ionomycin (10 μM) triggers a rapid release of ER calcium stores [149]. The peak of the ionomycin-induced $[\text{Ca}^{2+}]_i$ can be used as a measure of ER calcium (Figure 3.17B). In Figure 3.17C and D, we compare the peak ionomycin-induced calcium release (presented as YC 3.60 ratio change) in HEK293 cells exposed to various cadmium concentrations for 4 or 24 h. There was no significant effect of low cadmium exposure (1 – 10 μM) for 4 or 24 h on ER calcium stores compared to controls. A decrease of ER calcium store content was only observed in cells treated with 30 μM cadmium chloride for 4 or 24 h with decreases of 36% and 57% respectively (Figure 3.17C and D).

In summary, these data suggest that low concentrations of cadmium (1 – 10 μM), sufficient to induce transcription of cadmium-responsive genes, do not interfere with calcium signaling mechanisms nor deplete ER calcium stores. Significant effects on ER calcium stores were observed with 30 μM cadmium, an exposure condition that may have non-specific effects since it is a toxic exposure condition.

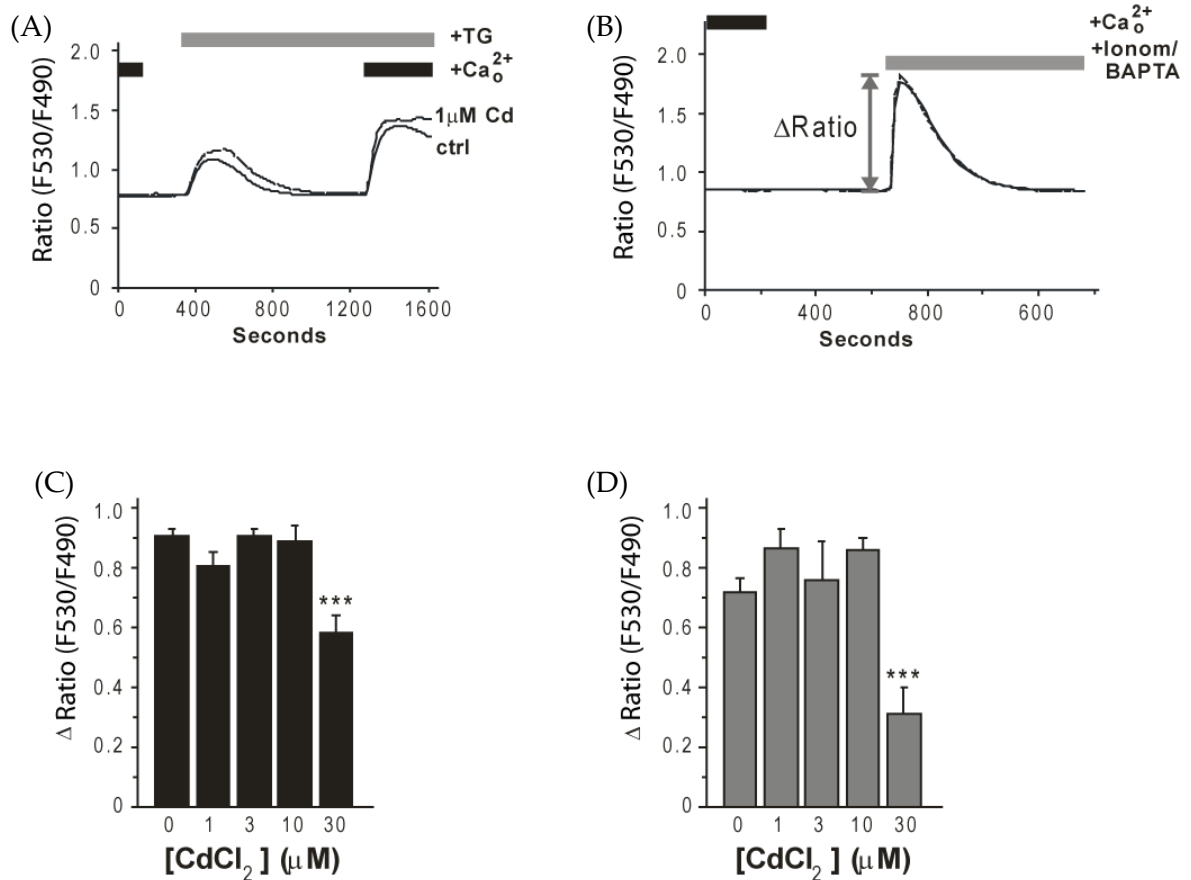


Figure 3.17: In Ca²⁺-free HBSS, thapsigargin and ionomycin were used to assess calcium homeostasis and ER Ca²⁺-store content in cells exposed to cadmium. (A) Trace represents the [Ca²⁺]_i measured in HEK293 cells stably expressing the YC 3.60 under control conditions (solid) or after a 4 h exposure to 1 μM CdCl₂ (broken). Application of thapsigargin (TG) in Ca²⁺-free HBSS produced an increase in [Ca²⁺]_i as a result of SERCA pump inhibition and activated SOCE. (B) Trace represents the [Ca²⁺]_i measured in HEK 293 cells stably expressing the YC 3.60 following ionomycin exposure in Ca²⁺-free HBSS under control conditions (solid) or after a 4 h exposure to 1 μM CdCl₂ (broken). (C) Mean peak values of ionomycin response in HEK293 cells following exposure to 0, 1, 3, 10 and 30 μM CdCl₂ for 4 h or (D) 24 h. Data were expressed as the mean ± SE and were analyzed by one-way ANOVA followed by Dunnett's post-test. Asterisks indicate a significant difference between control and cadmium treated groups (***) *P* < 0.001).

3.8 Low levels of cadmium exposure do not significantly alter cAMP/ calcium responsive gene expression

To determine whether cadmium could influence the transcriptional activity of cAMP/ calcium-responsive genes we utilized a pathway specific PCR array from SABiosciences that consists of 84 genes relevant to calcium signaling (Table 3.5). Consistent with our observations that low-level cadmium exposure does not significantly alter ER calcium stores or calcium homeostasis, we found that 1 μM cadmium exposure for 4 or 24 h did not significantly alter the transcriptional activity of many of the cAMP/ calcium-responsive genes (Table 3.6 and 3.7). However, exposure to 30 μM cadmium for 4 or 24 h did significantly influence the transcriptional activity of many of the cAMP/ calcium-responsive genes (Table 3.6 and 3.7) an affect that may result from 30 μM cadmium depleting ER calcium stores and effecting downstream signaling processes. To test this, we compared the effects of 4 and 24 h exposure of 30 μM cadmium with those of the SERCA pump inhibitor, thapsigargin. In comparing genes up-regulated by exposure to 30 μM cadmium for 4 or 24 h to genes up-regulated following thapsigargin exposure for 4 h (Table 3.8 and Figure 3.18), we found that 10 genes were affected under all 3 conditions (Table 3.9). Most of these genes were involved in apoptosis, differentiation, or mitogenesis. Fifteen of the 17 genes induced by thapsigargin exposure were also found to be up-regulated in response to a 24 h exposure to 30 μM cadmium. Yet, there were still many other calcium-responsive genes differentially expressed following a 24 h exposure to 30 μM cadmium. These results

suggest a majority of the calcium-responsive genes differentially expressed at the highest level of cadmium exposure are most likely not induced solely due to alterations in ER store content.

