CHCA-1 is a copper-regulated Ctr1 homolog required for normal development, copper accumulation, and copper-sensing behavior in Caenorhabditis elegans

Sai Yuan\(^1\), Anuj Kumar Sharma\(^1\),*, Alexandria Richart\(^1\), Jaekwon Lee\(^2\), and Byung-Eun Kim\(^{1,3,\#}\)

\(^1\)Department of Animal and Avian Sciences, and \(^3\)Biological Sciences Graduate Program, University of Maryland, College Park, Maryland 20742, USA
\(^2\)Redox Biology Center, Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68588, USA
\(^1\)Present address: Department of Physics, Princeton University, Princeton, New Jersey 08544, USA

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#Corresponding author contact information:
Department of Animal and Avian Sciences, and Biological Sciences Graduate Program, University of Maryland, College Park, MD 20742, USA. Tel.: 301-405-3977; Fax: 301-405-7980; E-mail: bekim@umd.edu

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**ABSTRACT**

Copper (Cu) plays key roles in catalytic and regulatory biochemical reactions essential for normal growth, development, and health. Dietary Cu deficiencies or mutations in Cu homeostasis genes can lead to abnormal musculoskeletal development, cognitive disorders, and poor growth. In yeast and mammals, Cu is acquired through the activities of the Ctr1 family of high-affinity Cu transporters. However, the mechanisms of systemic responses to dietary or tissue-specific Cu deficiency remain unclear. Here, taking advantage of the animal model *Caenorhabditis elegans* for studying whole-body Cu homeostasis, we investigated the role of a *C. elegans* Ctr1 homolog, CHCA-1, in Cu acquisition and in worm growth, development, and behavior. Using sequence homology searches, we identified ten potential orthologs to mammalian Ctr1. Among these genes, we found that chca-1, which is transcriptionally upregulated in the intestine and hypodermis of *C. elegans* during Cu deficiency, is required for normal growth, reproduction, and maintenance of systemic Cu balance under Cu deprivation. The intestinal Cu transporter CUA-1 normally traffics to endosomes to sequester excess Cu, and we found here that loss of chca-1 caused CUA-1 to mislocalize to the basolateral membrane under Cu overload conditions. Moreover, animals lacking chca-1 exhibited significantly reduced Cu avoidance behavior in response to toxic Cu conditions compared with wild-type worms. These results establish that CHCA-1–mediated Cu acquisition in *C. elegans* is crucial for normal growth, development, and Cu-sensing behavior.

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**INTRODUCTION**

Copper (Cu) is a crucial micronutrient involved in a wide range of catalytic reactions. It serves as a cofactor in cuproenzymes necessary for mitochondrial ATP synthesis, redox reactions, iron homeostasis and neuropeptide biogenesis (1,2). Given its redox properties, Cu accumulation can also be toxic to organisms due to generation of reactive oxygen species (ROS) (1,3). Organisms have adopted conserved Cu homeostatic mechanisms to regulate Cu uptake, detoxification,
and distribution at both the cellular and systemic levels.

The intestinal epithelium is the main site for dietary Cu absorption. Following reduction from Cu(II) to Cu(I) by a metalloreductase, Cu enters cells via the high-affinity Cu transporter 1 (Ctr1). Several metallochaperones such as CCS, Cox17, and Atox1 then deliver Cu to Cu/Zn superoxide dismutase (SOD1), cytochrome c oxidase (CCO), and ATP7A/ATP7B, respectively. ATP7A and ATP7B are P-type ATPases that transport Cu to Cu-dependent enzymes in the secretory pathway and export Cu out of the cell. Upon export from the basolateral membrane of intestinal cells by ATP7A, Cu is delivered to the liver, the main Cu storage tissue, and other peripheral tissues as needed (1,4).

The first Cu transporter gene (Ctr) was found in yeast, leading to the discovery of several Ctr family members in humans, mice, fruit flies, and other species by homology searches and functional complementary studies (5). While Ctr proteins mediate Cu uptake, they are expressed in different tissues during distinct developmental stages, or as determined by Cu availability. All characterized Ctr proteins contain three putative transmembrane domains (TMD) with a Met-X3-Met domain in the second TMD. Electron crystallography revealed that human Ctr1 (hCtr1) formed trimers at the plasma membrane (6-8). The extracellular N-terminal Met-rich domain, the Met-X3-Met motif and the cytosolic C-terminal Cys-His-Cys motif are potential Cu binding sites necessary for effective Cu transport (9-11). Ctr1 localizes to the plasma membrane and intracellular vesicles, and its protein stability is regulated by Cu at the post-translational level (12,13). Human Ctr2 facilitates truncation of the Ctr1 ectodomain via a cathepsin protease (14,15). Cu-dependent regulation of Cu importers also occurs at the transcriptional level, as S. cerevisiae Ctr1 and Ctr3 and D. melanogaster Ctr1B transcripts are induced under Cu deprivation via the transcription factors Mac1 and MTF-1, respectively (16,17).

Cu acquisition via Ctr1 is vital to maintaining cellular and systemic Cu homeostasis. Depleting Ctr1 in mice and zebrafish results in lethality at the embryonic stage (18,19). Conditional knockout of this gene in the mouse intestine leads to severe Cu deficiency in peripheral tissues, cardiac hypertrophy, severe viability defects, and accumulation of non-bioavailable Cu in the intestine (20). Cardiac-specific Ctr1 knockout mice exhibit elevated intestinal ATP7A expression and serum Cu, and decreased hepatic Cu storage (21). However, the mechanisms underlying the systemic response to dietary or tissue-specific Cu deficiency remain unelucidated.

We have chosen Caenorhabditis elegans (C. elegans), a tractable, multi-tissue organism to explore Cu homeostasis and investigate these unknowns at the systemic level. C. elegans has been proven to be amenable to nutrient absorption and distribution studies with zinc, iron and heme (22-25). Several players in Cu trafficking have been delineated in C. elegans, including metallothionein, CUC-1, and CUA-1 (26,27). CUA-1 has been functionally characterized as a key intestinal Cu exporter responsible to maintain systemic Cu homeostasis (28). However, mechanisms by which C. elegans acquires Cu have yet to be characterized. In this study, ten Ctr ortholog genes are identified in C. elegans; of these, CHCA-1 is functionally characterized. We show that chca-1 is transcriptionally regulated by Cu in both the intestine and hypodermis, and is essential for normal Cu level, growth, and reproduction. Furthermore, we found that altered Cu balance caused by the loss of CHCA-1 resulted in defects in Cu-responsive behavior. Identification of this Ctr-like gene in C. elegans, which appears to be essential for normal Cu balance, illustrates the importance of Cu delivery via CHCA-1 for normal metazoan development and behavioral phenotypes.

