CeHMT-1, a Putative Phytochelatin Transporter, Is Required for Cd2⁺ Tolerance in *Caenorhabditis elegans*.

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Phytochelatins (PCs), (γ-Glu-Cys)₅Gly polymers that were formerly considered to be restricted to plants and some fungal systems, are now known to play a critical role in heavy metal (notably Cd²⁺) detoxification in *Caenorhabditis elegans*. In view of the functional equivalence of the gene encoding *C. elegans* PC synthase 1, *ce-pcs-1*, to its homologs from plant and fungal sources, we have gone on to explore processes downstream of PC fabrication in this organism. Here we describe the identification of a half-molecule ATP-binding cassette transporter, CeHMT-1, from *C. elegans* with an equivalent topology to that of the putative PC transporter SpHMT-1 from *Schizosaccharomyces pombe*. At one level, CeHMT-1 satisfies the requirements of a Cd²⁺ tolerance factor involved in the sequestration and/or elimination of Cd²⁺-PC complexes. Heterologous expression of *ce-hmt-1* in *S. pombe* alleviates the Cd²⁺-hypersensitivity of hmt-1 mutants concomitant with the localization of CeHMT-1 to the vacuolar membrane. Suppression of the expression of *ce-hmt-1* in intact worms by RNA interference (RNAi) confers a Cd²⁺-hypersensitive phenotype similar to but more pronounced than that exhibited by *ce-pcs-1* RNAi worms. At another level, it is evident from comparisons of the cell morphology of *ce-hmt-1* and *ce-pcs-1* single and double RNAi mutants that CeHMT-1 also contributes to Cd²⁺ tolerance in other ways. Whereas the intestinal epithelial cells of *ce-pcs-1* RNAi worms undergo necrosis upon exposure to toxic levels of Cd²⁺, the corresponding cells of *ce-hmt-1* RNAi worms instead elaborate punctate refractive inclusions within the vicinity of the nucleus. Moreover, a deficiency in CeHMT-1 does not interfere with the phenotype associated with *CePCS-1*-deficiency and vice versa. Double *ce-hmt-1*/*ce-pcs-1* RNAi mutants exhibit both cell morphologies when exposed to Cd²⁺. These results and those from our previous investigations of the requirement for PC synthase for heavy metal tolerance in *C. elegans* demonstrate PC-dependent, HMT-1-mediated heavy metal detoxification not only in *S. pombe* but also in some invertebrates while at the same time indicating that the action of CeHMT-1 does not depend exclusively on PC synthesis.

The toxicity of supraoptimal levels of the essential heavy metals copper and zinc and of trace or higher levels of the nonessential heavy metals cadmium, arsenic, mercury, and lead is thought to result from the displacement of endogenous co-factors from their cellular binding sites, thiol-capping of essential proteins and peptides, and promotion of the formation of reactive oxygen species (1). In humans, the repercussions of heavy metal exposure can range from acute poisoning to progressive kidney, liver, and lung dysfunction and, in some instances, cancer. Although its true clinical and epidemiological significance remains to be determined, chronic exposure to heavy metals is often associated with muscular and neurologically degenerative conditions reminiscent of muscular dystrophy, multiple sclerosis, Alzheimer disease, and Parkinson disease (2–4).

Three classes of heavy metal-binding peptides are known to participate in the homeostasis and detoxification of heavy metals in most animals. These are the ubiquitous thiol tripeptide glutathione (GSH), a family of small (4–8 kDa) cysteine-rich proteins termed metallothioneins, and several higher molecular mass albeit sequence-unrelated metal-binding proteins. In all of the plants studied and in some fungi (as exemplified by *Schizosaccharomyces pombe* and *Candida glabrata*) and some animals (as exemplified by the model nematode *Caenorhabditis elegans*), a third class of cysteine-rich peptides termed phytochelatins (PCs)¹ has also been shown to be involved in heavy metal detoxification.