Table 3.5: Functional gene grouping of human cAMP/ calcium PCR Array. Fill color: red = genes whose promoters contain SRE or SRE-like elements; white = genes whose promoters contain CRE elements; green = genes whose promoters contain an SRE and CRE element; blue = genes whose promoters contain other calcium responsive elements; pink = housekeeping genes.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ADRB1	AHR	AMD1	AREG	ATF3	BCL2	BDNF	BRCA1	CALB1	CALB2	CALM1	CALR
B	CCNA1	CCND1	CDK5	CDKN2B	CGA	CHGA	CNN1	CREB1	CREM	CTF1	CYR61	DDIT3
C	DUSP1	EGR1	EGR2	ENO2	FGF6	FOS	FOSB	GCG	GEM	GIPR	HK2	HSPA4
D	HSPA5	IL2	IL6	INHBA	JUNB	JUND	KCNA5	LDHA	MAF	MIF	NCAM1	NF1
E	NOS2A	NPY	NR4A2	PCK2	PCNA	PENK	PER1	PLAT	PLN	PMAIP1	POU1F1	POU2AF1
F	PPP1R15A	PPP2CA	PRKAR1A	PRL	PTGS2	RB1	S100A12	S100A6	S100G	SCG2	SGK1	SLC18A1
G	SOD2	SRF	SST	SSTR2	STAT3	TACR1	TGFB3	TH	THBS1	TNF	VCL	VIP
H	B2M	HPRT1	RPL13A	GAPDH	ACTB	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Table 3.6: SuperArray analysis of cAMP/calcium signaling transcript expression in HEK293 cells. Genes whose expression was up-regulated following a 4 or 24 h exposure to 1 or 30 μ M CdCl₂.

Exposure	Gene Name	Fold Change	<i>P</i> value
4 h 1 μM CdCl₂	PENK	2.4512	0.023029
4 h 30 μM CdCl₂	FGF6	24.6539	0.005357
	DDIT3	9.0099	0.000048
	DUSP1	6.6408	0.000159
	PENK	4.664	0.000512
	ATF3	4.6518	0.000101
	PPP1R15A	4.6185	0.000054
	INHBA	4.3349	0.000586
	CNN1	4.2822	0.002495
	EGR1	3.9973	0.016708
	AREG	3.3761	0.002253
	HSPA5	3.0578	0.000963
	FOSB	3.0299	0.001182
	TACR1	2.8573	0.005705
	GEM	2.7639	0.000113
	SCG2	2.6789	0.029449
	EGR2	2.3043	0.018078
	FOS	2.1596	0.033391
	PTGS2	2.1378	0.056764
	JUND	2.1214	0.001347
24 h 30 μM CdCl₂	FOSB	1994.706	0.000001
	CHGA	1270.4422	0.000002
	EGR1	1247.6159	0.000005
	FGF6	740.5477	0.000597
	CGA	226.4632	0.000004
	TH	85.4584	0.00002
	EGR2	76.2662	0.000199
	PPP1R15A	70.6985	0.000005
	DDIT3	61.3558	0.000042
	AREG	57.1957	0.000005
	IL6	51.4587	0.000126
	FOS	51.0661	0.000006
	S100A6	46.5164	0.000114
	TACR1	43.5103	0.000079

Table 3.6: continued.

Exposure	Gene Name	Fold Change	<i>P</i> value
24 h 30 μ M CdCl ₂	GEM	42.7737	0.000025
	JUND	28.2554	0.006534
	PRL	27.6541	0.000204
	CNN1	26.1911	0.000112
	INHBA	25.5051	0.000085
	POU1F1	20.8117	0.000247
	CREM	20.704	0.000779
	PTGS2	20.6576	0.000475
	HSPA5	20.0538	0.000024
	CALB1	19.4826	0.003432
	JUNB	16.0708	0.000079
	SCG2	14.004	0.001741
	GIPR	12.922	0.00001
	CALB2	12.3142	0.000762
	SGK1	10.5842	0.000023
	NR4A2	9.6651	0.00006
	THBS1	8.3133	0.000252
	SSTR2	7.538	0.001687
	MAF	7.0063	0.001082
	NOS2A	6.3728	0.002331
	HK2	5.6116	0.000267
	TNF	5.5566	0.006301
	NPY	4.7459	0.000043
	DUSP1	4.5155	0.000003
	PENK	4.1311	0.005164
	CTF1	2.9341	0.021035
	BCL2	2.8517	0.000331
	CCNA1	2.7945	0.015029
	CALR	2.5418	0.003024
	ATF3	2.5353	0.001326
	PLAT	2.4699	0.046362
	PMAIP1	2.3666	0.002755

Table 3.7: SuperArray analysis of cAMP/calcium signaling transcript expression in HEK293 cells. Genes whose expression was down-regulated following a 4 or 24 h exposure to 1 or 30 μM CdCl₂.

Exposure	Gene Name	Fold Change	<i>P</i> value
4 h 1μM CdCl₂	TNF	-3.4233	0.012398
24 h 1 μM CdCl₂	TNF	-13.0436	0.000352
	FOS	-3.7234	0.003562
	PTGS2	-3.3062	0.023548
	THBS1	-2.3139	0.005863
	EGR1	-2.0611	0.020815
4 h 30 μM CdCl₂	TNF	-19.1092	0.000215
	JUNB	-2.3095	0.000264
	ADRB1	-2.1577	0.000071
	PER1	-2.0993	0.00208
	PLN	-2.0285	0.09175
24 h 30 μM CdCl₂	CREB1	-5.9272	0.052032
	NCAM1	-2.4919	0.041313
	LDHA	-2.059	0.010214
	PRKAR1A	-2.0369	0.008681

Table 3.8: SuperArray analysis of cAMP/calcium signaling transcript expression in HEK293 cells treated with 2 μ M thapsigargin for 4 h.

Exposure	Gene Name	Fold Change	<i>P</i> value
4 h 2 μM Thapsigargin	DDIT3	21.2583	0.00017
	PTGS2	15.4315	0.011724
	HSPA5	7.9023	0.00148
	INHBA	7.8966	0.004143
	NR4A2	5.6134	0.004956
	IL6	5.0238	0.014326
	FOSB	4.9708	0.079038
	PPP1R15A	4.3266	0.001643
	GEM	4.2286	0.001231
	EGR1	4.1209	0.048005
	PCK2	3.8763	0.001097
	ATF3	3.5312	0.000724
	CGA	3.242	0.020191
	HK2	2.5842	0.133179
	PER1	2.541	0.018747
FOS	2.4214	0.003781	
TNF	2.2131	0.048366	

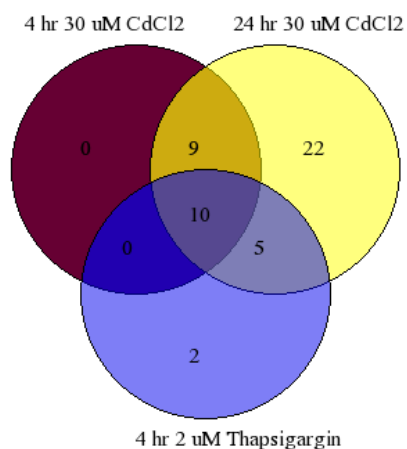


Figure 3.18: Venn diagram illustrating shared gene expression in HEK293 cells under the exposure of 30 μM CdCl₂ for 4 and 24 h and 2 μM thapsigargin for 4 h. The Venn diagram was generated using the following web application: <http://mcbc.usm.edu/genevenn/>.