RESULTS

Cu-responsive transcriptional regulation of Ctr1-like genes in C. elegans

To identify potential genes for Cu acquisition in C. elegans, a Basic Local Alignment Search Tool (BLAST) search was performed using the human Ctr1 protein sequence as a probe. Unlike many characterized organisms that contain two or three Ctr homologs (10,17,18,29), fifteen protein orthologs encoded by ten gene loci are predicted to be potential C. elegans Ctr genes. The BLAST result scores for the fifteen candidate
proteins demonstrate that, in general, worm Ctr candidates share 30-40% amino acid sequence identity with hCtr1 (Supplemental Table 1). Worm Ctr1 candidate proteins were further analyzed based on the conserved features of Ctr proteins, such as number of transmembrane domains and Cu-transporting motifs at the amino (N) and carboxyl (C) terminus, and the second predicted TMD. In general, C. elegans Ctr candidates have a shorter N terminus as compared to human and mouse Ctr1 (Supplemental Fig. S1). Candidate proteins expressed at F31E8.4, Y58A7A.1, F58G6.3, F58G6.7 and F58G6.9 gene loci are enriched with both methionine and histidine at the N-terminus. All fifteen candidates contain a Met-X3-Met domain within or close to one of the predicted TMD.

Ctr1 abundance is regulated by Cu availability. In yeast and mammalian cells, high Cu induces Ctr1 protein degradation, while Cu deprivation stabilizes the protein (13,30,31). In yeast, such regulation occurs at the transcriptional level. Ctr1 expression is induced under Cu-depleted conditions by the transcriptional factor Mac1 (32). To test whether worm Ctr mRNA abundance can be regulated by altered Cu status, qRT-PCR was performed for the ten candidate genes under optimal, high, and low Cu conditions in liquid axenic growth media. To determine the desired range of Cu concentration for worm growth, the C. elegans Habituation and Reproduction (mCeHR) axenic liquid culture (22) was further modified to contain minimal levels of Cu (“low Cu” mCeHR). Around 100 synchronized L1 stage worms were grown in axenic culture supplemented with various concentrations of copper chloride (CuCl2) or bathocuproinedisulfonic acid (BCS, a Cu(I) chelator) for 9 days; total worm number was counted for each condition on day 9. Supplementation with 10 µM Cu was most favorable for worm growth, while worms exhibited the defects in development or embryogenesis under both Cu-replete and Cu-deficient conditions, resulting in significantly decreased total populations (Fig. 1A). Either 10 µM Cu, 300 µM Cu, or 100 µM BCS was applied to generate normal, strong Cu overload and strong Cu deficiency conditions, respectively, to measure changes in gene expression. Synchronized L1 worms were cultured to the L4 stage, and levels of mRNA of each candidate gene under high and low Cu conditions were calculated by normalizing to expression levels under 10 µM Cu condition. Under Cu deprived conditions, expression of only F58G6.3, F58G6.7 and F58G6.9 was significantly elevated (Fig. 1B). These three genes, together with other candidate genes such as F01G12.1, Y58A7A.1 and K12C11.6, had expression suppressed by 300 µM Cu (Fig. 1C).

**Importance of Ctr1 candidate genes for growth, reproduction, and Cu accumulation in worms**

Our recent studies have shown that limited availability of dietary Cu causes developmental defects in worms, and that depletion of the Cu exporter CUA-1 in the intestine inhibits Cu distribution to peripheral tissues, resulting in reduced growth and brood size (28). To test whether our potential Ctr genes were required for worm growth in a Cu-dependent manner, L1 stage worms were grown on NGM media plates seeded with OP50 bacteria. Plates were supplemented with either 150 µM CuCl2, 50 µM BCS, or nothing. Milder Cu conditions were used (as compared to the experiments in Figure 1) to more closely mimic physiologically-relevant conditions of Cu abundance and deprivation and to enable observation of intermediate growth phenotypes. Following 3 days of growth, F58G6.9- and F27C1.2-depleted P0 animals cultured with 50 µM BCS were found to be stage-delayed, as indicated by time of flight (TOF, worm length quantification), in comparison to vector-treated animals (Fig. 2A-D). There were no apparent growth defects observed in worms treated with RNAi against other Ctr-like genes under different Cu conditions (Fig. 2A-C) suggesting an important role of the proteins encoded by F27C1.2 and F58G6.9 in response to Cu deficiency in worms.

To determine the efficiency of knockdown, qRT-PCR was performed to test candidate gene expression levels after RNAi treatment. All candidate genes’ mRNA levels were significantly decreased after worms were fed RNAi bacteria. While F01G12.1, F31E8.4, and K12C11.3 transcripts exhibited a mild reduction (~80-60% of wild type expression), silencing of all other Ctr candidates was highly effective (less than 30% of wild type expression) (Supplemental Fig. S2).
To further test whether Ctr candidates are important for normal Cu levels in worms, each gene was individually silenced, and whole-body metal levels were examined by ICP-MS. When providing synchronized L1 (P0) with sufficient Cu (10 µM) for two generations, F1 worms lacking a number of the candidate genes displayed decreased Cu accumulation (73 - 80% of that of vector) (Fig. 2E). To identify the gene most strongly associated with Cu accumulation in C. elegans, a Cu-pulse assay was conducted on each RNAi-treated candidate by pre-culturing worms in Cu-limited conditions followed by a 12-hour (h), 50 µM CuCl₂ pulse (Fig. 2F). Following BCS treatment, all worms had extremely low systemic Cu concentrations, in a range of 0.03-0.06 µg/g (data not shown). When calculating the level of Cu acquired during the Cu pulse, F58G6.3-, F58G6.7-, and F58G6.9-depleted worms displayed significant defects in restoration of Cu levels. Of these, the most defective were the F58G6.9 RNAi animals, which only accumulated 40% of the Cu measured in vector-treated worms (Fig. 2G). Depleting a number of other candidate genes decreased body Cu accumulation under prolonged Cu treatment (Fig. 2E). However, these conditions did not significantly impact Cu restoration from a Cu-deficient state within the time frame assayed, suggesting that these genes, when functioning independently, are not required for effective Cu uptake.

Our results narrowed down the Ctr-like gene list, leading to F58G6.9 as the strongest candidate Cu importer in worms. To further explore the role of F58G6.9 in worm regeneration, we measured brood size in F58G6.9-silenced worms under Cu-limited conditions. P₀ worms after one generation of F58G6.9 RNAi under 50 µM BCS supplementation showed substantially smaller brood sizes compared with control animals. Similarly, worms treated with F58G6.9 RNAi exhibited severe defects in generating embryos when treated with 100 µM BCS (Fig. 2H). Given that the F58G6.9 gene showed significantly elevated transcript levels under low Cu conditions, and that it is required for normal growth, reproduction, and Cu accumulation in a low-Cu environment, we focused on the F58G6.9 gene as a potential Ctr candidate and named it Ctr1 Homolog required for Copper Accumulation-1 (CHCA-1).