PCs, which constitute a family of short-chain heavy metal-binding peptides with the general structure (γ-EC)ᵢXₙₙₙ, where *n* = 2–11, are derived from GSH and related thiols in a γ-glutamylcysteinyl transpeptidation reaction catalyzed by phytochelatin synthases (EC 2.3.2.15) (1, 5, 6). It is now almost 15 years since the partial purification of the enzyme capable of catalyzing PC synthesis (5), yet it is only in the last several years that its molecular identity has been determined by the cloning and characterization of genes encoding PC synthases (PCSs). Originally isolated from *Arabidopsis thaliana*, *S. pombe*, and wheat (*Triticum aestivum*), and latterly from *C. elegans*, these genes (designated *AtPCS1*, *SpPCS*, *TaPCS1* and *ce-pcs-1*, respectively) encode 40–50% sequence-similar 41–55-kDa polypeptides that contribute to heavy metal detoxification by catalyzing the *de novo* synthesis of PCs (7–12).

In those systems that have been studied in sufficient detail, namely the fission yeast *S. pombe* and plants, PCs thiol coor-

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¹ The abbreviations used are: PC, phytochelatin; ABC, ATP-binding cassette; Ce (prefix), *C. elegans*; DAPI, 4',6-diamidino-2-phenylindole; ds (prefix), double-stranded; EMM, Edinburgh minimal medium; GFP, green fluorescent protein; HMT, heavy metal tolerance factor; Sp (prefix), *S. pombe*; NBF, nucleotide binding fold; NGM, nematode growth medium; NTE, N-terminal extension; PCS, phytochelatin synthase; RNAi, RNA interference; RT, reverse transcription; TMD, transmembrane domain.
dinate and chelate heavy metals to promote their removal from the cytosol by vacuolar sequestration (1, 13, 14). In the most thoroughly characterized of these systems, *S. pombe*, the vacuolar sequestration of heavy metal-PC complexes has been inferred to be catalyzed by a vacuolar membrane-localized “half-molecule” ATP-binding cassette (ABC) transporter, SpHMT1 (*S. pombe* heavy metal tolerance factor 1) (15, 16).

Canonical, “full-molecule” ABC proteins consist of two transmembrane domains (TMDs), each of which contains 4–6 transmembrane α-helices, two nucleotide binding folds (NBFs), each of which contains the Walker A and B boxes, and the ABC signature motif. So-called half-molecule ABC transporters, by contrast, contain only one TMD and one NBF (17). In addition to these core domains, some members of both types of ABC transporters possess a hydrophobic N-terminal extension (NTE) and a linker (L0) sequence contiguous with the TMD or the NBF. In the case of SpHMT1, the mature 90.5-kDa polypeptide species contains an NTE containing five hydrophilic minima and an L0 linker sequence contiguous with one TMD and one NBF (15).

The precise mechanism of action for SpHMT1 is unknown. On the one hand, it has been demonstrated that *S. pombe hmt1*-mutants are hypersensitive to Cd\(^{2+}\) in the growth medium (15) and that vacuoles isolated from *S. pombe* overexpressing plasmid-borne SpHMT1 mediate the MgATP-dependent, vanadate-inhibitable uptake of Cd-PC complexes and apo-PCs (16). On the other hand, the relevance of what has been inferred from investigations of *S. pombe* to the equivalent processes in plants is contentious. HMT-1 homologs have yet to be isolated from or even detected in the genomes of vascular plants (18), although an MgATP-energized, vanadate-inhibitable transport pathway for PCs, analogous to that identified in *S. pombe*, has been characterized in vacuolar membrane vesicles purified from oat roots (19).

Here we report the identification and functional characterization of a half-molecule ABC transporter from *C. elegans* that is crucial for Cd\(^{2+}\) tolerance in the intact organism. In so doing, we establish that an HMT-1-dependent pathway for the detoxification of Cd\(^{2+}\) is not restricted to fungi and plants but is also operative in some animals. That said, the results of these investigations also reveal a versatility for CeHMT-1 in the intact worm and possibly its equivalents in other organisms that has not been considered previously. In particular, whereas the basic structural and functional properties of CeHMT-1 are consistent with it being an ortholog of SpHMT1, comparisons of the phenotypes of *ce-hmt-1* and *ce-pcs-1* single RNAi worms and *ce-hmt-1;ce-pcs-1* double RNAi worms yield findings that invoke auxiliary roles for CeHMT-1 in Cd\(^{2+}\) detoxification that do not depend only on upstream PC synthesis.