Table 3.9: Genes whose expression changed following a 4 and 24 h exposure to 30 μM CdCl₂ and a 4 h exposure to 2 μM thapsigargin.

Gene	Description
DDIT3	transcription factor that promotes cell death during ER stress
PTGS2	responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis
HSPA5	involved in the folding and assembly of proteins in the ER during stress
INHBA	member of the transforming growth factor-beta superfamily that may acts as both a growth/differentiation factor and a hormone
FOSB	part of the transcription factor complex AP-1 and regulates cell proliferation, differentiation, and transformation
PPP1R15A	helps mediate apoptosis following stressful conditions
GEM	belongs to the RAD/GEM family of GTP-binding proteins, could play a role receptor-mediated signal transduction
EGR1	transcriptional regulator of genes required for differentiation and mitogenesis
ATF3	member of the CREB protein family and mediates pro-apoptotic effects of p38
FOS	part of the transcription factor complex AP-1 and regulates cell proliferation, differentiation, and transformation

3.9 Cadmium exposure alters the *C. elegans* defecation cycle:

To describe more fully the effects of cadmium exposure on calcium signaling as well as to illustrate cadmium's effect on a biological process that is regulated by calcium oscillations; we monitored the defecation cycle in *C. elegans*.

In *C. elegans* defecation is a highly rhythmic behavior that is the result of a stereotypical motor program (DMP) that is initiated roughly once a minute. Calcium oscillations throughout the intestinal cells are thought to regulate the initiation of the defecation cycle and posterior body wall muscle (pBoc) contractions. To further investigate the mechanistic relationship between cadmium and calcium, we investigated the effects of cadmium on the length and periodicity of the *C. elegans* defecation cycle. We observed that cadmium exposure increased the frequency of the calcium oscillations (Figure 3.19) and significantly shortened the defecation cycle (Figure 3.20 and Table 3.10). Under control conditions, the mean pBoc period was 43 s and following cadmium exposure the mean was reduced to 35 s. The low value of the coefficient of variance (CV) indicated that pBoc cycles remained rhythmic in cadmium treated worms (Table 3.10). Figure 3.19A and B are an example of typical calcium oscillations measured in transgenic nematodes expressing the YC6.1 under control conditions or after exposure to 100 μ M cadmium for 5 h. The magnitude of the calcium oscillations in cadmium exposed nematodes was not significantly different from untreated nematodes but the frequency of the oscillations increased. Mutants deficient in calcium signaling usually

exhibit defecation cycles that are lengthened and no longer rhythmic, therefore suggesting that cadmium exposure does not alter calcium signaling in *C. elegans*.

In support of this, we found that when *C. elegans* were exposed the SERCA pump inhibitor, thapsigargin (TG, 5 μ M), the mean defecation cycle was lengthened to 81 s and the pBoc cycles became highly arrhythmic as indicated by the high CV value (Figure 3.20B and Table 3.10). This response is not a consequence of general ER stress since exposing worms to 5 μ g/ μ l of tunicamycin for 5 h, which induces ER stress by inhibiting glycosylation of newly synthesized proteins stress, had no significant effect on the defecation cycle (Figure 3.20B and Table 3.10). In summary, the data suggest that cadmium alters the defecation cycle independent of changes in calcium signaling.

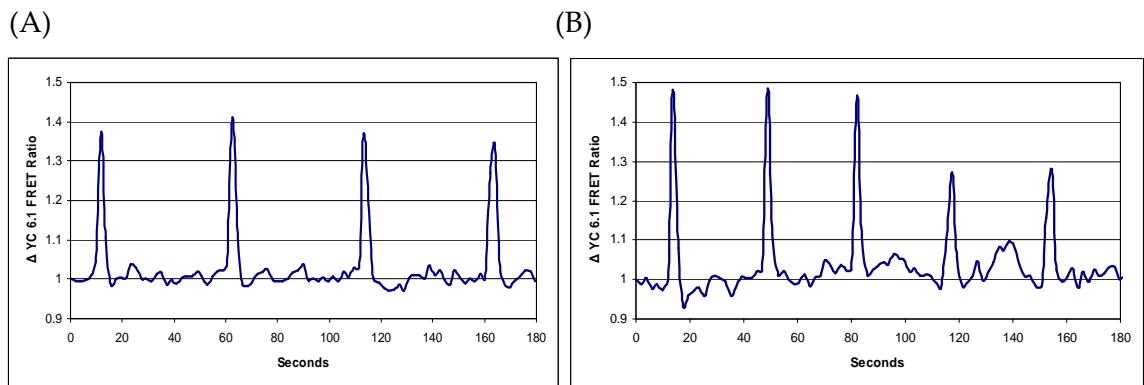


Figure 3.19: Intestinal Ca²⁺ dynamics during defecation in (A) control nematodes and (B) nematodes exposed to 100 μ M CdCl₂ for 5 h.

