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The Intestinal Copper Exporter CUA-1 Is Required for Systemic Copper Homeostasis in Caenorhabditis elegans

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Copper plays key catalytic and regulatory roles in biochemical processes essential for normal growth, development, and health. Defects in copper metabolism cause Menkes and Wilson’s disease, myeloneuropathy, and cardiovascular disease and are associated with other pathophysiological states. Consequently, it is critical to understand the mechanisms by which organisms control the acquisition, distribution, and utilization of copper. The intestinal enterocyte is a key regulatory point for copper absorption into the body; however, the mechanisms by which intestinal cells transport copper to maintain organismal copper homeostasis are poorly understood. Here, we identify a mechanism by which organismal copper homeostasis is maintained by intestinal copper exporter trafficking that is coordinated by which organisms associate with other pathophysiological states. Consequently, it is critical to understand the mechanisms by which organisms control the acquisition, distribution, and utilization of copper. The intestinal enterocyte is a key regulatory point for copper absorption into the body; however, the mechanisms by which intestinal cells transport copper to maintain organismal copper homeostasis are poorly understood. Here, we identify a mechanism by which organismal copper homeostasis is maintained by intestinal copper exporter trafficking that is coordinated with extraintestinal copper levels in Caenorhabditis elegans. Specifically, we show that CUA-1, the C. elegans homolog of ATP7A/B, localizes to lysosome-like organelles (gut granules) in the intestine under copper overload conditions for copper detoxification, whereas copper deficiency results in a redistribution of CUA-1 to basolateral membranes for copper efflux to peripheral tissues. Worms defective in gut granule biogenesis exhibit defects in copper sequestration and increased susceptibility to toxic copper levels. Interestingly, however, a splice isoform CUA-1.2 that lacks a portion of the N-terminal domain is targeted constitutively to the basolateral membrane irrespective of dietary copper concentration. Our studies establish that CUA-1 is a key intestinal copper exporter and that its trafficking is regulated to maintain systemic copper homeostasis. C. elegans could therefore be exploited as a whole-animal model system to study regulation of intra- and intercellular copper trafficking pathways.

Copper is essential for catalytic and regulatory functions in a wide range of biochemical reactions involved in mitochondrial respiration, connective tissue formation, and iron metabolism (1, 2). Copper deficiency is associated with pathologies that include anemia, neutropenia, and cardiomyopathy (1, 3). Additionally, if its homeostasis is not properly regulated, copper can be extremely toxic due to its stimulation of free radical production. Organisms finely tune copper homeostasis through a combination of absorption, distribution, and efflux in multiple tissues. In many species, a key aspect of copper homeostasis is facilitated by membrane-bound copper efflux pumps. Mammals have two primary copper exporters that are structurally related, ATP7A and ATP7B P-type ATPases. In tissue culture models, both of these proteins deliver copper to the lumens of the secretory machinery for incorporation into various copper-dependent enzymes at basal or low intracellular copper concentrations. At elevated cellular copper levels, ATP7A traffics to the plasma membrane to remove excess copper from cells, and ATP7B relocates to the plasma membrane and endosomes to excrete copper (2, 4). In humans, mutations in the genes encoding ATP7A and ATP7B result in severe systemic copper deficiency (Menkes disease) and hepatic/neuronal hyperaccumulation of copper (Wilson’s disease), respectively (1). However, to date, copper-responsive steady-state distributions of ATP7A/B have been studied predominantly at the cellular level in tissue culture models, and regulation of their trafficking by dietary copper has not yet been thoroughly elucidated within an intact animal model (4–6).

Although the optically transparent roundworm Caenorhabditis elegans has emerged as a highly amenable model of micronutrient metabolism (7–9), the C. elegans model system has been relatively unexploited for questions related to copper homeostasis, despite the fact that these worms have a defined and highly versatile intestinal capacity for nutrient absorption (10–12). For these reasons, we utilized C. elegans, which has been widely used in the study of the metabolism of other metals, including iron, heme, and zinc (8, 13–17). Although mammals have two copper exporters, serving complementary functions in different tissues such as the gut and liver, lower metazoans (including nematodes (C. elegans) and insects (Drosophila melanogaster)) have only a single homolog of ATP7A/B (2, 18, 19). The protein product of C. elegans cua-1 shares very high sequence similarity with human ATP7A/B. Previous studies have used yeast to demonstrate that cua-1 has copper efflux functions, as the gene encoding C. elegans CUA-1 could rescue a yeast strain lacking the CCC2 gene, which encodes a functional counterpart (20). In worms, transcriptional reporters...
have been used to detect cua-1 expression in several tissues, including the intestine. An essential role for cua-1 for copper transport in C. elegans has been suggested, as deletion of cua-1 results in embryonic lethality (18, 21). However, how CUA-1 in the intestine responds to systemic copper status is not understood, and its intracellular location and copper responsiveness have not been determined.