**MATERIALS AND METHODS**

**Yeast Strains and Growth Conditions**—The *S. pombe* strains used in these studies were the wild type strain Sp223 (15, 16) and the isogenic hmt1* mutant strain LK100 derived from Sp223 by ethyl methanesulfonate mutagenesis (15) (a kind gift from Dr. David Ow, University of California, Berkeley, CA). Both strains were grown routinely at 30 °C in YE (0.5% yeast extract) medium supplemented with 2% dextrose and 225 mg/liter each leucine, adenine, and uracil. *S. pombe* transformants were selected for uracil prototrophy in Edinburgh minimal medium (EMM), which, in addition to leucine, adenine and dextrose, contained 14.7 mM potassium hydroxide phosphate, 15.5 mM Na\(_2\)HPO\(_4\), 93.5 mM NaCl, 0.26 mM MgCl\(_2\), 4.99 mM CaCl\(_2\), 0.67 mM KCl, 14.1 mM Na\(_2\)SO\(_4\), 80.9 mM boric acid, 23.7 mM MnSO\(_4\), 13.9 mM ZnSO\(_4\), 7.4 mM FeCl\(_3\), 2.47 mM molybdate, 6.02 mM potassium iodide, 1.60 mM CuSO\(_4\), 47.6 mM citric acid, 2.40 mM pantothenic acid, 81.2 mM nicotinic acid, 55.5 mM inositol, and 40.8 μM biotin. When assaying Cd\(^{2+}\) tolerance, the growth media were supplemented with CdCl\(_2\) as indicated.

**Isolation and Heterologous Expression of ce-hmt-1**—The cDNA corresponding to the coding sequence for CeHMT-1 was amplified from *C. elegans* N2 strain RNA by reverse transcription (RT) PCR. Total RNA was extracted in TRIzol R Reagent (Invitrogen), according to the manufacturer’s recommendations, and the first strand was synthesized by standard procedures using a SuperScript preamplification system (Invitrogen). The primers for amplification of the 2.4-kb ce-hmt-1 open reading frame (5′-CANAATTGCCGCTAGATGGGTTTTACCA-3′ and 5′-TCAAGGAAAGTCTCCGGGCGGATGCAAG-3′) were designed to generate XhoI and Smal restriction sites at the 5′- and 3′-termini, respectively, of the amplification product. For heterologous expression, the ce-hmt-1 cDNA (GenBank accession numbers AF497513 and AF497514) was subcloned into the XhoI and Smal restriction sites of the *S. pombe*-Escherichia coli shuttle vector pREP4X (American Type Culture Collection) to place it under the control of the thiamine-repressible promoter of the *S. pombe* nmt-1 (no message in thiamine) gene. The resulting pREP4X-ce-hmt-1 construct was expressed in LK100 *S. pombe* cells. As a control, pREP4X vector lacking the ce-hmt-1 insert was transformed into Sp223-derived LK100 progenitor cells. In all cases, *S. pombe* cells were transformed using the lithium acetate procedure (20), and the transformants were selected for uracil prototrophy in EMM.