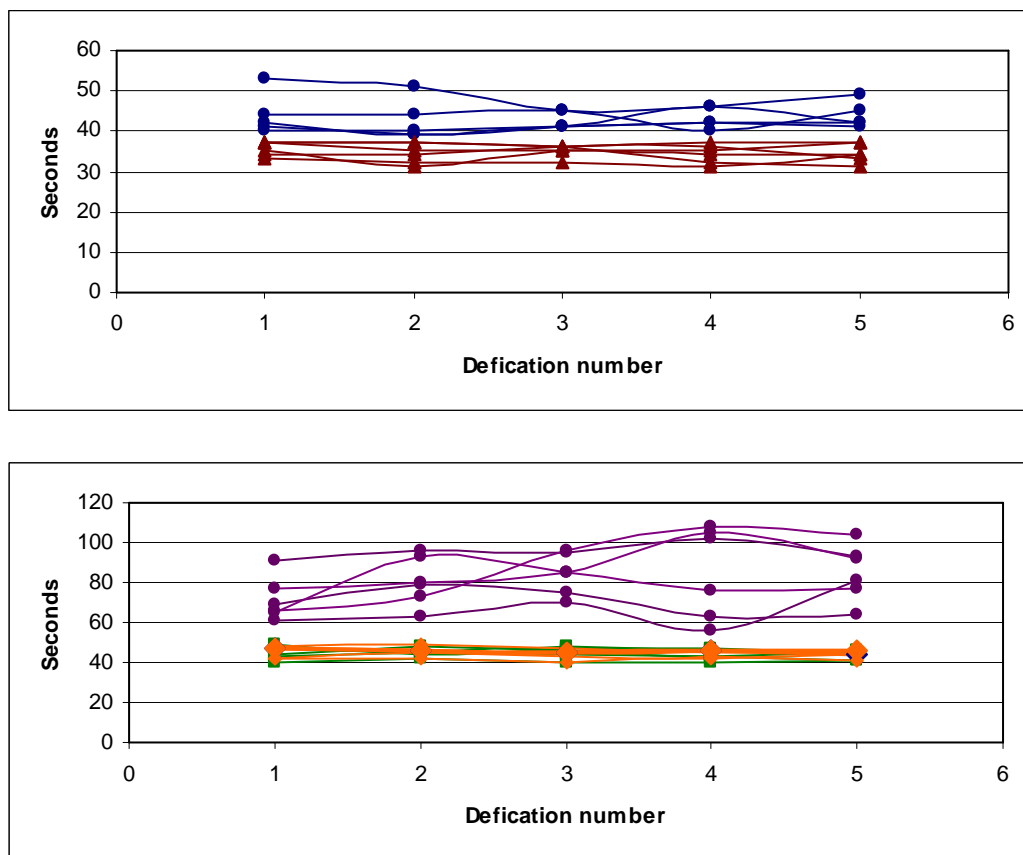


Figure 3.20: pBoc cycle period in control nematodes or nematodes exposed to 1% DMSO vehicle control, 100 μM CdCl₂, 5 μM thapsigargin, or 5 $\mu\text{g}/\mu\text{L}$ tunicamycin. (A) The DMP was determined for control nematodes (blue) and nematodes exposed to 100 μM CdCl₂ for 5 h (B) The DMP was determined for nematodes exposed to 1% DMSO vehicle control (green), 5 μM thapsigargin (purple), and 5 $\mu\text{g}/\mu\text{L}$ tunicamycin (orange) for 5 h.

Table 3.10: Cycle period and rhythmicity. Values are means \pm SD calculated from 5 worms. pBoc, posterior body wall muscle contraction and CV, coefficient of variation.

Condition	pBoc Period, s	pBoc CV, %
Control	43.2	7.6
DMSO	43.9	2.5
5 h 100 μM CdCl ₂	34.7	4.4
5 h 5 $\mu\text{g}/\mu\text{l}$ Tunicamycin	44.7	2.2
5 h 5 μM Thapsigargin	81.3	11.6

4. Discussion

A better understanding of the early cellular events associated with cadmium exposure will offer insight into the processes involved in sensing cadmium-stress and the subsequent signaling pathways important for mediating transcriptional changes necessary for defense against cadmium toxicity. Thus, the main goal of this study is to understand the molecular mechanisms by which cadmium influences gene expression.

To achieve this goal, two cadmium-responsive genes of unknown biological function were characterized and assessed for their role in the defense against cadmium toxicity in *C. elegans*. The evaluation of these two cadmium-responsive genes in *C. elegans* leads us to reevaluate the role of calcium in regulating cadmium-induced transcription in mammalian cells. There is a large body of literature indicating that cadmium can alter $[Ca^{2+}]_i$, yet the precise assessment of the effects of cadmium on $[Ca^{2+}]_i$ are unresolved [68]. Our data indicates that modulation of $[Ca^{2+}]_i$ is unlikely the primary mechanism by which cadmium regulates transcription at low-levels of exposures. In accordance with our cell culture data, we find in *C. elegans* that low, non-toxic concentrations of cadmium do not affect $[Ca^{2+}]_i$.

4.1 Characterization of two cadmium-responsive genes in *C. elegans*, *numr-1* and *numr-2*

The carcinogenic potential of cadmium is well established, yet the molecular mechanisms by which cadmium can affect signaling pathways to elicit a specific cellular

stress-response have not been well defined. Although cadmium-induced gene expression on a whole-genome scale has been previously documented in *C. elegans*, many of the differentially expressed genes identified in these analyses are genes of unknown function [60, 112]. In this study, we report the molecular characterization of two cadmium-inducible genes, *numr-1* and *numr-2*. *numr-1* was originally identified from previous work in our laboratory using whole genome analysis to characterize cadmium responsive genes in *C. elegans* [60]. Based on sequence homology we identified an identical *C. elegans* gene, F08F8.1, which we designated as *numr-2*. *numr-1* and *numr-2* respond to a variety of metal stress conditions and calcium mobilization but *numr-1* and *numr-2* do not respond to more general stress conditions such as oxidative stress, heat shock, pathogenic infection, and starvation. This suggests that *numr-1* and *numr-2* are specific stress responsive genes.

ProSite Analysis was used to identify potential motifs within NUMR-1/-2.

ProSite analysis predicted a nuclear localization signal (NLS), an arginine-rich region, a histidine-rich region, plus two potential Casein Kinase II phosphorylation sites (Table 3.1). Casein Kinase II can be activated in cells exposed to cadmium [62]. Additionally, the predicted NLS of NUMR-1/-2 may be functional since the NUMR-1 translational fusion is found to be localized in the cellular nuclei of all cells in which it is expressed.

Constitutive expression of *numr-1/-2* is observed throughout the intestinal cells during early larval stages of development and this pattern of expression decreases as the

animal develops into an adult, at which point constitutive expression of *numr-1/-2* is observed in sensory neurons in the head, a few cells around the vulva, and the posterior end of the worm (Figure 3.2B-F and Figure 3.3). The high level of constitutive *numr-1/-2* expression during development is consistent with the identification of a highly conserved zinc-finger transcription factor, Sp1, binding motif within the *numr-1/-2* promoters. Sp1 is considered a potent transcriptional activator that has been found to be essential for the developmental process of a large number of tissues both in humans and in *C. elegans* [123, 150]. Thus, Sp1 could be important for regulating *numr-1/-2* expression during early larval stages of development.

In adult nematodes *numr-1/-2* is expressed in the egg-laying muscles of the vulva. In accordance with the expression of *numr-1/-2* in the egg-laying muscles of the vulva, nematodes harboring either of the two *numr-1* mutant alleles, *ok2239* and *tm2775*, demonstrate an increased propensity to bag (Figure 3.10A). Bagging is an egg-laying-defective phenotype that arises from defects in vulva formation, alterations in the functional activity of the egg-laying muscles, or defects in the hermaphrodite specific (HSN) neurons which provide serotonergic stimuli to the vulva muscles [81]. Thus, the importance of *numr-1* expression for proper egg-laying behavior, suggests a role for *numr-1* in certain muscular functions. Furthermore, nematodes harboring either of the two *numr-1* mutant alleles also show decreased feeding levels when compared to wild-type nematodes (Figure 3.10B). Since feeding is also considered a muscular function, this

data further supports a role for *numr-1* expression in certain muscular functions. Since the expression pattern of *numr-1* and *numr-2* is identical, we assume we would observe similar phenotypes in a *numr-2* deletion allele. During the preparation of this dissertation we received a *numr-2* deletion allele and plan to investigate these phenotypes in the *numr-2* allele as well.

In adult nematodes *numr-1* and *numr-2* are constitutively expressed in a pair of chemosensory neurons of the head, most likely a pair of the amphid wing neurons (Figure 3.4). The amphid wing neurons mediate either attractive or repulsive odor responses and calcium fluxes are thought to regulate at least a subset of these responses [151]. Unfortunately, the exact molecular mechanisms that link odor detection in olfactory neurons to chemotaxis behavior generated by downstream interneurons are largely unknown. One study suggests that odor sensitivity can be enhanced by overexpressing the UNC-86 POU transcription factor in the AIZ interneurons [128]. Since the *numr-1/-2* promoter contains an UNC-86 binding site and is expressed in sensory neurons, *numr-1/-2* may help orchestrate a connection between odor detection and a specific behavioral response.

Additionally, since *numr-1/-2* is constitutively expressed in a pair of chemosensory neurons in the head, and cadmium exposure causes *numr-1/-2* expression to increase in the intestine, one might speculate that *numr-1/-2* is part of a peripheral signaling pathway that plays a role in sensing environmental stress and transfers a

signal from the neurons to the intestine to mediate a specific stress response. However, the specific neuronal events concerning *numr-1/-2*, as well as a more detailed phenotypic analysis of the exact biological role of *numr-1/-2* require further study.