CHCA-1 is required for normal Cu level and development

We tested functional complementation by worm CHCA-1 in yeast cells defective in the high affinity Cu transporters, Ctr1 and Ctr3, on non-fermentable carbon sources (33). Expression of CHCA-1 protein with a carboxyl-terminal FLAG epitope tag (CHCA-1-2xFLAG) in the heterologous system failed to restore yeast growth (Supplemental Fig. S3). To test functional consequence of loss of the endogenous CHCA-1 in vivo system, we exploited an established Cu-responsive CUA-1-trafficking reporter animal model. Our recent studies have demonstrated that the intestinal CUA-1.1 Cu exporter maintains systemic Cu homeostasis by altering its subcellular localization (28). It localizes to the basolateral membrane during Cu deficiency, and upon Cu overabundance, re-distributes to lysosome-like organelles, called gut-granules, to protect peripheral tissues from Cu toxicity. As ICP-MS assays only measure whole body Cu levels, we silenced chca-1 in transgenic worms expressing CUA-1.1::GFP from the constitutive, intestine-specific vha-6 promoter (34) (BK015 strain) to evaluate whether the depletion of chca-1 yields any change in intestinal Cu status. Importantly, when given sufficient dietary Cu, worms lacking chca-1 showed CUA-1.1::GFP localized to the intestinal basolateral membrane with significantly decreased distribution to the gut granules when compared with vector-treated animals (Fig. 3A). Individual silencing of all other Ctr candidate genes did not change CUA-1.1 localization in the intestine (Supplemental Fig. S4), suggesting the CHCA-1 is a major player in intestinal Cu homeostasis. Our studies have also shown that endogenous CUA-1.1 expression is induced in the hypodermis under dietary Cu restriction (28). Upon depleting chca-1 in transgenic worms expressing CUA-1.1::GFP driven by its own promoter (BK017 strain), CUA-1.1 expression was significantly enhanced in the hypodermis under basal culture conditions at a comparable level to that of the transgenic strain grown in 25 µM BCS (Fig. 3B). Together, Cu dependent CUA-1.1 distribution and expression in worms lacking chca-1 was similar to dietary Cu deficient worms, suggesting a role for CHCA-1 in Cu acquisition in C. elegans.
To further characterize CHCA-1 and to verify the RNAi-based findings, a chca-1 mutant animal (chca-1 (tm6506) IV) was obtained from the National Bioresource Project (35). The tm6506 allele found in this mutant contains a 464-bp deletion which begins in the 5’ UTR and spans the second intron of the F58G6.9 gene; this deletion is predicted to affect the entire N terminus and first TMD (Fig. 4A, Supplemental Fig. S5A). Worms homozygous for the tm6506 allele exhibited defects in growth under basal and Cu-replete conditions (10 µM CuCl₂) and showed a severe growth phenotype under BCS treatment within one generation (P₀). However, the growth defects were fully rescued by Cu supplementation at 25 µM (Fig. 4B and C). To test the metal specificity of CHCA-1-dependent growth, 50 µM BCS and several metal sources (CuCl₂, ZnCl₂, FeCl₂, and MnCl₂) were mixed together in the growth media, and the growth of the tm6506 mutant strain and its wild-type outcrossing brood mate (WT) were compared after 3 days of culture. Only Cu supplementation rescued the growth defect observed in chca-1 mutant worms, suggesting a function for this gene in Cu-specific regulation (Fig. 4D). Compared to WT animals, chca-1 (tm6506) IV worms contained significantly lower Cu levels under Cu deficiency (ranging from 15 µM BCS up to 10 µM supplemented Cu conditions), while Fe levels were not affected in mutant worms (Supplemental Fig. S5B); this defect could only be rescued by supplementing the media with 25 µM CuCl₂ (Fig. 4E). Consistent with our RNAi-based results (Fig. 2G), the mutant worms also demonstrated defects in dietary Cu acquisition upon Cu-pulse (Fig. 4F).

Intestinal CHCA-1 is critical for Cu-dependent growth and Cu accumulation

To further understand the role of CHCA-1 in Cu homeostasis, transgenic worms expressing GFP driven by the endogenous chca-1 promoter were used to examine tissue-specific expression of this gene. While the GFP reporter in this strain was barely detectable under Cu-replete conditions, it was induced in the intestine and hypodermis upon severe dietary Cu deprivation (Fig. 5A). Tissue-specific CHCA-1 knockdowns were performed to determine the contribution of CHCA-1 in these two tissues to its systemic role in Cu accumulation. This was performed using rde-1 mutant animals which are RNAi-resistant. In a whole-animal rde-1 knockout background, knockdown is only effective in a tissue expressing rde-1 cDNA (36). In this study, the intestine- and hypodermis-specific RNAi-sensitive strains (VP303 and NR222, respectively) were used to deplete CHCA-1, and their growth was compared to whole-body CHCA-1 silencing in the N2 strain. Whole animal RNAi-resistant (WM27) and muscle-specific RNAi-sensitive (WM118) strains served as negative controls. After normalizing the growth of CHCA-1 RNAi worms to vector in both P₀ and F₁ generations following RNAi, worms depleted for intestinal CHCA-1 in 100 µM BCS exhibited comparable growth and reproduction defects as to those exposed to whole-body CHCA-1 RNAi depletion. On the other hand, worms lacking CHCA-1 only in the hypodermis did not reveal growth defects until the F₂ generation under the same Cu-deficient conditions (Fig. 5B and C). The loss of intestinal CHCA-1 was also able to phenocopy the decreased Cu levels observed in a whole animal CHCA-1 knockdown (Fig. 5D and E). These results suggest a dominant function of intestinal CHCA-1 for Cu-dependent growth under a Cu-limited condition. Meanwhile, CHCA-1 depletion in both the intestine and hypodermis resulted in similar effects on Cu accumulation when worms were exposed to 25 µM CuCl₂, a Cu-abundance condition (Supplemental Fig. S6A).

To determine which tissue, when lacking CHCA-1, most significantly contributes to aberrant CuA-1.1 localization in the intestine (Fig. 3A), BK014 transgenic worms (Pvha-6♂:/CuA-1.1::GFP) were crossed each with the WM27, VP303, and NR222 strains. Compared to a whole-body knockdown effect (Supplemental Fig. S6B, d and e), loss of CHCA-1 solely in the intestine or hypodermis was not sufficient to increase CuA-1.1::GFP basolateral membrane distribution, nor to reduce its gut granule expression in the presence of 10 µM Cu (Supplemental Fig. S6B, n, o, s, t). These results suggest that both intestinal and hypodermal CHCA-1 play important roles in regulating Cu level in C. elegans, and raise a possibility that CHCA-1 in the hypodermis could function to compensate Cu deficiency in the intestine and/or at the organisinal level.