Here, we report the importance of copper in C. elegans development and a distinct role for CUA-1 in mediating copper transport. Expression of cua-1 specifically in the intestine is sufficient to rescue the embryonic lethal phenotype of the cua-1 mutant. A CUA-1 translational fusion protein primarily localizes to the basolateral membrane of the intestine under basal and copper-deficient conditions but redistributes to lysosome-related organelles (gut granules) in response to elevated copper levels in the diet. Moreover, copper injection into the pseudo-coelom, a fluid-filled body cavity between the intestine and the body wall, leads to a significant endosomal re-localization of intestinal CUA-1 even in dietary copper-deprived worms. Our studies show that intestinal CUA-1 is a key component regulating copper supply and detoxification to maintain systemic copper homeostasis in the intact animal.

Results

Dietary Copper Levels Affect Worm Growth—Living organisms have an optimal range of copper concentrations, and dietary excess or deficiency of copper causes various diseases and developmental defects in a broad range of organisms (1, 2). To determine the dietary copper requirements of C. elegans grown on NGM2 plates fed with the Escherichia coli strain OP50, we followed worm growth over 8 days under copper restriction using the copper(I)-specific chelator bathocuproinedisulfonic acid (BCS) or with copper supplementation using CuCl2. Worm growth showed a biphasic curve over dietary copper levels (Fig. 1A). C. elegans displayed maximal growth in the low micromolar range of supplemental copper and impaired growth at either end of the copper spectrum, 100 μM BCS and 150 μM copper, with smaller brood size and delayed development. Large amounts of copper (∼200 μM) resulted in growth arrest at the L3 stage, likely due to copper toxicity. Monitoring of animal development revealed that at optimal copper (∼2 μM), worms became gravid adults in 3 days, whereas most animals grown at high or low copper conditions only reached the L3 to young adult stage at this time point (Fig. 1B).

To quantify the effect of dietary copper on each animal within a mixed population, we used a COPAS Biosort instrument. Animal development was significantly delayed by supplementation of high doses of either copper or BCS (Fig. 1C). To complement the analysis of how dietary copper influences worm growth, whole-animal copper content was measured using inductively coupled plasma-mass spectrometry (ICP-MS). Worms cultured with high copper (150 μM) had only a 5-fold increase in copper content as compared with animals grown on 10 μM copper supplementation, whereas the relative levels in the diet. Moreover, copper injection into the pseudo-coelom, a fluid-filled body cavity between the intestine and the body wall, leads to a significant endosomal re-localization of intestinal CUA-1 even in dietary copper-deprived worms. Our studies show that intestinal CUA-1 is a key component regulating copper supply and detoxification to maintain systemic copper homeostasis in the intact animal.

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Copper exporter gene duplication to ATP7A occurred following the branching off of the vertebrates, which is suggested that the copper exporter gene duplication to ATP7A/B are highly conserved in CUA-1 (Fig. 2B and supplemental Fig. S2B). The genomic structure of cua-1 consists of 16 exons, and it is predicted to encode two splice isoforms. The first isoform, cua-1.1 (Y76A2A.2a), contains all exons, whereas the cua-1.2 (Y76A2A.2b) isoform lacks the first two exons, which encode the first metal-binding domain (Fig. 2B, A and B).

To ascertain the physiological function of cua-1, worms were analyzed for growth and developmental phenotypes after knockdown of cua-1. Without copper supplementation, animals treated with cua-1 RNAi at the L1 stage became gravid adults in 4 days but failed to lay eggs. For worms grown in the presence of 100 μM BCS, treatment with cua-1 RNAi resulted in near total lethality, whereas vector control worms only showed a delayed F1 hatching (Fig. 2C). Together, these results indicate that cua-1 is required for normal growth in C. elegans. To further explore the importance of cua-1 in fecundity, total brood size was quantified. Regardless of RNAi conditions, addition of 100 μM BCS resulted in reduced brood sizes, indicating that dietary copper uptake is essential for normal reproduction (Fig. 2D). In mammals, cytosolic copper is delivered to either ATP7A or ATP7B by the copper chaperone ATOX1. In C. elegans, CUC-1 is a putative ATOX1 homolog that has been previously identified by yeast complementation assay (18).
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Depletion of cua-1 or cuc-1 by RNAi resulted in a decrease in brood size as compared with vector control RNAi (Fig. 2D). Double knockdown of cua-1/cuc-1 led to a severe egg-laying defect, suggesting that a CUC-1/CUA-1 copper relay and transport pathway is conserved in C. elegans, analogous to the ATOX1-ATP7A/B pathway in mammals.

To investigate the physiological significance of maternal copper availability to progeny, RNAi against cua-1 with either 0 or 10 μM supplemental copper was conducted for 3 days until parental animals (P₀) reached adulthood, and their synchronized L1 progeny (F₁) were cultured under identical RNAi knockdown conditions in the presence or absence of BCS for 2 days. Worms treated with cua-1 RNAi whose parents were grown in 0 μM supplemental copper displayed delayed growth rates in the presence of BCS as compared with those treated with vector control (data not shown). When mothers were provided with 10 μM supplemental copper, the growth rate of F₁ progeny treated with control RNAi was faster than that of progeny from mothers that received no supplemental copper (Fig. 2E). However, the growth rate of the cua-1 knockdown progeny was not strongly affected when the mothers were cultured in 10 μM supplemental copper (Fig. 2E). These results suggest that viability of embryos is both dependent upon maternal copper status and upon cua-1 activity.