**RT-PCR Analysis of Expression of ce-hmt-1 in *C. elegans***—For RT-PCR analysis of the expression of ce-hmt-1, N2 worms were cultured on solid nematode growth medium (NGM) for 3 days using standard procedures. Mixed stage worm pellets collected from the plates were then inoculated into liquid S-medium supplemented with *E. coli* OP50 and CdCl\(_2\) and cultured with gentle shaking at 20 °C. After 6 and 24 h of incubation, the worms were collected, washed free of bacteria in M9 buffer, and frozen in liquid nitrogen. Total RNA was extracted using TRIzol Reagent (Invitrogen) as described above. The first cDNA strand was synthesized from total RNA (1 μg) using an Advantage RT-PCR kit according to the manufacturer’s instructions (Clontech). Amplification was by PCR using the ce-hmt-1-specific oligonucleotide primer pair 5′-TGAAGGTGTGATAGTAATGGC-3′ and 3′- TTCAATGCTTGGTCTCTTTCT-5′ to yield a 1097-bp amplification product. The primers were designed to preclude amplification from any contaminating genomic DNA that might have been present in the cDNA preparations. To verify that equivalent amounts of RNA had been amplified for the same RNA template, the cDNA was also subjected to RT-PCR using an oligonucleotide primer pair 5′-CTTCCACCCACCGCCT-GACCGTG-3′ and 3′-CGGAATCCAGTCGGAGTACTTG-5′ specific for the *C. elegans* actin ce-act-4 gene. In all cases, PCR was performed for 18, 25, 30, or 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C. The PCR products were resolved by electrophoresis on 1.2% (w/v) agarose gels.

**Double-stranded RNA Synthesis**—For the *in vitro* synthesis of ce-hmt-1 RNA, 958 bp of the coding sequence corresponding to the NTE of the CeHMT-1 polypeptide was PCR-amplified from the ce-hmt-1 cDNA using the primer pairs 5′-AAAAACAGGCGCCGCAATGG-GCTTTTACCATTTCTCG-3′ and 3′-GTGTGGGCGCTGCGTCAAACA-TTACCGT-5′ and subcloned into plBluevector script (Invitrogen). RNA was transcribed in vitro from the T7 promoter of a plasmid containing an oligonucleotide transcription kit (Stratagene). After DNase digestion of the template, the sense and antisense preparations were gel-purified, mixed in an equimolar ratio in 1 μM EDTA buffered to pH 7.5 with Tris-HCl, heated for 1 min in boiling water, and annealed at room temperature as described for ce-pcs-1 (12). To ensure that the ce-hmt-1 dsRNA preparations from the annealing step were devoid of residual single-stranded RNA, the mixtures were treated with RNase T1 (22). The dsce-hmt-1 RNA product was purified by phenol/chloroform extraction, reconstituted in water, diluted to a concentration of 500 ng/μl with injection buffer (23), and stored at −80 °C for injection.

**dsRNA Microinjection and Phenotypic Screens**—The ce-hmt-1 RNAi experiments and phenotypic screens were performed as described for ce-pcs-1 (12). In all cases, the dsce-hmt-1 RNAi- or control (injection buffer alone)-injected worms were first cultured for 12–18 h on NGM plates seeded with *E. coli* OP50 before transfer to plates supplemented with Cd\(^{2+}\). Heavy metal sensitivity was screened in two ways. In the first procedure, individual worms were transferred sequentially onto NGM plates supplemented with 0, 25, 50, and 100 μM CdCl\(_2\). Egg laying was allowed to proceed for 6 h at each Cd\(^{2+}\) concentration, after which eggs from the next generation were transferred to another round of egg laying. In this way, it was determined that 25 μM CdCl\(_2\) was sufficient to cause severe morphological and developmental changes in the CeHMT-1-deficient worms but not in the controls. In the second screen, individual injected worms were transferred to NGM plates supplemented with 0, 5, 10, 25, 50, or 100 μM CdCl\(_2\) and allowed to lay eggs for 6 h, after which they were transferred for another 6 h onto plates containing 25 μM CdCl\(_2\). Only the progeny of worms that...
exhibited the CeHMT-1-deficient phenotype upon transfer to plates containing 25 μM Cd2+ (100% of the progeny from worms injected with dsce-hmt-1 RNA) were scored at the other concentrations. The phenotypes of the worms were examined 4–6 days after hatching.