CHCA-1 localizes to intracellular vesicles in the intestine
Given the importance of enterocytes in regulating dietary Cu uptake, we generated transgenic worms expressing a CHCA-1::GFP fusion protein driven by the intestine-specific vha-6 promoter. This fusion protein localizes to vesicles throughout the intestine under basal, Cu-deficient, and Cu-replete conditions (Fig. 6A and B). In addition, CHCA-1::GFP expression levels were not altered by Cu availability (Fig. 6C). While CUA-1.1 co-localized with a fluorescent Cu probe (CF4) in gut granules (28), CHCA-1::GFP did not co-localize with the CF4 or gut granules, the latter of which are auto-fluorescent (Fig. 6B, Supplemental Fig. S7A). To test whether GFP-tagging could lead to CHCA-1 protein mislocalization, a 2xFLAG tag, followed by a stop codon and an SL2 splicing leader sequence, were inserted between CHCA-1 and GFP (P_{vha-6}::CHCA-1-2xflag::SL2::GFP). Both GFP and FLAG tags in this study were placed at the C-terminus of CHCA-1 to avoid N-terminal truncation observed in mammalian Ctr1 (37). The immunofluorescence analysis showed similar vesicle localization of FLAG-tagged CHCA-1 protein (Supplemental Fig. S7B). The function of intestinal CHCA-1 was then tested by crossing the transgenic worms expressing WT CHCA-1 (P_{vha-6}::CHCA-1::SL2::GFP) driven by an intestinespecific promoter onto a tm6506 mutant background. Transgenic worms expressing WT CHCA-1 in the intestine in the mutant background significantly rescued tm6506 growth during Cu deficiency (Fig. 6D). These results suggest a role for vesicular CHCA-1 in mediating Cu acquisition in the worm intestine, and that CHCA-1 protein abundance, in contrast with mammalian Ctr1, is not regulated by Cu status.

**CHCA-1 is required for behavioral avoidance of potentially toxic levels of Cu**

Animals navigate complex natural environments containing both dangerous and valuable items, such as predators and food. *C. elegans* must approach and obtain nutrients while avoiding various threats, which include toxic levels of Cu. Worms detect threats via primary sensory neurons that then propagate such information through an interneuron network to ultimately reach pre-motor command interneurons that direct controlled locomotion. Thus far, several ciliated sensory neurons (ASH, ASE, ADL, ASI, and ADF) are known to correlate with Cu sensing and/or avoidance behavior (38,39). We studied whether altered Cu status in worm affects its Cu avoidance behavior. To quantify levels of avoidance, assays were performed on rectangular plates containing a gradient of CuCl₂, from no Cu supplementation on one end, to toxic Cu levels on the other end. After production, assay plates were kept in a cold chamber for a defined period of time to allow for Cu diffusion and concentration gradient formation, and marked in sections (Fig. 7A). With Cu supplementation, plates generated with the method described above contain a sharp Cu gradient ranging from section 3 to section 5, as measured by ICP-MS performed on agar samples from the gradient plates (Supplemental Fig. S8A).

Stage-synchronized L4 young adult worms were seeded in the middle of the bacteria-free Cu gradient plates, and worm distribution after 2.5 h was visualized. During quantification, each plate was evenly divided into five sections (with section 5 representing the highest Cu level area), and a formula was used to calculate the avoidance index (AI) as described in the Methods.

We observed that wild type N2 worms on the Cu gradient plates (10 mM Cu) had a strong tendency to avoid high Cu-containing areas (Fig. 7B) as compared to normal distribution of worms on non-Cu plates. Changing the maximum concentrations found in the Cu gradient from 1 mM to 10 mM led to N2 worms exhibiting enhanced avoidance (Fig. 7C) For further assays, 8 mM or 10 mM Cu gradients were chosen in order to achieve clear resolution under tested conditions. On 8 mM Cu gradient plates, *che-2* mutant animals (CB1033), of which sensory cilia formation are defective (40), lacked Cu avoidance behavior compared with N2 worms, supporting the importance of the chemosensory axis in Cu sensing. Meanwhile, *chca-1*(RNAi) worms demonstrated decreased levels of avoidance (Fig. 7D), suggesting that CHCA-1 is required for a role in sensing and avoiding toxic Cu concentrations. To test whether altered systemic Cu levels in *chca-1*(RNAi) worms result in changes to avoidance behaviors, both Cu-deprived and Cu-overloaded worms were generated by pre-culturing N2 worms in 100 µM BCS or 100 µM Cu conditions, and along with N2 worms cultured in basal media, placed on Cu gradient and non-Cu plates. The Cu-deficient worms exhibited less avoidance
compared with non-pre-cultured worms, which recapitulated the behavior of chca-1(RNAi) worms. However, interestingly, Cu-overloaded worms also revealed less avoidance of toxic Cu (Fig. 7E). These results suggest that C. elegans Cu sensing behavior is correlated with abnormal Cu levels, although the precise mechanisms that determine this behavior remain elusive.

Cu is known to be crucial for the neuropeptide maturation process through the Cu-dependent peptidyl-glycine alpha-amidating monooxygenase (PAM) (41). To test whether neuropeptides are important for Cu-sensing behavior in worms, using RNAi hypersensitive strains that allow mRNA silencing in neurons (42), the proprotein convertase egl-3 and the carboxypeptidase egl-21 were silenced individually (43,44) and a significantly decreased avoidance behavior was observed (Fig. 7F). Furthermore, depletion of all predicted PAM orthologs in C. elegans (pgal-1, pghm-1 and pamn-1) caused reduced Cu avoidance (Fig. 7G). Gene silencing efficiency was tested by qRT-PCR performed in parallel to the RNAi experiments. After RNAi treatment, mRNA levels of che-2, egl-3, egl-21 and PAMs were decreased (Supplemental Fig. S8B). Taken together, our results suggest that Cu-sensing and/or corresponding behavior of C. elegans is associated with the Cu-dependent neuropeptides maturation in neurons that requires CHCA-1 activity and balanced body Cu levels.

**DISCUSSION**

Cu acquisition via Cu transporter (Ctr) family proteins is critical for survival during fluctuations in environmental Cu levels. Our studies reveal that, similar to yeast, fish, and mammals, worms lacking CHCA-1 have lower systemic Cu levels and exhibit profound growth and reproductive defects under low Cu availability. Our results with worm Ctr candidates also suggest that the pathway for Cu trafficking to the secretory pathway via the Ctr1-Atox1-ATP7A/B axis in mammals is conserved in worms. C. elegans can thus be exploited as a facile whole live animal system to isolate novel components regulating Cu homeostasis, as well as providing additional insight into known components.

For example, the fact that C. elegans CHCA-1 is predicted to lack a third TMD, as well as lacking a C-terminal cysteine or histidine residue, calls into question the minimal structural requirements for a high affinity Cu transporter. Additionally, while yeast Cu metabolism components are regulated transcriptionally, and mammalian Cu metabolism components are generally regulated at the post-translational level, worms exhibit characteristics of both. Ctr homolog genes, such as F58G6.3 and F58G6.7, are transcriptionally regulated by Cu. The worm ATP7A/B homolog, CUA-1, is regulated both transcriptionally and post-translationally (28). C. elegans may thus shed light on the evolutionary history of Cu metabolism regulation. Importantly, we show differing contributions of CHCA-1 to systemic Cu metabolism depending on whether it is expressed in the intestine or in the hypodermis. Thus, C. elegans also provides insight into coordination of Cu homeostasis in multi-tissue organisms.

Uncovering ten Ctr candidate genes in C. elegans was unexpected, as other model organisms contain fewer Ctr homologs. Individually silencing these candidate genes did not severely affect worm growth or Cu accumulation under basal or Cu replete conditions (Fig. 2A and 2E). While non-Ctr ortholog metal importers could contribute to dietary Cu uptake in C. elegans, it is also likely that several Ctr genes function redundantly, or that Ctr proteins form higher-order heteromultimers which serve as fully active Cu transporters (45). Considering that CHCA-1 is dominantly enriched only in the intestine and hypodermis, it is possible that other Ctr proteins in C. elegans are expressed in different tissues, and/or are required under different Cu conditions or during specific developmental stages.