The ability of an organism to adapt and respond to environmental stress is imperative for survival; hence there is a strong correlation between enhanced stress tolerance and extended longevity [152]. The results of this study demonstrate that *numr-1/-2* overexpression is capable of increasing longevity and resistance to cadmium exposure. This is based on two major findings. First, overexpression of *numr-1/-2* results in nematodes that are more resistant to cadmium stress and longer lived than control nematodes expressing the selectable marker alone (Figure 3.11). Second, feeding *numr-1/-2(RNAi)* to *numr-1(ok2239)* animals resulted in increased sensitivity to cadmium exposure (Figure 3.12B). The minor but significant effect of the reduction of *numr-1/-2* expression on sensitivity to metal exposure may suggest that *numr-1/-2* is one of many numerous down stream effector molecules that exert a small but specific form of protective influence in response to cadmium stress. An upstream signaling regulator would be expected to have a much larger effect on life span and stress response. For example, down-regulation of any single DAF-16 target gene has a much smaller effect on lifespan than direct inhibition of DAF-16 itself [153]. Thus, *numr-1/-2* may function as a downstream effector in a pathway important for cadmium-stress response.

Alternatively, since cadmium exposure can cause *C. elegans* to bag and *numr-1*

expression is required for normal egg-laying behavior, we speculate that overexpression of NUMR-1/-2 may stimulate egg-laying even in the presence of cadmium, preventing bagging, and thus extending life span.

Exposing adult nematodes to cadmium, results in increased *numr-1/-2* expression throughout the intestine (Figure 3.5A and Figure 3.8B). Intestinal *numr-1/-2* expression is consistent with the finding of a highly conserved GATAA motif within the *numr-1/-2* promoters. Like *numr-1/-2*, three other well-characterized cadmium-responsive genes in *C. elegans*, *mtl-1*, *mtl-2*, and *cdr-1*, contain GATA elements within their promoters and are inducible in the intestine following cadmium exposure [132, 154]. A recent study by McGhee et al. (2009) showed that the GATAA motif is largely over-represented in the promoters of intestinally expressed genes (>80%) and that the ELT-2 GATA-type transcription factor regulates the expression of the majority of these genes [125]. It has also been suggested that ELT-2 plays a critical role in regulating components of the *daf-2/daf-16* pathway [125]. A DAF-16 binding motif was identified in the promoters of *numr-1/-2*. Furthermore, McGhee et al. (2009), have proposed a model in which ELT-2 may combine with SKN-1 to control expression of some SKN-1 downstream target genes [125]. Interestingly, the *numr-1/-2* promoter also contains a SKN-1 binding motif. The presence of both a SKN-1 and DAF-16 binding motif suggests a potential role for *numr-1/-2* in stress-response since these two transcription factors are known to play important roles in mediating stress-resistance in *C. elegans* [86, 126, 155].

The intestinal cells of *C. elegans* are the primary site of metal detoxification [132, 154]. Thus, *numr-1/-2* expression throughout the intestine following cadmium treatment is consistent with a role in metal stress response. Interestingly, copper exposure resulted in increased *numr-1/-2* expression solely throughout the pharynx (Figure 3.5B and Figure 3.8A). The variation in expression pattern could be a result of differences in the toxicity of the metal exposures since very low exposures to cadmium were found to only induce *numr-1/-2* expression in the pharynx, at least initially. It is unlikely that the differences in expression are a result of variations in the distribution of the two metals throughout the nematode since copper accumulates throughout the intestine and the pharynx [156]. Interestingly, it has been shown that copper exposure stimulates calcium influx into the sensory neurons of the pharynx in *C. elegans* [151]. We have shown that *numr-1/-2* responds to changes in intracellular calcium and thus *numr*'s response to copper may be the result of calcium influx into the sensory neurons following copper exposure. This may offer a reason as to why copper exposure only stimulates *numr-1/-2* expression throughout the pharynx and not the intestine.

We also identified a mammalian NFAT binding motif within the *numr-1/-2* promoter. NFAT is a highly calcium-responsive transcription factor [129] and *numr-1/-2* was found to respond to alterations of intracellular calcium levels. However, a *C. elegans* NFAT homolog has yet to be identified.

4.2 Activation of *numr-1/-2* transcription

We next turned our attention to the goal of trying to better understand the signaling events involved in initiating *numr-1/-2* expression in response to cadmium exposure. To this end, we investigated *numr-1/-2* expression induced by other stress conditions such oxidative stress, starvation, endoplasmic reticulum stress, heat shock, intracellular calcium mobilization, and pathogenic infection.

We find that stress conditions such as oxidative stress and heat shock do not alter *numr-1/-2* expression (Table 3.3 and Table 3.4). Interestingly, when we investigated the effects of various forms of ER stress, we find that exposure to TG but not tunicamycin increases *numr-1/-2* expression (Figure 3.13B and C, respectively). TG is a potent ER SERCA pump inhibitor that depletes ER calcium stores, and activates store-operated calcium entry, whereas tunicamycin inhibits glycosylation of newly synthesized proteins. This suggests that a very specific form of ER stress selectively regulates *numr-1/-2* expression.

The depletion of ER calcium stores alters intracellular $[Ca^{2+}]_i$, and it has been proposed that cadmium can increase intracellular calcium levels [68]. This raised the intriguing possibility that the expression of *numr-1/-2*, at least in part, is regulated by alterations in intracellular calcium levels. In support of this idea, we found that *numr-1/-2* transcription greatly increased in nematodes exposed to a calcium ionophore (A23187) or fed *sca-1(RNAi)*, which is the *C. elegans* SERCA pump homolog (Figure 3.13D and

Figure 3.14B, respectively). Taken together, this data shows that *numr-1/-2* responds to mobilization of intracellular calcium and SERCA pump inhibition. However, the data also indicates that the accumulation of unfolded proteins does not increase *numr-1/-2* expression since heat shock nor tunicamycin exposure increases *numr-1/-2* expression.

Since several studies in cell culture systems suggest that cadmium may alter intracellular calcium levels [68]. Therefore, we hypothesized that cadmium exposure might influence *numr-1/-2* expression through alterations of $[Ca^{2+}]_i$. Thus, to address the questions we had of how cadmium may influence intracellular $[Ca^{2+}]_i$ we used a cell model system, HEK293 cells.