Evaluation of the Gene Specificity of dsce-hmt-1 RNA Action—To evaluate the specificity of the action of dsce-hmt-1 RNA, parallel RNAi experiments were performed on two of the closest CeHMT-1 homologs, the half-molecule ABC transporters Haf-7 (Y50E8A.16, GenBankTM accession number CAB60586), and Haf-9 (ZK484.2, GenBankTM accession number T32865). For this purpose, cDNAs encoding the idio-
typic NTEs of ZK484.2 and Y50E8A.16 were generated by RT-PCR-
amplification using the primer pairs 5'-ATGAGTTCTTCTCTTTACAC-
and 3'-CTCTTCGGCACTCTTGGCATAAGATTAG-5' for ZK484.2 and the primer pairs 5'-ATGATGGCTCTCTCTCTTTACAC-
and 3'-CTCTTCGGCACTCTTGGCATAAGATTAG-5' for Y50E8A.16. Synthesis of the corresponding dsRNAs, their injection, and an assessment of the Cd2+ sensitivity of the progeny of the injected worms were performed as described for ce-hmt-1.

Subcellular Localization of Heterologously Expressed CeHMT-1 in C. elegans—To determine the subcellular localization of heterologously expressed CeHMT-1 in C. elegans, a construct was generated containing the full-length Ce-hmt-1 cDNA fused in-frame with egfp, the gene encoding the red-shifted variant of the 33-kDa green fluorescence protein (GFP). This construct pREP4X-ce-hmt-1::egfp, was used to transform LK100 cells. Vacuoles were visualized by incubating the transformants with acridine orange (0.05 μg/ml), a fluorescent lipophilic basic dye that accumulates in acidic compartments. Nuclei were visualized after incubating the transformants with 4′,6-diamidino-2-phenylindole (DAPI, 100 ng/ml) (Molecular Probes) in Tris-buffered saline for 30 min before being subjected to three cycles of washing in the same buffer. Log phase LK100/pREP4X-
ce-hmt-1::egfp cells and LK100/pREP4X cells stained with acridine orange or DAPI were examined at 100× magnification under a Leica DAS microscope equipped with a DMR camera, UV light source, and GFP-, rhodamine- and DAPI-specific filter sets.

Chemicals—All of the general reagents were obtained from Fisher, Research Organics, Inc., and Sigma

RESULTS

Identification and Cloning of ce-hmt-1—The discovery of a functional PCS gene, ce-pcs-1 in C. elegans (11, 12), and the results of earlier investigations suggesting that in S. pombe its homolog SpPCS acts upstream of SpHmt1 (15, 16) prompted a systematic search of worm sequence databases for HMT-1 homologs. Knowing that the C. elegans genome contains some 50 open reading frames for ABC transporters, of which 29 fall into the half-molecule-transporter category (24), and that even ABC transporters with disparate capabilities can have a relatively high degree of sequence similarity (18), putative C. elegans HMT-1 homologs were identified according to two structural criteria in addition to overall sequence similarity. The first criterion was the possession of a forward orientation, half-molecule ABC transporter structure consisting of a single TMD contiguous with a single NBF, in that order. The second was the presence of an NTE containing five hydrophilicity minima.

Application of these criteria disclosed only one gene whose putative translation product satisfies the structural requirements of an SpHmt1 equivalent. Designated ce-hmt-1 for C. elegans heavy metal tolerance factor 1 (alias haf-5 according to the nomenclature of Ref. 24), this gene encodes a 90.7-kDa polypeptide, CeHMT-1, that is 51% sequence similar (34% sequence identical) to SpHmt1. CeHMT-1 consists of a single TMD containing six putative transmembrane spans and an NBF containing a Walker A box motif (LILVLD) (Fig. 1). As inferred from application of the TopPred II computer algorithms, CeHMT-1 possesses a 223-amino acid residue NTE in tandem with the TMD and NBF consisting of a 189-amino acid residue membrane-spanning domain (MSD0) encompassing five hydrophilicity minima (25) and a 34-amino acid residue linker region (L0) (Fig. 1).