While studies demonstrate that mammalian Ctr1 constitutively cycles from endosomal compartments to the plasma membrane in many cultured cell lines (30), and endogenous mouse Ctr1 is localized to both the apical membrane and intracellular compartments of intestinal epithelial cells in mouse models (20), our data demonstrate that CHCA-1 localizes mainly to intracellular vesicles with minimal localization to the apical membrane. Reports in C. elegans have identified lysosome-like compartments known as gut granules in the
intestine that could serve as a buffering subcellular organelle by transporting metals in the compartment under Cu or Zn overload conditions (28,46). It is plausible that Cu stored in gut granules could be recycled by CHCA-1 upon a Cu-starvation stock. However, CHCA-1::GFP was not found to co-localize with gut granules. These results suggest that CHCA-1 may function to transport Cu across from the lumen of an as-yet-unidentified intracellular compartment, while other Cu importers drive Cu transport across the plasma membrane. Further studies will be necessary to ascertain whether CHCA-1 is important for the mobilization of Cu from endosomes.

In addition to the intestine, a significant induction of *chca-1* expression in the hypodermis is also observed under Cu deprivation. Similarly, *cua-1* abundance also increases in the hypodermis during Cu deficiency, for which regulation may also occur at the transcriptional level (28). The hypodermis is known to play a role in Fe (47) and heme homeostasis in worms (48); as such, these findings strengthen its potential role in Cu homeostasis. It is plausible that hypodermal CHCA-1 acquires Cu to incorporate into the secretory pathway through CUA-1 activity. Alternatively, CHCA-1 in the endosomes together with CUA-1 in the plasma membrane may function to recycle stored Cu in the hypodermis to peripheral tissues in response to systemic Cu deficiency, as the worm hypodermis is known to store other nutrients (49).

Dwelling in the soil, *C. elegans* encounters a complicated and mercurial environment requiring flexible responses to pathogen exposure, gas composition, and temperature transitions, as well as undesirable nutrient concentrations. Behavioral studies in worms under varying environmental stimuli have led to important discoveries, establishing the sensing and signaling axis towards CO$_2$/O$_2$ and temperature (50-54). High concentrations of Cu are used as a chemical repellent and several Cu chemosensory neurons have been identified (38,39). In this study, CHCA-1 was shown to be required for sensing and avoiding Cu, possibly via downstream effects of systemic Cu scarcity, as worms lacking CHCA-1 or with limited dietary Cu sources both revealed significantly decreased avoidance to toxic levels of Cu. Further experiments suggest that a neuropeptide maturation process, which is Cu-dependent in many organisms, is involved in worm Cu sensing and avoiding (Fig. 7F and 7G). Cu deficient worms may have an increased capacity to uptake Cu and to be resistant to toxic Cu levels, resulting in decreased avoidance in the time frame of our assays. Alternatively, this altered behavior may be due to defects in biogenesis of neuropeptides required for Cu sensing, or altered signal transmission to downstream interneurons or motor neurons.

An unanticipated observation from our studies was that dietary Cu-overload worms also showed reduced avoidance of toxic Cu. It is reported that acute Cu exposure induces ASH neuron activity, but repeated Cu stimulation leads to the reduction in the avoidance response and in ASH neuronal activity. This may result in changes to receptor activity and any downstream signaling pathways (55). Another explanation could be the failure of sensing caused by Cu toxicity during pre-culture in high Cu conditions (56). Is there a Cu-specific receptor on the neuronal cell surface that elicits subsequent behavioral responses? Could there be a Cu-sensing olfactory receptor? While the vertebrate olfactory system has a single receptor gene expressed in each sensory neuron (57), worms have limited numbers of chemosensory neurons, with multiple receptors expressed in one sensory neuron. There are ~1300 receptor genes found based on phylogenetic analysis. While electrical and hormonal signals are commonly used for neuronal signaling, neuropeptides, many of which are thought to be Cu-dependent, function as crucial signaling regulators as well (58,59). Identification of the essential component in the signaling events will provide leads for future studies seeking to understand Cu-responsive decision-making and behavior in *C. elegans*.

**EXPERIMENTAL PROCEDURES**

**Worms Strains and Culture**

*C. elegans* were cultured at 20°C on nematode growth medium (NGM) plates seeded with *E. coli* OP50 for general maintenance or with *E. coli* HT115 dsRNA-expressing bacteria for
RNAi experiments (60). Bristol N2 was used as the wild-type C. elegans strain. Mutant and transgenic strains were outcrossed with N2 to obtain wild-type backgrounds, and a wild-type brood mate animal was used following crossing in mutant and transgenic animal growth/avoidance assays. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The CHCA-1 (tm6506) IV strain was obtained from the National Bioresource Project (35) and outcrossed with N2 six times prior to use to establish heritability; CB1033 (che-2 (e1033) X) was obtained from CGC and outcrossed six times before use for the same purpose. A list of transgenic worms used in this study can be found in Supplemental Table 2. Transgenic animals in a CHCA-1 (tm6506) mutant background, as well as multiple transgene-presenting strains, were generated with standard mating methods; genotypes were confirmed by PCR and/or DNA sequencing. The CHCA-1 (tm6506) genotyping primers were: 5′-GTATCTAGTCCGATAAGAAG-3′ and 5′-TTGAAGCAAAAAACAAAGTGCC-3′.

**Yeast Strain and Spotting Assay**

The MPY17 *Saccharomyces cerevisiae* strains used in this study contained a scCtr1 and scCtr3 double deletion (10). Genes were tagged at the carboxyl terminals with a 2x FLAG sequence and inserted into a pYES3 vector (61). Plasmids containing either FLAG gene insertions or FLAG tag only were transformed into *Ctr1ΔCtr3Δ* yeast. Yeast strains were maintained in a synthetic complete medium (SC) lacking uracil for plasmid selection. Spotting assays were conducted on YPD (1.5% agar, 2% bacto-peptone, 2% glucose, 1% yeast extract) and YPEG (1.5% agar, 2% bacto-peptone, 3% glycerol, 2% ethanol, 1% yeast extract) media. Cells with an OD600 of 0.2 (7 µL) were spotted onto growth media in a series of 10-fold dilutions. Expression was induced by adding 0.4% galactose into the media. Pictures were taken following incubation at 30°C for 5 days following spotting.

**RNA Interference (RNAi)**

HT115 (DE3) bacterial strains containing plasmids expressing dsRNA against *F27C1.2, F31E8.4, Y58A7A.1, F58G6.7, F58G6.9, K12C11.6,* and *K12C11.7* genes were obtained from the Ahringer and ORFeome feeding libraries (62,63). The empty vector L4440 was used as a control. Portions of *F01G12.1, K12C11.3* and *F58G6.3* DNA constructs were cloned into the L4440 vector and transformed into HT115 bacteria. Each construct was sequenced using the primer 5′-AGCGAGTCAGTGAGCGAG-3′, and evaluated by the E-RNAi online tool (e-rnaig.org) to determine the RNAi target. NGM growth media with 12 µg/mL of tetracycline, 50 µg/mL carbenicillin, and 2 mM IPTG (isopropyl-β-D-galactopyranoside) were used for RNAi experiments.