CUA-1 Expression Is Regulated by Dietary Copper—To determine whether cua-1 is transcriptionally regulated by dietary copper, wild-type N2 worms were cultured with no supplementation, with 150 μM copper, or with 100 μM BCS, and mRNA levels were measured by quantitative real time PCR (qRT-PCR). Levels of cua-1 mRNA were modestly elevated under copper-limited conditions compared with copper supplementations (Fig. 3A). To further analyze cua-1 expression and localization, we generated transgenic animals expressing CUA-1::GFP translational fusions under the control of an endogenous promoter using the CUA-1.1 isoform, as this isoform is the full-length form of the CUA-1 gene. P_cua-1::CUA-1.1::GFP transgenic animals showed similar tissue distribution patterns to the previously reported transcriptional reporter worms, which was primarily intestine, neurons, hypodermis, and pharynx (18), although the intestinal expression was concentrated mainly in the anterior of the intestine (Fig. 3B).

The strain VC672 contains a deletion in cua-1(ok904), spanning exons 13–15 (Fig. 2A; supplemental Fig. S2C), which is genetically balanced due to the embryonic lethality of cua-1 mutant worms (21, 23). The cua-1(ok904) deletion removes a region containing the last two transmembrane helices as well as an ATP-binding motif and a dileucine-based sorting signal (Fig. 2B). The cua-1(ok904) mutant is rescued with the transgene P_cua-1::CUA-1.1::GFP, indicating that the CUA-1.1::GFP translational fusion protein is functional (Fig. 3C). To further confirm whether the CUA-1.1::GFP translational fusion protein can transport copper, we exploited previously established assays in Atpt7a⁻/⁻ and Atpt7a⁻/- mouse embryonic fibroblasts (MEFs) (24). Atpt7a⁻/- MEFs transiently transfected with CUA-1.1::GFP showed increased expression of copper chaperone for superoxide dismutase (CCS), indicating decreased levels of cellular copper (25, 26) as compared with Atpt7a⁻/- MEFs transfected with an empty vector (supplemental Fig. S3A).

Over-accumulated copper in Atpt7a⁻/- MEFs was rescued by ectopic expression of CUA-1.1::GFP (supplemental Fig. S3B), further indicating that CUA-1 can export copper. Moreover, these results demonstrate that a C-terminal GFP tag does not significantly interfere with CUA-1 function.

Unexpectedly, when transgenic worms (P_cua-1::CUA-1.1::GFP) were maintained at low copper concentrations (50 μM BCS), stronger CUA-1.1::GFP expression was observed in the hypodermis, whereas GFP expression levels were not altered in other tissues such as the intestine and neurons (Fig. 3C). Indeed, quantification of CUA-1.1::GFP by COPAS Biosort and immunoblotting assay showed significantly enhanced CUA-1.1::GFP levels under low copper conditions, suggesting that copper-dependent regulation in the hypodermal cells contributes to the overall steady-state abundance of CUA-1.1 (Fig. 3, D and E).

To determine the contribution of each tissue to the embryonic lethal phenotype of cua-1 mutant worms, we carried out tissue-specific RNAi experiments. Mutant rde-1 worms are resistant to RNAi, but restoring tissue-specific expression of the wild-type rde-1 cDNA in these mutants confers RNAi sensitivity to a specific tissue (27). We depleted cua-1 in wild-type N2, WM27 (rde-1 mutant, RNAi insensitive), VP303 (P_nix-2::RDE-1, intestine only RNAi), WM118 (P_vma-5::RDE-1, muscle only RNAi), and NR222 (P_lin-2c::RDE-1, hypodermis only RNAI) worm strains (13). Knockdown of cua-1 in the intestine resulted in reduced fecundity similar to that of whole-body RNAi (Fig. 3F), indicating that intestinal CUA-1 is crucial for the survival of worms. Although significant effects were not observed when cua-1 was knocked down in muscle, depletion of cua-1 in the hypodermis caused a severe reduction in brood size under low dietary copper conditions (Fig. 3F). Copper supplementation (10 μM) was able to rescue the reduced brood sizes phenotype caused by cua-1 RNAi in each of these strains (Fig. 3F). These results imply a critical role of both intestinal and hypodermal CUA-1 in worm growth under copper-deficient conditions.

Intestinal Expression of cua-1.1 Is Sufficient to Rescue the Lethal Phenotype of cua-1(ok904)—Given that targeted depletion of cua-1 in the intestine caused similar phenotypes as whole-body RNAi, we examined the subcellular localization of CUA-1.1 by driving the expression of CUA-1.1::GFP from the strong constitutive intestine-specific vha-6 promoter (28). P_vha-6::CUA-1.1::GFP localized to basolateral membranes and to intracellular compartments, reminiscent of basolateral sorting and recycling endosomes in the C. elegans intestine (Fig. 4A) (29). Importantly, the embryonic lethal phenotype of cua-1 (ok904) mutant can be rescued by intestine-specific expression of CUA-1.1::GFP (Fig. 4, B and C) suggesting that the intestine is the key site of copper regulation in C. elegans. These results are further corroborated by the fact that RNAi depletion of either cuc-1 or cua-1 in P_vha-6::CUA-1.1::GFP, cua-1(ok904) transgenic animals grown in low copper results in a lethal phenotype that can be rescued by copper supplementation. These observations suggest that a CUC-1/CUA-1 copper delivery pathway from intestine to peripheral tissues is essential for worms under dietary copper restriction (Fig. 4, B and C). To further assess the significance of intestinal CUA-1 function