The cDNA corresponding to ce-hmt-1 was isolated by an RT-PCR of total RNA extracted from mixed stage worms using primers designed to span the entire coding region. After confirming the fidelity of the 2.4-kb amplification product (GenBankTM accession number AAM33381) by sequencing, it was determined that the gene encodes a protein that is equivalent to SpHmt1, its heterologous expression in hmt-1 mutant S. pombe cells should alleviate Cd2+ hypersensitivity. Therefore, to gain an initial indication of its functional capabilities, ce-hmt-1 was subcloned into the E. coli-S. pombe shuttle vector pREP4X under control of the thiamine-repressible plasmid-borne promoter of the nmt-1 gene and transformed into S. pombe hmt-1 mutant strain LK100; thiamine-repressible suppression of the Cd2+-hypersensitive hmt-1 phenotype was then assayed.
These experiments demonstrated that expression of the pREP4X-ce-hmt-1 construct confers increased Cd\(^{2+}\)-tolerance versus empty vector-transformed LK100 controls regardless of whether growth is monitored as colony formation by serially diluted inocula on solid media containing Cd\(^{2+}\) after 8 days (Fig. 2A) or as cell density after 72 h of growth in liquid media containing Cd\(^{2+}\) (Fig. 2B). As determined from the concentrations of Cd\(^{2+}\) required to inhibit growth in liquid medium by 50%, plasmid-borne ce-hmt-1 increases the tolerance of LK100 cells by at least 10-fold (Fig. 2B). The effects seen are specifically attributable to expression of the ce-hmt-1 gene insert in that repression of the nmt-1 promoter when the growth media are supplemented with thiamine abolishes the Cd\(^{2+}\)-tolerance that is otherwise conferred by pREP4X-ce-hmt-1 (Fig. 2C).

**Heterologously Expressed CeHMT-1 Localizes to the Vascular Membrane of S. pombe—SpHMT1 localizes to the vascular membrane of S. pombe and is considered to catalyze the vacuolar sequestration of Cd-PC complexes (16). Growing heterologously expressed CeHMT-1 is able to suppress the Cd\(^{2+}\) hypersensitivity of SpHMT1-deficient S. pombe mutants, the intracellular distribution of CeHMT-1 in this system was examined. For this purpose, the full-length coding sequence of ce-hmt-1 was fused in-frame with the coding sequence for the red-shifted variant of GFP, enhanced GFP (EGFP), and cloned behind the nmt-1 promoter of pREP4X.

When transformed into S. pombe LK100, pREP4X-ce-hmt-1:egfp yields cells in which the bulk of the green fluorescence is associated with the fusion product localizes to the vacuole periphery (Fig. 3A). Fluorescence microscopy reveals intense fluorescent green circular structures in the transformants that coincide with the membranes bounding the vacuoles as determined by differential interference contrast microscopy (Fig. 3A) and the distribution of acidine orange, a fluorescent pH gradient indicator that undergoes preferential accumulation in acidic compartments (Fig. 3B). Neither of these fluorescence signals derives from the nucleus, because DAPI staining of the same cells yields a punctate distribution (Fig. 3C). These results of these manipulations were unequivocal. It was demonstrated that whereas the progeny of the control injection buffer-injected worms and the CeHMT-1-deficient worms in media devoid of heavy metals are indistinguishable and develop into normal-sized, gravid, fertile adults, the latter are markedly more sensitive than the former to exposure to Cd\(^{2+}\).
as the concentration of Cd\textsuperscript{2+} required for a 50% decrease in the number of individuals that reach adulthood, the progeny of ce-hmt-1 RNAi worms are at least 4-fold more sensitive to Cd\textsuperscript{2+} than the progeny of ce-pcs-1 RNAi worms (Figs. 4 and 5). Furthermore, the progeny of ce-hmt-1 and ce-pcs-1 RNAi worms undergo distinguishable morphological changes upon exposure to heavy metals (Fig. 6). As exemplified by worms hatched and cultured for 4 days on media containing toxic concentrations of CdCl\textsubscript{2}, the intestinal cells of the progeny of dsce-pcs-1-injected worms undergo necrosis as noted previously (12). In contrast, the same cells in the progeny of dsce-hmt-1-injected worms fabricate spherical refractive inclusions of ~20 μm in diameter in the immediate vicinity of the nucleus (Fig. 6). These inclusions are absent from the larvae immediately after hatching on media containing Cd\textsuperscript{2+} but are apparent after ~24 h and persist throughout the worm’s lifetime.