**BLAST and Topology Prediction**

The human Ctr1 protein sequence was used as a query sequence in a search using PSI-BLAST. Non-redundant protein sequences (nr) were chosen for the database, and results were filtered to include only hits on the *Caenorhabditis elegans* (taxid:6239) genome. Candidates had an E-value cut-off of less than 10⁻⁵. TMD of various organisms’ Ctr homologs were predicted by TMHMM 1.0. Clustal Omega was used to generate sequence alignment.

**Axenic Media Growth**

The axenic liquid media used in this study (‘low Cu’ mCeHR) is modified from the mCeHR media described previously (64) by removing extra Cu supplementation in the salt solution. 20 µM hemin is added for every culture condition. N2 worms grown in “low Cu” mCeHR media were synchronized and hatched overnight in M9 buffer. Approximately 100 L1 stage worms were seeded into a 10 mL liquid media-containing flask with indicated CuCl₂ or BCS concentrations. Flasks were incubated on a rotating platform at 20°C for 9 days. On day 9, animals were collected, samples were centrifuged (800 xg, 1 min) to collect worm pellet, and then washed twice with M9 buffer. To count the number of worms, the tube was vortexed to mix, a prescribed amount of sample was drawn up and placed on a slide, and worm counts were calculated. Each condition was tested and counted in triplicate.

**qRT-PCR**

For assays conducted in axenic media, N2 worms were maintained in 10 µM CuCl₂ “low Cu”
mCeHR, and then synchronized and split into flasks containing 10 μM CuCl₂ (optimal), 300 μM CuCl₂ (high Cu), or 100 μM BCS (low Cu) media. Worms were grown in each condition until the population reached the mid-L4 stage. Worms were collected after washing twice with M9 buffer and resuspended in 1 ml Trizol (Invitrogen), then lysed in Lysing Matrix Tubes (MP Biomedicals) by FastPrep-24 (MP Biomedicals) homogenizer. Total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel), and 1μg RNA was used for cDNA synthesis (SuperScript III First Strand synthesis kit, Invitrogen). Real-time PCR was performed with SYBR Green Taq 2x Mix (BioRad) with three biological replicates and two technical replicates. Fold change values were calculated using the 2^ΔΔCt method, with all values normalized to pmp-3 (Fig. 1b and 1C) or gpd-2 (Supplemental Fig. S2 and S8B) expression. Primers are listed in Supplemental Table 3.

**Quantification of Cu-dependent Worm Growth, Length, Brood Size, and Cu Levels**

Assays were performed as follows, unless specified in figure legends. For worm growth assays and length quantification, stage-synchronized L1 worms (P₀) were grown on RNAi-expressing bacteria until vector-fed control worms reached the L4 stage. P₀ worms were washed off the culture media with M9 buffer and 100 μL of worms in M9 buffer was transferred into a 96-well culture dish. Animals’ body length (TOF), density (extinction), and fluorescence intensity were quantified using a COPAS Biosort system FP-250 (Union Biometrica). To perform these assays on F₁ worms, 20 P₀ worms were transferred to fresh plates at the L4 stage and allowed to lay eggs for 12 h after reaching the gravid adult stage. P₀ worms were removed from the plate, and F₁ progeny were analyzed as above upon reaching the L4 stage.

For brood size analysis, synchronized L1 worms were cultured on dsRNA-expressing bacteria for 50 h until reaching gravid adult stage. Individual worms were then transferred onto fresh plates to allow egg-laying. After 3 consecutive days of egg-laying, the brood size, including both hatched and unhatched embryos was counted.

For Cu-pulse experiments, a mixed stage population was cultured on BCS-supplemented NGM plates for 5 days prior to synchronization. BCS-treated worms were then bleached to generate synchronized L1 animals which were then cultured on BCS plates for 48 h, washed off, and split evenly onto BCS- and Cu-supplemented plates for 12 h prior to worm pelleting. Restored Cu levels for each experimental condition were normalized to BCS-treated samples. See below for ICP-MS measurements.

**ICP-MS**

Metal contents of worms were measured using ICP-MS as described previously (20). Values were normalized to wet weight of worms. For sample preparation, synchronized L1 worms were grown on NGM plates seeded with OP50 or HT115 RNAi bacteria and supplemented with the indicated amounts of copper or BCS until worms reached L4 stage. Worm pellets were collected and washed extensively with M9 buffer, transferred to acid-washed tubes, and frozen at -80 °C. At least three independent biological replicates were analyzed.

**Generation of Transgenic Worms**

Transgene-expressing plasmids were generated using a Multisite Gateway Three-Fragment Vector Construction kit (Invitrogen). Promoter, ORF, and UTR regions were amplified separately and recombined into the plasmid. The unc-54 3’ UTR region is used in all constructs in this study. Together with the unc-119 rescue plasmid, the transgene-expressing plasmid was then introduced into unc-119 (ed3) III worms using a PDS-1000 particle delivery system (BioRad) bombardment system. To generate worm strains expressing multiple transgenes, one worm strain expressing a single transgene was crossed with another transgenic worm using methods previously described (65).

**Staining with the Cu Probe CF4**

Stage-synchronized L4 worms expressing CHCA-1::GFP in the intestine were used for the CF4 assay. Worms were washed three times with M9 buffer, and around 400 worms were suspended in 100 μL M9 buffer. CF4 Cu probe (28) was then added to the buffer at a concentration of 25 μM. Worms were stained in the dark at room temperature for 2 h, and then transferred on normal NGM plates outside the bacterial lawn. These plates were kept in the dark for 2 h, and
then the worms were collected and washed three times with M9 buffer and imaged via confocal microscopy.

**Cu-responsive Behavior Assay**

The Cu-avoidance assay in this study utilized rectangular Cu gradient plates. To make Cu gradient media, 4-well rectangular plates were tilted on their lids and 2 mL Cu-containing media (with indicated concentrations of CuCl$_2$ in 1.7% agar, 3 mg/mL NaCl, 5 µg/mL cholesterol, and 2.5 mg/mL bacto-peptone) was added to one third of the plate length. Upon solidification, plates were brought flat and 10 mL NGM agar was added on top. For control experiments with non-Cu containing plates, plates received 12 mL of NGM agar alone. After upper layer solidification, plates were kept at 4°C for 16 h to allow Cu diffusion prior to conducting the assay. Five sections were drawn on the bottom of the plates (indicating low to high Cu) for future quantification of worms per section as delineated by approximate concentration. Synchronized L4 worms were washed three times with M9 solution to remove bacteria, and 40 µL of worm-M9 solution was pipetted in the middle section of the plate (section three). Following a 2.5 h drying period, images of the plates were captured by camera and animals in each section were counted using Image J software. Each assay included at least 150 animals, and at least three independent experiments were performed for each condition. Avoidance behavior of the high Cu regions (sections four and five) were denoted by an Avoidance Index (AI) derived from the following formula:

\[
\text{Avoidance Index (AI)} = \frac{\text{Percentage of worms in section } [(4+5) - (1+2)]}{\text{Percentage of worms in section } [(4+5) + (1+2)]}
\]

Worms in section three were not calculated, as not all worms translocated to different sections in the given time frame.