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under varying copper availability, F1 progeny of N2 wild-type, P\text{vha-6}::CUA-1.1::GFP, and P\text{vha-6}::CUA-1.1::GFP; cua-1(ok904) worms derived from P0 worms exposed to 10 \mu M copper were grown to the L4/young adult stage under different dietary copper conditions and then analyzed by COPAS Biosort. Transgenic animals expressing CUA-1.1::GFP under control of the vha-6 promoter exhibited a reduced growth phenotype when exposed to high copper and enhanced growth under copper restriction in a dose-dependent manner (Fig. 4, C and D). We attribute the copper hypersensitivity to the constitutive overexpression of CUA-1.1 in the intestine, which would lead to the export of copper into the worm body.
that intestinal CUA-1 is crucial for copper delivery to extra-intestinal tissues, depletion of cua-1 by RNAi in both wild-type and \( P_{\text{vha-6}}::\text{CUA-1.1::GFP; cua-1(ok904)} \) worms resulted in improved growth under toxic copper conditions and growth inhibition under copper restriction as compared with vector RNAi (Fig. 4, C–E). To further understand the role of cua-1 in copper metabolism, we measured total worm copper content using ICP-MS. In the presence of 50 \( \mu \text{M} \) copper, either cua-1 or cuc-1 RNAi knockdown in wild-type worms resulted in \(~30\%\) lower total copper content than in control worms, whereas worms without copper supplementation exhibited similar total copper contents under both RNAi conditions (Fig. 4F). These results suggest that accumulation of copper under high dietary copper conditions is dependent on cua-1.
Intestinal CUA-1.1 Distribution Is Regulated by Copper Levels—Copper-responsive fluorescent probes have been used to visualize the distribution of labile copper in a variety of model systems (30–33). After conducting several pilot studies with a number of different copper probes in *C. elegans*, we selected the copper(I) probe CF4 based on its high specificity (see under “Experimental Procedures” for details). To investigate the dynamics of copper levels in the intestine, we pre-exposed worms expressing CUA-1.1::GFP in the intestine to either 50 μM CuCl₂ or 50 μM BCS followed by incubation with CF4. We observed that worms exposed to supplemental copper accumulated more fluorescence puncta in the intestine than worms treated with BCS, although a control CF4 probe, which lacks the copper-binding atoms but retains the same lipophilic dye platform, displayed weaker staining (Fig. 5A). These results indicate that CF4 detects labile copper levels in vesicles of the intestine in *C. elegans*.

To test whether dietary copper alters CUA-1.1 localization, transgenic animals harboring a *cua-1.1* translational reporter driven by the *vha-6* promoter were grown under varying copper concentrations and then imaged using confocal microscopy. In the presence of 50 μM BCS (Fig. 5B; supplemental Fig. S4) or no supplemental copper (data not shown), intestinal CUA-1.1::GFP localized predominantly to basolateral membranes (Fig. 5B, panels a and f), as well as low levels being detectable in intracellular compartments. We identified these compartments as Golgi, as some of them overlapped with the Golgi marker MANS::mCherry (supplemental Fig. S4). Strikingly, in the presence of 25 μM supplemental copper, CUA-1.1::GFP was redistributed to a cellular compartment that was distinct from...
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the Golgi (Fig. 5B, panels g and l; supplemental Fig. S4). CUA-1.1::GFP-containing vesicles overlapped with the auto-fluorescence of gut granules (Fig. 5B, panel f), an intestine-specific lysosome-related organelle, indicating that copper may be concentrated at these sites (34, 35).

To examine the relationship between CUA-1.1 and copper, transgenic animals expressing CUA-1.1::GFP were cultured with the copper probe CF4. In worms cultured with 25 μM copper, CF4 and CUA-1.1::GFP fluorescence overlapped almost completely, suggesting that CUA-1.1 localizes to the gut granules that concentrate copper in response to toxic levels of environmental copper (Fig. 5B, panel k). Additionally, intestinal CUA-1.1::GFP localization shifted from punctate staining to larger vesicles as worms were exposed to increasing concentrations of dietary copper (Fig. 5C). CUA-1.1::GFP expression in the intestine driven by the endogenous cua-1 promoter rather than the vha-6 promoter also showed similar trafficking in response to changes in dietary copper (supplemental Fig. S5 A). Worms in which cua-1 had been depleted by RNAi showed dramatically decreased levels of CF4 fluorescence in the intestine as compared with control worms (Fig. 5B, panels i and u) suggesting that CUA-1.1 acts to export copper to gut granules. In summary, CUA-1.1 mainly resides on the basolateral membranes under basal and copper-deficient conditions, but it localizes to gut granules in response to increasing dietary copper. Note that these changes are not due to copper-responsive elevation in protein abundance of intestinal CUA-1.1 (P_<ruby>_vha-6::<rtm>CUA-1.1::GFP<rtm></ruby>) in transgenic worms, as immunoblotting and COPAS Biosort analysis showed no significant differences in GFP levels when these worms were grown at varying copper levels (supplemental Fig. S6, A and B).