4.3 The effects of low dose cadmium exposure on intracellular calcium homeostasis and signaling

Our primary aim of the following section is to better understand the molecular mechanisms involved in regulating gene expression following low, non-toxic cadmium exposures and particularly whether calcium signaling is involved. The reason for studying low, non-toxic cadmium concentrations is that recent evidence indicates that alterations in gene expression following cadmium exposure occur at concentrations below those leading to measurable toxic levels [24, 58, 157]. For instance, Cui et al. (2007), have recently demonstrated that over 100 genes are differentially expressed in *C. elegans* following exposure to cadmium under conditions that do not induce the transcription of many general stress response genes [60]. Therefore, it is important to understand the molecular mechanisms underlying the early effects of cadmium

exposure on gene expression. Cadmium's ability to alter gene expression at early time points has been well-documented but the mechanisms regulating the transcriptional changes have not been well elucidated. Furthermore, there is a need to clarify the role of calcium as a second messenger in mediating cadmium-induced transcription

Modulations in $[Ca^{2+}]_i$ have been proposed to play an important role in mediating cadmium-induced transcription. From our work in *C. elegans* we know that *numr-1/-2* responds to both cadmium and calcium. Therefore, we hypothesize that changes in intracellular calcium levels following cadmium exposure is important in regulating cadmium-induced *numr-1/-2* expression. While several mechanisms have been proposed by which cadmium may alter $[Ca^{2+}]_i$, the effects of cadmium on calcium signaling remain unclear. A possible reason for this ambiguity could be due in part to several technical issues. In particular, the interpretation of data arising from the use of BAPTA-based fluorescent calcium indicators could be problematic since cadmium can bind with very high affinity [74] and elicit fluorescence signals that could be interpreted as a change in $[Ca^{2+}]_i$. Furthermore, to determine a role of calcium in regulating cadmium-induced gene expression, some studies pretreated cells with calcium chelators such as BAPTA. However, since these calcium chelators can bind cadmium with a higher affinity over calcium [158] the observed decrease in the cadmium-induced gene response may simply be due to a reduction in the effective cadmium concentration, rather than an effect on calcium signaling.

We found that exposing HEK293 cells to cadmium concentrations in the range of 1-10 μM (4 and 24 h) results in a classic cadmium-induced gene response and does not decrease cell viability (Figure 3.15 and Figure 3.16C, respectively). At 30 μM cadmium, a concentration at or below levels used in other studies, there is a significant decrease in cell viability, raising the problem that any effects induced by cadmium under these conditions maybe non-specific. An aim of this project was to control for these technical issues as well as to investigate the effects of low, non-toxic concentrations of cadmium on gene expression and determine if these effects of cadmium are mediated by effects on calcium signaling.

Exposing HEK293 cells to 1 μM cadmium for 4 h was sufficient to increase steady-state mRNA abundance of three well-characterized cadmium-inducible genes: *mt-1*, *c-fos*, and *grp-78* (Figure 3.15). This cadmium exposure had no significant effect on cell viability. Based on these results, subsequent calcium homeostasis and signaling experiments were performed using HEK293 cells exposed to 1 μM cadmium for 4 or 24 h. To replicate previous studies and gain an understanding of how toxic-levels of cadmium exposure may interact with calcium signaling systems, we also investigated the effects of exposing HEK293 cells to 30 μM cadmium.

For the assessment of cadmium-mediated alterations on intracellular calcium homeostasis, we used the protein based calcium ion sensor the YC 3.60 stably expressed in HEK293 cells. An advantage to using the YC 3.60 is that unlike BAPTA-based

fluorescent calcium indicators, such as fura-2 or fura-5F, YC 3.60 is insensitive to Cd²⁺ ions (Figure 3.16D) and thus enables cadmium-induced alterations on [Ca²⁺]_i to be investigated without the danger of Cd²⁺ ions interfering with the readings. Using this approach, we found that low-dose cadmium exposures did not interfere with calcium homeostatic mechanisms nor deplete ER calcium store content (Figure 3.17). Only high concentrations of cadmium (30 μM) depleted ER calcium stores, a condition that has a significant effect on cell viability. This observation is similar to that reported by Biagioli et al. (2007) showing a significant depletion of ER calcium stores in murine fibroblasts NIH 3T3 cells treated with 15 μM cadmium for 12 h [75].

Cadmium increases the activity of all three MAPK pathways as well as the calcium/calmodulin-dependent protein kinase II (CaMK-II) [63, 72, 120, 142, 159]. Since MAPKs and CAMK-II are considered integrators of calcium signaling we investigated whether cadmium exposure could alter the transcriptional activity of a range of calcium responsive signaling pathways or calcium responsive genes. Thus, we utilized a cAMP/calcium signaling focused array that contained 84 genes known to be responsive to calcium or cAMP signaling (<http://www.sabiosciences.com/>).

Our data indicated that only toxic concentrations of cadmium (30 μM) were capable of significantly altering the number of differentially expressed cAMP/ calcium-responsive genes (Table 3.6 and 3.7). Exposure to 1 μM cadmium for 4 h, which is sufficient to induce cadmium-responsive gene expression, only altered the

transcriptional activity of 2% of the genes found on the calcium array; whereas, exposure to 30 μ M cadmium for 24 h resulted in the differential expression of 60% of the genes. It was not surprising to observe such a large effect on the transcriptional activity of calcium-responsive genes following exposure to 30 μ M cadmium for 24 h, because this time point also corresponds with a substantial decrease in cell viability and significantly depleted ER calcium stores.

Since we observed exposure to 30 μ M cadmium for 4 or 24 h causes ER calcium pool depletion we compared those effects on calcium/ cAMP responsive genes to those induced by thapsigargin exposure, which also causes ER calcium pool depletion (Table 3.8 and Figure 3.18). We found that 10 genes were significantly up-regulated under all three conditions (Table 3.9) and that 15 of the 17 thapsigargin-induced genes were also found to be up-regulated under the most toxic cadmium exposure. Yet, there were still 31 genes that were significantly up-regulated at our most toxic cadmium concentration that were not induced under a survivable ER stress condition, suggesting that there are many other cellular processes that are disrupted at this high level of cadmium exposure.

4.4 The effects of cadmium exposure on calcium oscillations and defecation in C. elegans

To gain further insight into the mechanistic relationship between cadmium and calcium, we investigated the effects of cadmium on calcium oscillations and a biological process known to be regulated by calcium oscillations in a whole organism, the nematode *C. elegans*. Defecation is a highly rhythmic behavior initiated once a minute.

The initiation of the defecation cycle and pBoc is regulated by calcium oscillations in the intestinal cells. The intestine is also the primary site for metal detoxification and *numr-1/-2* induction following cadmium exposure. Therefore, we were interested in the effects that a non-toxic cadmium exposure may have on calcium oscillations in the intestine and the pBoc cycle. We observed that cadmium exposure significantly shortened the defecation cycle but did not alter the overall rhythmic nature of this behavior (Table 3.10). Mutants that exhibit a similar phenotype as that observed in the cadmium treated worms most commonly have deficiencies in lipid synthesis and vesicle transport [160, 161]. This is in contrast to mutants deficient in calcium signaling, which usually exhibit defecation cycles that are lengthened and no longer rhythmic [162, 163]. Furthermore, the magnitude of the calcium peaks during calcium oscillations in cadmium exposed nematodes was not significantly different from those observed in control nematodes. The frequency of the calcium waves was increased in cadmium exposed nematodes but the calcium oscillations were still rhythmic (Figure 3.19). Mutants with defects in calcium signaling exhibit calcium oscillations that are arrhythmic and occur with reduced amplitude [95]. This suggests that cadmium alters the defecation cycle independent of changes in calcium homeostasis.

4.5 Summary

This project presents data describing how non-toxic concentrations of cadmium are sufficient to induce gene expression, but do not alter calcium homeostasis or calcium

signaling. Therefore, calcium signaling is most likely not the primary mechanism regulating cadmium-induced gene transcription at low levels of exposure.

In *C. elegans*, *numr-1* and *numr-2* are both highly cadmium-inducible genes that are sufficient to increase resistance to cadmium exposure. Proper levels of *numr-1* expression are important for certain muscular functions such as feeding and egg laying. It is interesting that both of these behaviors are regulated by fluctuations in intracellular calcium levels and that *numr-1/-2* responds to these types of changes.