**Immunofluorescence and Western Blot**

The antibodies applied in the worm immunofluorescence assays are rabbit anti-FLAG (Rockland) at 1:300, and Alexa594 goat anti-rabbit IgG (ThermoFisher) at 1:300. For each condition, 75 µg of protein was loaded into gels. Transgenic animals expressing CHCA-1-2xFLAG::SL2::GFP in the intestine were stage-synchronized and L4 worms were fixed and stained with antibody as previously described (66). Worms applied with secondary antibody only served as negative controls. For the yeast Western blots, rabbit anti-FLAG (Rockland) at 1:2000, and mouse anti-PGK1 (Molecular Probes) at 1:1000 were applied as primary antibodies.

**Statistical Analysis**

Statistical significance was calculated by one-way ANOVA, two-way ANOVA, or a t-test with Prism GraphPad version 6 (GraphPad, San Diego, CA). ANCOVA was performed using SPSS Statistics version 23 (IBM). Data values were presented as mean ± SEM. Asterisk marks indicate significance at p values < 0.05.

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**CONFLICT OF INTEREST**
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

S.Y. and B.-E.K. conceived the study. S.Y. conducted most of the experiments, analyzed the data, and wrote most of the paper with B.-E.K. A.K.S. carried out the majority of Cu avoidance and yeast assay. A.R. performed qRT-PCR assay. J.L. conducted ICP-MS analysis. All authors reviewed the results and approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1. Transcriptional regulation of *C. elegans* Ctr1 candidate genes by Cu
A) Cu-dependent *C. elegans* growth in axenic media. Total worm numbers were calculated for three independent experiments. Asterisks indicate significant difference from optimal 10 µM CuCl2 condition (One-way ANOVA, Dunnett post hoc test, \(*p < 0.01, ****p < 0.0001\)). B) and C) qRT-PCR analysis of *C. elegans* Ctr candidate genes under Cu-limited (B) and high Cu (C) conditions. Worms were synchronized and supplemented with 100 µM BCS, 10 µM CuCl2 or 300 µM CuCl2 from L1 to L4 stage in axenic media. Individual gene expression was normalized first to *pmp-3*, then to its own expression under Cu-optimal conditions. Two independent experiments were conducted under each condition. Asterisks indicate significant difference from indicated gene expression levels under optimal conditions. (Two-way ANOVA, Sidak post hoc test, \(*p < 0.05, ****p < 0.0001\)). Error bars, mean ± SEM.

Figure 2. Requirement of Ctr1 candidate genes for growth, reproduction, and Cu accumulation in worms
A-C) Cu-dependent growth assay under basal (A), high Cu (B) and limited Cu (C) conditions. Synchronized L1 stage N2 worms were cultured on RNAi plates until the vector-treated worms reached L4 stage. Worm length (TOF) was quantified using a COPAS BioSort system, and worm length under each RNAi condition was normalized to vector TOF. ~400 individual animals were analyzed under every condition. Values with asterisk are significantly different from vector (one-way ANOVA, Dunnett post hoc test, \(*p < 0.05, **p < 0.01, ***p < 0.001\) ). D) Representative images of *P. pacificum* expressing bacteria. E) Cu levels in *C. elegans* were measured by ICP-MS after exposure to 10 µM Cu for two generations. Values with asterisk are significantly different from vector with three independent trials (One-way ANOVA, Dunnett post hoc test, \(*p < 0.05\) ). F) A schematic presentation of Cu-pulse assays. *C. elegans* worms were cultured in axenic media supplemented with 25 µM BCS for 5 days prior to synchronization. Synchronized L1 animals were cultured on 50 µM BCS NGM agar plates expressing indicated dsRNA for 72 h. Animals were then washed, aliquoted, and re-plated on fresh 50 µM BCS or 50 µM CuCl2 plates for 12 h. Restored Cu levels are indicated by normalizing Cu-treated worms to BCS-cultured animals. G) Restored Cu levels under indicated RNAi treatments. Values with asterisk are significantly different from vector (One-way ANOVA, Dunnett post hoc test, \(****p < 0.001, ****p < 0.0001\) ). H) Brood size analysis of F58G6.9 RNAi animals. Error bars indicate mean ± SEM of five independent experiments. Values with asterisk are significantly different from vector under same culture condition (Two-way ANOVA, Sidak post hoc test, \(*****p < 0.0001\) ). Error bars in this figure represent mean ± SEM.

Figure 3. Depletion of CHCA-1 gene by RNAi decreases intestinal Cu availability in *C. elegans.*
A) Synchronized L1 stage BK015 transgenic worms [Pchsa-6::CUA-1.1::GFP::unc-54 3′ UTR; cua-1 (ok904)] were cultured on NGM agar plates seeded with *E. coli* expressing dsRNA against CHCA-1 or vector. CUA-1.1::GFP localization in L4 worms was examined using confocal microscopy. Scale bar, 15 µm. B) CHCA-1-depleted worms display increased endogenous CUA-1.1::GFP expression in the hypodermis. BK017 [Pcua-1::CUA-1.1::GFP::unc-54 3′ UTR; cua-1 (ok904)] transgenic animals were maintained on 10 µM CuCl2 plates prior to synchronization. L1 animals were then re-plated for CHCA-1 RNAi. After 60 h culture, CUA-1.1::GFP expression levels in L4 animals were examined using confocal microscopy. Scale bar, 50 µm.

Figure 4. Characterization of the CHCA-1 (tm6506) IV strain
A) Schematic of human Ctr1 and *C. elegans* CHCA-1 gene loci, with blue-colored ORFs and grey-colored UTR regions. Note that CHCA-1b1 and CHCA-1b2 isoforms differ at the 5′ UTR region but express identical proteins. The deleted region in the tm6506 allele is indicated by the red bar. Scale bar indicates 100-base pairs (bp). B) and C) Growth of tm6506 animals under various CuCl2 or BCS-
supplemented conditions. Worms homozygous for the tm6506 allele and their outcrossing wild type brood mates (WT) were cultured from synchronized L1s for 72 h. B) Representative images of animals growing under indicated conditions. C) Worm growth quantification using a COPAS BioSort system. Error bars indicate mean ± SEM of around 75 worms. Values with asterisk are significantly different from WT animals under same Cu or BCS concentrations (Two-way ANOVA, Dunnett post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001). D) WT and tm6506 mutant worms grown on 50 µM BCS plates or 50 µM BCS plus indicated concentrations of metals. Error bars indicate mean ± SEM of around 100 individual animals. Means with different letters are significantly different at p = 0.05 (Two-way ANOVA, Tukey’s post hoc test). E) Cu levels of tm6506 and WT animals. Synchronized L1 animals were cultured on indicated concentrations of Cu or BCS-supplemented media for 60 h and then pelleted for ICP-MS. For each condition, 3 or 4 samples were analyzed. Values with asterisk are significantly different from those of WT animals (t-test for each treatment condition, *p < 0.05, ***p < 0.001, ****p < 0.0001). F) Cu-acquisition capacity of tm6506 worms. WT or tm6506 worms pre-treated with 15 µM BCS were washed, and separately cultured on fresh 15 µM BCS or 50 µM CuCl2 NGM plates for 12 h, followed by ICP-MS analysis. Three independent samples were assayed for each condition. Asterisks indicate that Cu levels in tm6506 worms post-pulse are significantly different from those in the WT strain (ANOVA, Bonferroni post hoc test, p = 0.016).