We next expressed CUA-1.1::GFP in the specialized epithelial cells of the C. elegans hypodermis using the hypodermis-specific dpy-7 promoter (34). Confocal microscopy studies in transgenic worms expressing P_<ruby>_dpy-7::<rtm>CUA-1.1::GFP<rtm></ruby> showed that in hypodermal tissues, plasma membrane localization of CUA-1.1::GFP was not affected by dietary copper, suggesting that copper-responsive trafficking of CUA-1.1 protein is intestine-specific (supplemental Fig. S5B).

To further explore whether the intracellular localization of intestinal CUA-1.1::GFP is altered by elevated copper status in the pseudocoelom, ~100 μg of CuCl₂ per g of worm (wet weight) in M9 buffer was injected into the pseudocoelom of adult worms harboring the CUA-1.1::GFP transgene, which had been preincubated with 50 μM BCS. Interestingly, we observed a punctate pattern of CUA-1.1::GFP in the intestine of animals injected with copper, whereas M9-injected controls showed predominantly basolateral membrane localization (Fig. 5D), implying the existence of a systemic copper homeostasis mediated by the trafficking of intestinal CUA-1.1.

CUA-1.1 Is Required for Copper Detoxification in Intestine—To further investigate the dynamics of CUA-1.1 localization in response to sub-toxic doses of copper supplementation, we assayed CUA-1.1 colocalization with LysoTracker, a lysosome-specific fluorescent dye (35, 36). In the presence of 50 μM copper, LysoTracker was observed in intestinal vesicles surrounded by membrane-bound CUA-1.1::GFP (Fig. 6A). Upon RNAi knockdown of cua-1, the number and morphology of LysoTracker-positive compartments did not change. However, RNAi knockdown pgg-2, which encodes an ABC transporter that is required for gut granule biogenesis and localizes to the gut granule membrane (35, 37), significantly reduced LysoTracker staining and prevented CUA-1.1::GFP-containing vesicle formation, whereas CUA-1.1::GFP was detected on the basolateral membrane (Fig. 6A). These results further suggest that CUA-1.1 localizes to the membranes of gut granules, and gut granules are the destination of copper sequestered by CUA-1.1 in the intestine when animals are exposed to high dietary copper.

To determine whether copper sequestration into gut granules by CUA-1.1 contributes to copper detoxification, we tested copper sensitivity by measuring the growth rate of C. elegans in the presence of increasing concentrations of copper. When compared with control worms, pgg-2 depleted worms showed increased sensitivity to high copper, as they displayed a dose-dependent decrease in growth rate in response to copper (Fig. 6B). To quantify copper sequestration defects in gut granule-deficient worms, we measured total copper content in worms by ICP-MS. pgg-2 mutant worms cultured in 50 μM copper displayed reduced total copper content as compared with wild-type worms (Fig. 6C). Consistent with previous reports, pgg-2 mutant worms displayed lower zinc content independent of copper supplementation (supplemental Fig. S7, A and B) (8). These results indicate that copper deposition accounts for a significant proportion of total body copper in C. elegans and that gut granules are at least partially required for copper detoxification.

CUA-1 Isoforms Function Coordinate to Maintain Systemic Copper Levels—RNAseq assays with synchronized populations of worms treated with different copper or BCS levels revealed the existence of cua-1.2 variant as annotated (Fig. 2A), although relative levels of the two isoforms have not been established.³ To examine whether intestinal CUA-1.2 also retains the capacity to traffic in response to a high copper diet, we generated transgenic worms expressing a CUA-1.2::GFP translational fusion driven from the intestinal vha-6 promoter. Confocal microscopy analysis showed that CUA-1.2::GFP is localized to the basolateral membranes irrespective of dietary copper concentration (Fig. 7A). Additionally, CUA-1.2::GFP did not colocalize with autofluorescent gut granules under high copper conditions suggesting CUA-1.2 functions constantly at basolateral membranes. Because CUA-1.1 has an additional 122 amino acid sequences at the N terminus end compared with CUA-1.2, it is plausible that copper-dependent trafficking of CUA-1.1 to gut granules is dependent on trafficking motifs within this N-terminal segment.

Discussion

Dietary copper availability can fluctuate widely depending upon an organism’s immediate environment, as would be the case in C. elegans, which lives in soil. In this study, we show that CUA-1.1 is expressed in intestinal cells and normally localizes to the basolateral membrane and intracellular compartments, such as the Golgi. When worms are exposed to higher copper

³ B.-E. Kim, unpublished data.
levels, redistribution of intestinal CUA-1.1 promotes copper sequestration into lysosome-related organelles called gut granules. Defects in gut granule biogenesis lead to decreased copper accumulation and increased susceptibility to toxic copper levels. Together, these results suggest that copper homeostasis is regulated by altering the localization of intestinal CUA-1.1 to either the basolateral membrane for delivery of copper to peripheral tissues or to the gut granule membrane to prevent copper toxicity (Fig. 7B).