Our studies suggest that *numr-1/-2* responds to both cadmium and calcium and not necessarily cadmium's ability to alter intracellular calcium levels. Preliminary data suggest that cadmium does not alter calcium levels under exposure conditions sufficient to increase *numr-1/-2* transcription. Our studies also indicate that *numr-1/-2* responds to alterations of intracellular calcium based on the thapsigargin and calcium ionophore studies. In addition, knockdown of the *C. elegans* SERCA pump homolog increased *numr-1-2* transcription. The fact that *numr-1/-2* may have two distinct stimuli that regulate its transcription is not unprecedented.

In our mammalian cell culture system we show that non-toxic cadmium exposures, sufficient to induce gene expression, do not affect calcium homeostasis or calcium signaling. However, toxic concentrations of cadmium did adversely affect calcium homeostasis and did significantly affect the transcriptional activity of many

calcium responsive genes. These findings are consistent with previous studies indicating that high cadmium concentrations alter intracellular calcium levels.

The amount of data indicating that cellular response to cadmium exposure begins prior to typically measured toxicity markers is increasing rapidly. Thus, there is a need to understand the early cellular events involved in mediating a response to low-level cadmium exposure. In addition, the known signaling pathways that are activated in response to cadmium exposure do not account for all the genes that are differentially expressed following cadmium exposure. Furthermore, the precise molecular mechanism regulating the activity of these known signaling cascades have not been well elucidated. The data presented in this study suggests that alterations on intracellular calcium levels is not a primary mechanism for regulating cadmium-induced gene expression at low levels of exposure and the old paradigm that 'cadmium alters intracellular calcium levels' is oversimplified.

The results from these studies add to the body of our knowledge in regards to the molecular mechanisms involved in the regulation of cadmium-responsive gene expression. Defining the regulatory mechanisms associated with cadmium-induced transcription, will allow for better assessment of the risk associated with cadmium exposure and provide more detail for mechanism-based risk assessment.

5. Future Directions

5.1 Defining a biological role for *numr-1/-2*

The regulation of transcription has been extensively studied, yet little is known about the proteins and mechanisms involved in regulating mRNA processing. RNA-binding proteins have begun to emerge as important regulators of many post-transcriptional steps in gene expression [164]. In fact, the eukaryotic genome encodes for hundreds of RNA-binding proteins and only a small fraction of these proteins have been characterized [165]. NUMR-1/-2 contains an arginine rich domain and previous studies have shown that arginine-rich regions are important for RNA binding [118, 119]. This suggests that NUMR-1/-2 may play a role in RNA processing. In further support of this idea, NUMR-1/-2 was found to co-localize with HSF-1 in discrete punctate nuclear structures following cadmium treatment. In cell culture, these structures have been termed nuclear stress granules and have been shown to be dynamic nuclear structures that form in response to various types of stress conditions such as, heat shock and heavy metal exposure [166]. The precise role of these nuclear granules are unclear but a number of RNA processing factors have been reported to localize to nuclear stress granules [167]. Other studies in RNA processing suggest that each RNA binding protein has a small yet specific purpose [164]. In agreement with this, in *C. elegans* many RNA binding proteins have very tissue-specific mutant phenotypes [168]. Thus, if NUMR-1/-2 is an RNA binding protein that plays a role in mRNA processing, it may explain the

weak yet significant phenotypes we observe with decreased *numr-1/-2* expression. It would be interesting to try to assess the potential RNA processing capabilities of NUMR-1/-2 and identify potential targets, which would be important for understanding the role of *numr-1/-2* in stress response and certain neuromuscular functions. It would also be of interest to try to determine what other proteins co-localize with NUMR-1/-2 and HSF-1 during times of stress. This would offer further insight into mechanisms of cadmium induced gene expression and a functional role for *numr-1/-2*.

5.2 Determine if cadmium activates *numr-1/-2* independent of calcium mobilization

One of the goals of this project was to better understand the relationship between cadmium exposure, calcium mobilization, and the subsequent effect on gene expression. Our cell culture data clearly shows that low levels of cadmium exposure activate transcription independent of calcium mobilization. However, it is not as clear in *C. elegans* with regards to the induction of *numr-1/-2*, which responds to both cadmium exposure and changes of intracellular calcium. It would be of interest to determine if cadmium activates *numr-1/-2* expression independently of calcium mobilization.

This goal could be achieved in several different ways. One possibility would be to isolate the intestine of *C. elegans* and see if we could first induce *numr-1/-2* expression in response to cadmium exposure in the isolated intestines. If we could, it would be interesting to try to inhibit various aspects of calcium signaling and homeostasis and see how this would influence *numr-1/-2* expression following cadmium exposure.

We could also perform an RNAi screen or a random mutagenesis assay and look for *numr-1/-2* mutants that do not respond to cadmium exposure and also look for *numr-1/-2* mutants that do not respond to changes of intracellular calcium levels. Once we identify the genes that produce *numr-1/-2* loss of function phenotypes in the presence of calcium and cadmium, we could see if these two phenotypes result from the knockdown of a common set of genes or two separate sets of genes. If a common set of genes is identified it would support the idea that cadmium activate *numr-1/-2* through calcium mobilization. If the sets of genes are independent of each other it would suggest that both cadmium and calcium activate *numr-1/-2* and cadmium's ability to activate *numr-1/-2* is independent of calcium mobilization.

Alternatively, we could perform a promoter bashing experiment in which we eliminate specific *cis* regulatory elements of the *numr-1/-2* promoters and examine the effect on cadmium- and calcium-induced transcription of *numr-1/-2*. Similar to the genetic experiments, the identification of regulatory sequences that independently activate *numr-1/-2* in response to cadmium and calcium would suggest that cadmium does not activate *numr-1/-2* through alterations of intracellular calcium levels. Identification of genes and regulatory elements that regulate *numr-1/-2* transcription in response to cadmium exposure would offer insight into signaling pathways important for mediating defense against cadmium exposure.

5.3 Potential disruption of fatty acid synthesis in *C. elegans* following cadmium exposure

Cadmium is considered a neurotoxin but little progress has been made in understanding the molecular mechanisms underlying this observed toxicity. Many neurological disorders are thought to result from various defects in lipid synthesis or metabolism [169]. Preliminary data in *C. elegans* suggest that disruption of lipid synthesis might be an early target of cadmium toxicity.

We observe that cadmium exposure for only 5 h significantly shortens the defecation cycle but does not alter the overall rhythmic nature of this behavior. Mutants that exhibit a similar phenotype as that observed in cadmium treated worms most commonly have deficiencies in lipid synthesis and vesicle transport [160, 161]. This is in contrast to mutants deficient in calcium signaling, which usually exhibit defecation cycles that are lengthened and no longer rhythmic [162, 163]. This would suggest that cadmium exposure alters defecation independent of calcium signaling, which leads us to postulate that cadmium exposure may alter lipid synthesis in *C. elegans* and thus alters the defecation cycle. In support of this idea, Cui et al. (2007) show that cadmium exposure significantly down-regulated the expression of several enzymes important for fatty acid metabolism but the biological consequence of the alteration in the mRNA levels of these genes has not been established [60]. Therefore, it would be of interest to investigate the relationship between cadmium exposure and fatty acid lipid synthesis. In *C. elegans* numerous genes and pathways involved in fat synthesis and storage have

been identified and are conserved in higher eukaryotes, thus making *C. elegans* a useful model to investigate mechanisms by which cadmium may interfere with lipid synthesis.