During the latter stages of preparing this manuscript for publication, two knock-out lines, VC252 and VC287, were obtained from the C. elegans Knock-out Consortium, each of which contains a partial deletion of the ce-hmt-1 gene. Both of these lines are of similar Cd\textsuperscript{2+} hypersensitivity and undergo the same morphological changes upon exposure to Cd\textsuperscript{2+} as the dsce-hmt-1 RNAi worms described here (data not shown), confirming that the Cd\textsuperscript{2+} hypersensitivity of and the morphological changes seen in CeHMT-1-deficient worms are the same whether CeHMT-1 function is disrupted by gene deletion or RNAi.

dsce-pcs-1 RNAi Does Not Influence the Cd\textsuperscript{2+} Hypersensitivity of dsce-hmt-1 RNAi Worms or the Assembly of Intracellular Inclusions—If CeHMT-1 acts downstream of PC synthesis and is solely responsible for the intracellular sequestration and/or cellular elimination of CdPC complexes, a deficiency in CeHMT-1 activity would be expected to cause the accumulation of CdPC complexes and/or their derivatives in the cytosol. Accumulation of such complexes in the cytosol might then provide the framework for and/or elicit formation of the refractive inclusions seen in Cd\textsuperscript{2+}-treated CeHMT-1-deficient worms. Cytosolic CdPC accumulation would, in turn, likely preclude salvage of PC constituents from their usual site of delivery, acidic vacuolysosomal compartments, and thereby exhaust cellular reserves of the PC precursor GSH. Because GSH is not only involved in metal chelation but also in alleviating some of the secondary oxidative consequences of heavy metal toxicity, this could account for why dsce-hmt-1 RNAi worms are more sensitive to Cd\textsuperscript{2+} than dsce-pcs-1 RNAi worms. A specific prediction therefore follows. Namely, if the extreme Cd\textsuperscript{2+} hypersensitivity of CeHMT-1-deficient worms and their facility for the elaboration of cytosolic inclusions depends exclusively on the synthesis of PCs, coincident suppression of ce-pcs-1 expression should abolish the ce-hmt-1 RNAI-specific phenotype.

In testing this hypothesis and refuting it through the generation of doubly CePCS-1- and CeHMT-1-deficient worms, results were obtained demonstrating that the morphological changes associated with the ce-hmt-1 RNAi phenotype neither depend obligatorily on a functional copy of ce-pcs-1 nor are alleviated by its suppression. Targeted suppression of the expression of both ce-pcs-1 and ce-hmt-1 neither diminishes the Cd\textsuperscript{2+} hypersensitivity associated with the loss of CeHMT-1 function nor interferes with assembly of the intracellular inclusions formed upon exposure to Cd\textsuperscript{2+}. Worms that are deficient in both CePCS-1 and CeHMT-1, like worms that are singly deficient in CeHMT-1, undergo developmental arrest and form nuclearly associated inclusions when grown on media containing Cd\textsuperscript{2+} (25 μM) (Fig. 6) but are indistinguishable from injection buffer-injected controls when grown on media lacking heavy metals (data not shown). Indeed, the intestinal cells of
CeHMT-1 has a special status in this respect. Its nearest equivalents, Haf-7 and Haf-9 from the same organism, do not contribute to heavy metal tolerance. Moreover, although a few full-molecule ABC transporters, namely one member of the multidrug resistance-associated protein (MRP-1) and two members of the P-glycoprotein subfamily (PGP-1 and PGP-3), have been shown to contribute to heavy metal tolerance in *C. elegans* (29), their phenotypic impact is low by comparison to that of CeHMT-1. *C. elegans* triple *mrr-1/pgp-1/pgp-3* mutants, for instance, exhibit a markedly less pronounced Cd$^{2+}$-hypersensitive phenotype (29) than their *ce-hmt-1* RNAi counterparts.