In *C. elegans*, which lack a liver, the intestine has been thought to perform functions associated with both the intestine and the liver (38). Worms encode only one copper exporter gene, *cua-1*, raising the question as to whether CUA-1 accomplishes some or all of the similar functions as ATP7A/B in the intestine and liver of mammals (20). We found that CUA-1 protein abundance in the intestine was not changed by dietary copper. Instead, both CUA-1 isoforms localize to basolateral membranes and intracellular compartments, including the Golgi under basal and copper-limiting conditions. The CUA-1.1 isoform alone redistributes to the gut granules when worms are exposed to high levels of copper, indicating that CUA-1 shares physiological features of mammalian ATP7A and ATP7B. Enrichment of CUA-1.1 to the membrane of gut granules rather than the basolateral membrane of the intestine suggests a distinct role for intestinal CUA-1.1 in detoxification of excess copper in *C. elegans*. Although relocation of CUA-1.1 and ATP7B to the gut granules and apical membrane, respectively, in polarized cells is not identical, the direction of trafficking toward preventing systemic copper toxicity is similar. Taken together, these data indicate that intestinal CUA-1 functions as copper exporters in worms like ATP7A/B in both the intestine and liver of mammals to maintain copper balance in the body.

Given that CUA-1.2 lacks a portion of the N-terminal intracellular domain of CUA-1.1 and is targeted constitutively to the basolateral membrane even under copper-loaded conditions, necessary trafficking information may exist in the first 122 amino acids of CUA-1.1 for copper responsiveness and correct targeting to gut granules. Several intriguing questions arise from this observation. What route does CUA-1.1 take to reach gut granules when intracellular copper is increased? Where within the first 122 amino acids is the crucial targeting signal? What cellular machinery recognizes the copper signal? One possible sorting complex is the biogenesis of lysosome-related
organelles complex-1 (BLOC-1), which is a known regulator of intracellular trafficking to lysosome-related organelles in mammals, Drosophila, and C. elegans (39–41). ATP7A is known to supply copper to melanosomes in a BLOC-1-dependent manner in mammalian cells, and BLOC-1 subunits are also required for the proper trafficking of gut granule cargo in worms (39, 42). Given that CUA-1.1 localizes to gut granules and that ATP7A localizes to melanosomes in response to elevated copper levels to promote copper detoxification in C. elegans.

We have determined that gut granules function to sequester excess copper via CUA-1.1 in the intestine. Ablation of cua-1 by RNAi does not interfere with the formation of gut granules, but copper was not readily detected by a copper probe in gut granules upon the loss of CUA-1.1. RNAi depletion of pgp-2 genes resulted in significantly reduced numbers of gut granules and increased sensitivity to copper toxicity. However, whether stored copper is capable of being reutilized under copper-limiting conditions was not determined. Studies by Kornfeld and co-workers (8) have shown that gut granules act to detoxify and store dietary zinc via the CDF-2 zinc exporter. When worms were exposed to high concentrations of dietary zinc, gut granules displayed a bilobed morphology, which was not observed in our studies with CUA-1.1 and high dietary copper. We speculate that gut granules may function as a central site of metal storage to prevent its cytotoxicity. This is specifically relevant because depletion of the metallothionein genes mtl-1 and mtl-2 by RNAi does not result in the expected enhanced susceptibility to copper toxicity (43, 44), raising the possibility that C. elegans may adopt a protective mechanism to withstand an environmental challenge of toxic copper by sequestering copper to an intracellular location via CUA-1.1.

Unexpectedly, we observed that CUA-1.1 expression is up-regulated in response to dietary copper deficiency in the hypodermis when expressed under the control of an endogenous promoter. Given that CUA-1.1 abundance was not altered under the dpy-7 promoter, and that cua-1 transcript levels increased under copper-limiting conditions, hypodermal cua-1 may respond to copper deficiency transcriptionally. MTF-1 is known to transcriptionally induce both ATP7 and metallothionein expression in Drosophila, but no MTF-1 homolog has been defined in C. elegans (45–48). MTF-1-independent metal-responsive transcription factors have been identified, suggesting the existence of other copper-responsive transcription factors that could regulate cua-1 expression in the hypodermis (43, 49). CUA-1 in the hypodermis may deliver copper to the secretory pathway for copper incorporation into copper-dependent enzymes. Another possibility is that the hypodermis acts as a copper storage compartment that can release copper to peripheral tissues by increasing CUA-1.1 expression when worms are in a copper-deficient environment. When CUA-1.1 is highly expressed in the hypodermis under dietary copper restriction, most CUA-1.1 localizes to plasma membranes. Notably, the hypodermis is known to act as a major fat storage site in worms by accumulation of lipid droplets (50, 51).