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Biography

Brooke E. Tvermoes

PERSONAL INFORMATION

DATE OF BIRTH DECEMBER 3, 1980
PLACE OF BIRTH PHOENIX, AZ

Education

Ph.D. (2003 to Present) • DUKE UNIVERSITY, Nicholas School of the Environment and Integrated Toxicology and Environmental Health Program – Durham, NC

National Institute of Health Graduate Partnerships Program Certificate (upon graduation) • NIEHS, National Institute of Environmental Health Sciences – Research Triangle Park, NC

Bachelor of Science in Chemistry with American Chemical Society Certification, *magna cum laude* (2003) NORTHERN ARIZONA UNIVERSITY – Flagstaff, AZ

Research Experience

Predoctoral IRTA (2007 to Present) • Investigating molecular mechanisms involved in response to cadmium exposure • NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES
Advisor: Dr. Jonathan Freedman

Graduate Research Assistant (2003-2007) • Investigating molecular mechanisms involved in response to cadmium exposure • DUKE UNIVERSITY, Nicholas School of the Environment and Integrated Toxicology and Environmental Health
Advisor: Dr. Jonathan Freedman

Undergraduate Research Assistant (2001-2003) • Investigating modes of action involved in chromium⁺⁶ carcinogenesis • NORTHERN ARIZONA UNIVERSITY – Flagstaff, AZ
Advisor: Dr. Diane Stearns

Teaching and Mentoring Experience

Summer of Discovery Mentor for Undergraduate Research (Summer 2007) • Benjamin Dickey • NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES, NC

Guest Lecturer (Spring 2007) • Graduate Course: Genetics – Oncogenes and the Molecular Basis of Cancer • NORTH CAROLINA CENTRAL UNIVERSITY, Durham, NC

Preparing Future Faculty Fellow (2006-2007) • DUKE UNIVERSITY, Durham, NC

Teaching Assistant (Spring 2006) • Graduate Course: Fate of Organic Compounds • DUKE UNIVERSITY, Durham, NC

Funding Awards

Predoctoral Intramural Research Training Award (2007 to Present) • NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES • Investigate the molecular mechanisms involved in response to cadmium exposure in *C. elegans*

Burroughs Wellcome Fund (2007) • \$2000.00 • NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES • Help fund the National Institute of Environmental Health Sciences' Career Fair

Beckman Award (2002-2003) • \$17,460 • NORTHERN ARIZONA UNIVERSITY • Investigating the effects of Vitamin C and E on DNA damage caused by chromium(III)trispiccolinate

Hooper Grant recipient (2001-2003) • \$7,000 • NORTHERN ARIZONA UNIVERSITY • Investigating types of DNA lesions induced by exposure to chromium(III)trispiccolinate

Publications

Molecular characterization of NUMR-1/-2: nuclear-localized, metal-responsive genes that confer increased resistance to cadmium toxicity and are essential for certain neuromuscular functions in *Caenorhabditis elegans*
Brooke E. Tvermoe, Windy A. Boyd, and Jonathan H. Freedman (manuscript)

Low-level cadmium exposures can induce transcription of cadmium-responsive genes independent of calcium mobilization in HEK293 cells

Brooke E. Tvermoes, Gary S. Bird, and Jonathan H. Freedman

Presentations

Oral Presentations

Society of Toxicology 48th Annual Meeting, March 2009, "The effect of cadmium exposure on calcium homeostasis and signaling pathways," Brooke E. Tvermoes, Gary S. Bird, Windy A. Boyd, and Jonathan H. Freedman

Duke University Integrated Toxicology Program Environmental Health Spring 2009 Seminar Series, January 2009, "Functional and molecular analysis of the novel metal responsive *Caenorhabditis elegans numr-1* and *numr-2*," Brooke E. Tvermoes, Gary S. Bird, Windy A. Boyd, and Jonathan H. Freedman

National Institute of Environmental Health Sciences Science Awards Day, November 2008, "The use of comparative genomics to investigate disruption of Ca²⁺ levels in the regulation of cadmium induced gene expression," Brooke E. Tvermoes, Gary S. Bird, Windy A. Boyd, and Jonathan H. Freedman

Aging Stress, pathogenesis, and heterochrony *C. elegans* topic meeting, August 2008, "Caenorhabditis elegans genes NUMR-1 and NUMR-2 confer increased resistance to cadmium toxicity and are essential for certain neuromuscular functions," Brooke Tvermoes, Windy Boyd, and Jonathan H. Freedman

Laboratory of Pharmacology and Chemistry and Laboratory of Molecular Toxicology Department Seminar, NIEHS, 2007, "Functional analysis of *numr-1*: A nuclear localized metal responsive gene that confers increased resistance to metal stress and influences lifespan in *Caenorhabditis elegans*," Brooke Tvermoes and Jonathan H. Freedman

16th International *C. elegans* Conference, 2007, "Functional analysis of *numr-1*: A nuclear localized metal responsive gene that confers increased resistance to metal stress and influences lifespan in *Caenorhabditis elegans*," Brooke Tvermoes and Jonathan H. Freedman

Poster Presentations

National Society of Toxicology Meeting, 2008, "*Caenorhabditis elegans* gene, *numr-1*, assembles into nuclear stress granules after cadmium treatment" Brooke Tvermoes and Jonathan H. Freedman

2nd Place Poster Award, National Society of Toxicology Meeting, 2007, "The molecular characterization of a cadmium inducible transformer-2-like protein from the nematode *Caenorhabditis elegans*" Brooke Tvermoes, Yuxia Cui, Windy Boyd, Daniel Snyder, and Jonathan H. Freedman

Superfund Basic Research Program Meeting, 2006, "The identification and characterization of a novel-metal inducible gene expressed in the AWA sensory neurons in the nematode *Caenorhabditis elegans*" Brooke Tvermoes, Yuxia Cui, Windy Boyd, Daniel Snyder, and Jonathan H. Freedman

Fourth Conference on Molecular Mechanisms of Metal Toxicity and Carcinogenesis, 2006, "Temporal and spatial expression patterns of F08F8.5: A novel metal-inducible gene from the nematode *Caenorhabditis elegans*" Brooke Baker, Yuxia Cui, Windy Boyd, Daniel Snyder, and Jonathan H. Freedman

North Carolina Regional Society of Toxicology Meeting, 2006, "Molecular Characterization of a Novel Metal Inducible gene, F08F8.5, from the nematode *Caenorhabditis elegans*" Brooke Baker, Yuxia Cui, Windy Boyd, Daniel Snyder, and Jonathan H. Freedman

Duke's Graduate Student Symposium, 2003, "Development of two model systems to study MTF-1 activation in response to cadmium exposure" Brooke Baker and Jonathan H. Freedman

National Society of Toxicology Meeting, 2003, "DNA Damage Induced by Chromium Picolinate" BE Baker, AM Luke, JA Hager, KK Wolf and DM Stearns

Professional Affiliations

Society of Toxicology (2003 to present)

Genetics Society of America (2007)

North Carolina Society of Toxicology (2006 to present)

American Association for the Advancement of Science (2005 to present)