Figure 5. Intestinal CHCA-1 plays an important role in Cu-dependent growth and Cu accumulation
A) Tissue-specific expression of the CHCA-1 gene. Transgenic animals expressing GFP driven by the 2.8 kb CHCA-1 promoter region were cultured on NGM plates containing 10 µM CuCl2 (a, b) or 200 µM BCS (c-f, same animal, different focus layers). Arrowhead indicates intestine, and arrow indicates hypodermis cells. a, c, e: bright field; b, d, f: fluorescence. Scale bar, 50 µm. B) and C) Cu-dependent growth following CHCA-1 gene depletion in specific tissues. N2, RNAi-resistant strains (rde-1, WM27) and tissue-specific rde-1 expressing strains (VP303, NR222, and WM118) were used to knock down the CHCA-1 gene in the indicated tissues (Int: intestine, Hyp: hypodermis, Mus: muscle). Synchronized L1s were cultured to the L4 stage (panel B, P = 0.016). Growth of CHCA-1-depleted worms was normalized to vector for each condition. CHCA-1 RNAi in N2 and VP303 strains on 100 µM BCS plates exhibited severe defects in P0 reproduction (U.D., under detection limit). Error bars indicate mean ± SEM of ~200 individual animals. Values with asterisk are significantly different from the same strain under basal conditions (Two-way ANOVA, Dunnett post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant). D and E) Cu accumulation in N2 and tissue-specific CHCA-1-depleted animals. Different strains of worms were pre-cultured on 50 µM BCS NGM plates and then half of population were separated and treated with Cu, as described above in Figure 2, F and G. D) Restored Cu levels after normalizing to BCS-cultured samples. Error bars represent mean ± SEM of four independent experiments. Values with asterisk are significantly different from vector (Two-way ANOVA, Sidak post hoc test, ***p < 0.001, ****p < 0.0001). Cu levels following BCS pre-culture were not significantly different among strains by Two-way ANOVA (data not shown). E) Percentage of Cu levels restored by CHCA-1 RNAi after normalizing to vector animals under the same conditions. Error bars, mean ± SEM of four independent experiments. Values with asterisk are significantly different from one another (One-way ANOVA, Dunnett post hoc test, *p < 0.05, **p < 0.01, ns = not significant).

Figure 6. Intestinal expression of CHCA-1::GFP
A) Intestinal expression of CHCA-1::GFP under basal conditions (Pvha-6::CHCA-1::GFP, unc-54 3′UTR). Scale bar, 50 µm. B) CHCA-1::GFP expression in the intestine under basal, Cu-deficient, and high Cu conditions. A DAPI channel was used to observe intestinal auto-fluorescence from gut granules. Scale bar, 15 µm. C) CHCA-1::GFP signal intensity was quantified under high, low, or replete Cu conditions using a COPAS BioSort system. At least 100 synchronized L4 CHCA-1::GFP-expressing worms were used.
following 2.5 days of Cu or BCS-supplemented cultures in each condition (One-way ANOVA, Dunnett post hoc test, ns = not significant). D) Intestinal expression of CHCA-1 partially rescued growth of CHCA-1 mutant animals. Transgenic animals expressing CHCA-1::SL2::GFP protein were crossed with tm6506 animals to generate an intestinal CHCA-1 expression animal in a whole body CHCA-1 mutant background. These transgenic animals, together with their wild-type brood mates (WT), as well as tm6506 animals, were quantified by TOF after 60 h culture from synchronized L1s in indicated conditions. Error bars indicate mean TOF ± SEM of ~150 individuals. Values with asterisk are significantly different from tm6506 worms cultured at the same condition (Two-way ANOVA, Tukey’s post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

**Figure 7. Worms lacking CHCA-1 exhibit reduced Cu sensing behavior**

A) Schematic of Cu avoidance assay: Cu gradient plates were made by adding a given concentration of CuCl₂ to one side of a rectangular plate. Non-Cu containing plates were used as controls. At least 150 animals were used on each plate with total three independent experiments. B) Representative results of N2 worm distribution on a non-Cu plate (left) or 10 mM Cu-gradient plate (right). Error bars, mean ± SEM of two independent experiments. Asterisk indicate the percentage of animals in the low Cu area (sections 1 and 2) is significantly different from the percentage in the high Cu area (sections 4 and 5) (Two-way ANOVA, Sidak post hoc test, **p < 0.01, ns = not significant). C) N2 worm avoidance index on Cu gradient plates with varied concentrations of Cu. Error bars indicate mean ± SEM of three independent experiments.-Asterisk values are significantly different from the avoidance index on non-Cu plates (One-way ANOVA, Dunnett post hoc test, ***p < 0.001, ****p < 0.0001). D) Avoidance index of CB1033 (che-2 (e1033) X) and N2 vector worms or CHCA-1 RNAi worms on 8 mM Cu gradient plates. Three independent experiments for CB1033 and six independent experiments for N2 were analyzed (One-way ANOVA, Dunnett post hoc test, *p < 0.05, ***p < 0.001). E) Worms were pre-cultured with 100 µM CuCl₂ or 100 µM BCS for one generation and assayed on 8 mM Cu gradient plates. (One-way ANOVA, Dunnett post hoc test, ***p < 0.001, ****p < 0.0001). F) Avoidance index of RNAi-hypersensitive worms (TU3335) lacking the egl-3 or egl-21 gene on 10 mM Cu gradient plates. Error bars indicate mean ± SEM of eight independent experiments under each condition. Values with asterisk are significantly different from each other (One-way ANOVA, Dunnett post hoc test, *p < 0.05, **p <0.01, ****p <0.0001). G) PAM genes (pgal-1, pghm-1 and pamn-1) were co-depleted by RNAi for two consecutive generations in TU3335 strain, followed by the avoidance assay on 10 mM Cu-gradient plates. Error bars indicate mean ± SEM of two independent experiments. Values with asterisk are significantly different (One-way ANOVA, Dunnett post hoc test, * p < 0.05, **p <0.01).
Figure 1
Figure 2
Figure 3
Figure 4

CHCA-1-Mediated Copper Homeostasis in Worms
Figure 5
Figure 6
Figure 7
CHCA-1 is a copper-regulated Ctr1 homolog required for normal development, copper accumulation, and copper-sensing behavior in *Caenorhabditis elegans*

Sai Yuan, Anuj Kumar Sharma, Alexandria Richart, Jaekwon Lee and Byung-Eun Kim

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