Organs communicate to ensure that intestinally derived micronutrients are distributed appropriately throughout tissues in the body, balancing cellular requirements against toxicity (52, 53). We injected copper into the pseudocoelom of adult worms expressing CUA-1.1::GFP that had been precultured with BCS. Interestingly, worms injected with copper showed a punctate distribution of CUA-1.1::GFP, implying that CUA-1.1 responds to copper status in the pseudocoelom and/or copper overload in peripheral tissues. In mammals, iron overload results in the liver producing elevated levels of secreted hepcidin that interacts with the ferroportin iron exporter on the basolateral membrane of intestinal epithelial cells as well as on macrophages. Hepcidin causes ferroportin to be internalized, resulting in decreased iron entry into the bloodstream (53, 54). In C. elegans, several neuropeptides are known to mediate intestinal function to regulate metabolism and development (55–57), although a hepcidin homolog has not been found. Cardiac copper deficiency caused by depletion of the Ctrl1 copper importer in the heart induced a significant up-regulation of ATP7A in the intestine of mice, which may lead to increased copper supply into circulation (58). This study suggested that cross-talk may take place between tissue types to coordinate systemic copper homeostasis. In C. elegans, although enter-
cyto-autonomous copper homeostasis regulation may result in a sufficient organismal copper balance in general, another possibility is that intestinal CUA-1.1 is regulated via an inter-organ communication network. A cellular component responsible for copper-dependent CUA-1.1 trafficking in the intestine may be the molecular link by which the enterocytes sense and respond to extraintestinal copper status. Future studies to characterize how intestinal copper acquisition and distribution are regulated at the systemic level may lead to the discovery of new pathways of organismal copper trafficking.

**Experimental Procedures**

**Worm Culture and Strains—** *C. elegans* were cultivated at 20 °C on NGM plates seeded with *E. coli* OP50 or HT115(DE3) as a food source. Strain information (including the Bristol N2 strain, transgenic strains, and deletion strains used in this study) is detailed in [supplemental Table S1](#). Several worm strains were obtained from the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health Office of Research Infrastructure Programs. The presence of the *cua-1 (ok904)* allele was confirmed by sequencing of the *cua-1* locus using the following primers: 5′-CCAGCTAACCACAATTTGGT-TTTCG-3′ and 5′-CGAATCCTTCTCTGTCATTTTC-3′. Genotypes of transgenic animals and mutant worms were confirmed by DNA sequencing or PCR. CuCl₂ was used as the source for copper supplementation in NGM dishes and media in all experiments.

**Worm Analysis Using COPAS Biosort or Microscopy—** Gravid hermaphrodites were bleached to release their eggs, which were allowed to hatch and arrest at the L1 stage in M9 buffer overnight. The resultant age-synchronized L1 larvae were cultured on NGM plates for ~2.5 days. Worms from each condition (~200 worms) were analyzed for time of flight (length), extinction (width), and GFP fluorescence using a COPAS Biosort FP-250 (Union Biometrica). To visualize live worms, animals were paralyzed in M9 buffer containing 10 mM sodium azide (NaN₃) and mounted on agarose pads. GFP, mCherry, copper probe CF4, autofluorescence, and Lysotracker fluorescence in worms were imaged using an SP5 X confocal microscope (Leica).

**ICP-MS—** Metal contents of worms and MEFs were measured using ICP-MS as described previously (59). Values were normalized to wet weight of worms or cells. For sample preparation, synchronized L1 worms were grown on 10-cm NGM plates seeded with OP50 or HT115 RNAi bacteria and supplemented with the indicated amounts of copper or BCS. Worm or cell pellets were collected and washed extensively with M9 buffer or PBS, respectively, transferred to acid-washed tubes, and frozen at ~80 °C. At least three independent biological replicates were analyzed.

**qRT-PCR—** Synchronized larvae were grown to the L4/young adult stage on NGM plates seeded with OP50 bacteria and supplemented with indicated concentrations of copper or BCS. Then the worms were extensively washed with M9 buffer and collected for RNA isolation. Briefly, worms were resuspended in TRIzol (Invitrogen) reagent followed by lysis using a FastPrep-24 (MP Biomedicals) homogenizer in Lysing Matrix Tubes (MP Biomedicals). Total RNA was isolated using TRIzol and treated with DNase I (Ambion), and cDNA was produced using SuperScript VILO Master Mix (Invitrogen). Real-time PCR was performed on an Agilent Mx3005P qPCR system thermocycler (Agilent Genomics) using SYBR Green JumpStart Taq ReadyMix (Sigma). Expression levels of *cua-1* were compared with an internal GAPDH (*gpd-2*) control, and the fold changes were determined using the 2−ΔΔCt method (60). The primers used for qPCR were as follows: *cua-1*, 5′-TGGCACA-ATCACCGAGGAC-3′ and 5′-CAATCGGTATGGCTGCG-3′; and *gpd-2*, 5′-TGCTCACGGGGAGACTAC-3′ and 5′-GCCTGAGTCAACGGCATAG-3′.

**Plasmid Construction and Transgenic Strain Generation—** To generate *C. elegans* expression plasmids, gene-specific gateway *attB* primers were used to amplify DNA sequences such as promoters, ORFs, and 3′-UTRs. Purified DNA fragments were recombined into donor vectors first and then into expression plasmids using Gateway recombination reactions (Invitrogen). For mammalian cell expression plasmids, the GFP-tagged ORF of CUA-1 was digested with NheI and BamHI and ligated into the pEFP-C1 vector (Clontech). Transgenic animals were produced by introducing transcriptional or translational reporters into *unc-119* worms using the PDS-1000 particle delivery system (Bio-Rad) for bombardment transformation (13, 15).