From investigations of the molecular basis of heavy metal detoxification in *S. pombe* it has been inferred that SpHMT1 acts downstream of PC synthase by catalyzing the transport of Cd-PC complexes into the vacuole (15, 16). Our finding that CeHMT-1-deficient worms, like CePCS-1-deficient worms, are Cd$^{2+}$-hypersensitive is consistent with the general applicability of such a scheme to other organisms that harbor genes for HMT-1-type ABC transporters. This being the case, the phenotypes of CeHMT-1-deficient and CePCS-1-deficient worms are nonetheless sufficiently different to necessitate auxiliary roles. Specifically, CeHMT-1-deficient worms are markedly more sensitive to Cd$^{2+}$ than CePCS-1-deficient worms, and, whereas the intestinal cells of the former elaborate refractive inclusions upon exposure to Cd$^{2+}$, the same cells of the latter do not, but instead undergo necrosis. Accordingly, closer inspection of the genetic interactions between *ce-hmt-1* and *ce-pcs-1* does not demonstrate a strict dependence of either on the other, as would be predicted for genes whose translation products are exclusively restricted to a common pathway. Suppression of *ce-pcs-1* expression in conjunction with *ce-hmt-1* does not interfere with formation of the intestinal inclusions, nor does it decrease Cd$^{2+}$ sensitivity to the level observed in single *ce-pcs-1* RNAi worms. Rather, the intestinal cells of double *ce-pcs-1;ce-hmt-1* RNAi worms manifest both phenotypes, namely necrosis and the formation of refractive inclusions after exposure to Cd$^{2+}$.

The most straightforward explanation for these observations is that CeHMT-1 is required for Cd$^{2+}$ detoxification, but not only in a PC-dependent manner. It is probably also involved in the transport of molecules other than PCs. For example, CeHMT-1-deficient and CePCS-1-deficient worms are nonetheless sufficiently different to necessitate auxiliary roles. Specifically, CeHMT-1-deficient worms are markedly more sensitive to Cd$^{2+}$ than CePCS-1-deficient worms, and, whereas the intestinal cells of the former elaborate refractive inclusions upon exposure to Cd$^{2+}$, the same cells of the latter do not, but instead undergo necrosis. Accordingly, closer inspection of the genetic interactions between *ce-hmt-1* and *ce-pcs-1* does not demonstrate a strict dependence of either on the other, as would be predicted for genes whose translation products are exclusively restricted to a common pathway. Suppression of *ce-pcs-1* expression in conjunction with *ce-hmt-1* does not interfere with formation of the intestinal inclusions, nor does it decrease Cd$^{2+}$ sensitivity to the level observed in single *ce-pcs-1* RNAi worms. Rather, the intestinal cells of double *ce-pcs-1;ce-hmt-1* RNAi worms manifest both phenotypes, namely necrosis and the formation of refractive inclusions after exposure to Cd$^{2+}$.

The most straightforward explanation for these observations is that CeHMT-1 is required for Cd$^{2+}$ detoxification, but not only in a PC-dependent manner. It is probably also involved in the transport of molecules other than PCs. For example, CeHMT-1 may contribute to the cellular sequestration and/or elimination of heavy metals coordinated with other ligands such as GSH and/or metallothioneins as well as PCs and/or is involved in alleviating the toxic effects of the reactive oxygen species formed when cells are exposed to heavy metals.

A corollary of the conclusion that CeHMT-1 has auxiliary functions is that the same may apply to other HMT-1-like transporters. Domain comparisons among the half-molecule ABC transporters in the protein databases of other animals disclose transporters that are HMT-1-like (for instance,
CG4225 from *Drosophila* and mammalian mitochondrial ABC protein 3 (MTABC3) from humans. This would not be expected from what is known of SpHMT1, because *Drosophila* and humans do not deploy a PC-dependent pathway for heavy metal detoxification (30). Three explanations capable of reconciling what ostensibly is a contradiction might follow from these findings and from the results of studies implicating mammalian mitochondrial ABC protein 3 in iron homeostasis (31). The first explanation is that the HMT-1s have undergone divergence such that representatives in different organisms serve different roles. The second is that, in addition to heavy metal detoxification in *C. elegans* or iron homeostasis in higher animals, these transporters are involved in other cellular processes. The third possibility is that the mammalian mitochondrial ABC protein 3 of humans and CG4225 from *Drosophila*, in addition to iron homeostasis, are involved in detoxification of heavy metals and/or the by-products of heavy metal action.

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