**RNA Interference (RNAi)—** RNAi bacteria strains against *cua-1 (Y76A2A.2)* and *gpd-2 (C34G6.4)* were obtained from the Ahringer feeding library (61), and *cuc-1 (ZK652.11)* was obtained from the ORFeome-based RNAi library (62). Bacteria transformed with the empty L4440 vector were used as a negative RNAi control. Synchronized L1 animals were grown on RNAi plates (NGM dishes containing 2 μM isopyropyl-β-d-galactopyranoside, 12 μg/ml tetracycline, and 50 μg/ml carbenicillin) that were seeded with HT115(DE3) bacteria expressing dsRNA for each gene.

**Cell Culture and Immunoblotting—** Atp7a+/− and Atp7a−/− MEFs were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Lonza) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals) and 100 units/ml penicillin/streptomycin (Lonza). The plasmid expressing CUA-1.1::GFP was transfected to Atp7a−/− MEFs using PolyJet (SignaGen Laboratories). All cells were cultured under 5% CO₂ at 37 °C. Cells at ~70% confluence were collected and washed three times with ice-cold PBS, pH 7.4. Cell pellets were suspended in about five times their volume in ice-cold cell lysis buffer (PBS, pH 7.4. 1% Triton X-100, 0.1% SDS, 1 mM EDTA) containing Halt protease inhibitor mixture (Thermo Scientific), briefly vortexed, and incubated for 1 h. Early stage adult worms grown from synchronized larvae were collected and washed with M9 buffer. Worms were resuspended in the same lysis buffer followed by disruption using a FastPrep-24 (MP Biomedicals) homogenizer in the presence of glass beads. The same lysis buffer was used for disruption of MEFs on ice for 30 min. Cell suspensions and worm lysates were centrifuged at 16,000 × g at 4 °C for 15 min. After centrifugation, the clarified lysates were used for immunoblotting, and protein concentrations were measured using the BCA protein assay kit (Thermo Scientific). Samples (100 μg/lane) were fractionated on a 4–20% gradient gel (Bio-Rad). The anti-ATP7A antibody (a gift
from Dr. Stephen G. Kaler, National Institutes of Health, Bethesda), anti-GFP (Covance), and anti-tubulin antibody (Sigma) were used at a 1:1000 dilution. The anti-CCS antibody (Santa Cruz Biotechnology) and anti-GAPDH antibody (Sigma) were used at 1:4000 dilution and 1:10,000 dilution, respectively. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Rockland Immunochemicals) was used as the secondary antibody for immunoblotting (1:5000 dilution). Immunoblots were detected using SuperSignal West Pico Chemiluminescent Substrate reagents (Thermo Fisher Scientific) using a chemidocumentation imaging system (Bio-Rad).

Staining with the Copper Probe CF4 and LysoTracker—The Copper Fluor-4 (CF4) sensor combines a piperidine-substituted rhodol with a trifluoromethyl-substituted bottom ring bearing a thioether receptor, along with a matched control Copper Fluor-4 (Control CF4) dye that lacks the copper-responsive receptor to help distinguish between copper-dependent and dye-dependent responses (30, 63). Replacement of the thioether-rich receptor arms for copper recognition in CF4 by isostructural octyl groups in control CF4 provides a mimic of the size, shape, and hydrophobicity of thioethers but do not bind copper, offering a matched pair of probes to disentangle copper-dependent fluorescence responses from potential dye-dependent ones. We used the Rhodol-based CF4 probe and control CF4 probe (both final concentrations of 25 μM) for staining copper(I) in intact worms and 2 μM LysoTracker Red DND-99 (Invitrogen) for detecting gut granules. Both chemicals were prepared in M9 buffer and dispensed onto NGM plates (8). L3 stage worms were cultured on these dishes for 12–16 h in the dark. Postincubation, the stained worms were transferred to seeded NGM plates containing copper concentrations equivalent to treatment conditions. After 2 h of incubation, worms were collected and washed three times with M9 buffer and imaged via confocal microscopy.

Copper Microinjection Experiments—To deliver copper directly to the basolateral side of the intestine and peripheral tissues, CuCl2 solution was microinjected into the pseudocoelom in the posterior region of young adult worms that had been grown in 50 μM BCS-treated NGM dishes. Histidine or CuCl2 with histidine (1:2 molar ratio) diluted in M9 buffer was injected to worms using an Eppendorf Femtojet microinjector attached to a Leica inverted microscope under specified settings (injection pressure = 30 p.s.i., compensation pressure = 4.5 p.s.i., injection time = 4 s). Worms were recovered on seeded plates for 6 h and then mounted on 2% agarose pads for imaging.

Bioinformatics and Statistics—Clustal Omega and ClustalW2 were used to generate a multiple sequence alignment and phylogenetic tree of a subset of cua-1 homologs (64). Transmembrane helices and copper-binding motifs of CUA-1 homologs were predicted using Trans-Membrane prediction using Hidden Markov Models and the Conserved Domain Database (22). Statistical significance was determined using a one-way or two-way ANOVA followed by Tukey’s post hoc test in GraphPad Prism, Version 6 (GraphPad Software). All data are presented as mean ± S.E., and p values less than 0.05 were considered to be statistically significant.