Abstract
Zinc is an essential trace element involved in a wide range of biological processes and human diseases. Zinc excess is deleterious, and animals require mechanisms to protect against zinc toxicity. To identify genes that modulate zinc tolerance, we performed a forward genetic screen for Caenorhabditis elegans mutants that were resistant to zinc toxicity. Here we demonstrate that mutations of the C. elegans histidine ammonia lyase (haly-1) gene promote zinc tolerance. C. elegans haly-1 encodes a protein that is homologous to vertebrate HAL, an enzyme that converts histidine to urocanic acid. haly-1 mutant animals displayed elevated levels of histidine, indicating that C. elegans HALY-1 protein is an enzyme involved in histidine catabolism. These results suggest the model that elevated histidine chelates zinc and thereby reduces zinc toxicity. Supporting this hypothesis, we demonstrated that dietary histidine promotes zinc tolerance. Nickel is another metal that binds histidine with high affinity. We demonstrated that haly-1 mutant animals are resistant to nickel toxicity and dietary histidine promotes nickel tolerance in wild-type animals. These studies identify a novel role for haly-1 and histidine in zinc metabolism and may be relevant for other animals.

Introduction
Zinc is a trace nutrient that plays critical roles in all biological systems. Zinc contributes to protein structure and enzymatic activity and functions in signal transduction processes [1,2,3]. The important role of zinc in biological systems is demonstrated by its impact on human health, since both zinc deficiency and excess can be deleterious. Zinc deficiency in humans causes a wide spectrum of symptoms that result from functional defects in the epidermal, gastrointestinal, central nervous, immune, skeletal, and reproductive systems, and inadequate dietary intake of zinc is a major worldwide problem [4,5]. Zinc deficiency is also associated with mutations in genes encoding human zinc transporters such as Zip4, the causative gene in acrodermatitis enteropathica [6]. Excess zinc is also deleterious. The mechanisms underlying toxicity caused by excess zinc are not well defined. However, excess zinc may displace other trace metals or bind low-affinity sites, leading to protein dysfunction [7].

In humans, zinc toxicity associated with excess dietary intake has been reported, but occurs rarely [8,9]. By contrast, pathological conditions that lead to focal disruptions of zinc metabolism may be more common. For example, zinc release from dying cells during ischemic brain injury is postulated to be a major contributor to cell death and functional deficits [10]. Zinc metabolism appears to modulate the pathology of Alzheimer’s disease, since precipitation and toxicity of the Aβ peptide that causes the disease are influenced by interactions with metal ions such as zinc [11,12,13]. Zinc-mediated toxicity is also associated with pancreatic islet cell destruction during diabetes [14]. Because zinc plays critical roles in human health and disease, it is important to understand the biological processes that mediate zinc metabolism and protect against zinc toxicity.

Organisms have evolved several strategies to promote zinc homeostasis and protect against zinc toxicity. One strategy is to regulate zinc uptake and excretion such that zinc uptake is downregulated and zinc excretion is upregulated in the presence of high levels of dietary zinc. For example, vertebrate cells downregulate zinc importers in the ZIP family in response to high levels of zinc [15]. A second strategy is to sequester zinc in an intracellular organelle, as illustrated by the import of zinc into the vacuole of S. cerevisiae [16]. A third strategy is chelation of zinc by small molecules such as glutathione or proteins. Dietary zinc causes vertebrate cells to upregulate expression of metallothionein, a small protein that can bind multiple zinc atoms [17].

The nematode C. elegans is a powerful model system that has been used to characterize fundamental and highly conserved biological processes such as RNA interference (RNAi) and apoptosis [18,19]. It has also been used to develop innovative
experimental techniques such as in vivo expression of green fluorescent protein [20]. C. elegans is a relevant model system for the study of metal biology, since it has been used to analyze zinc signaling, metal toxicity, and iron and heme metabolism [21,22,23,24,25,26,27]. We are using C. elegans to study zinc metabolism, since these animals have a simple and wellecharacterized anatomy, culture methods that permit precise control of dietary zinc are established, and powerful genetic approaches such as forward genetic screens are available [28,29].

To identify genes involved in C. elegans zinc metabolism, we conducted a forward genetic screen for chemically induced mutations that caused resistance to high levels of dietary zinc [28]. Nineteen mutations that confer significant resistance to dietary zinc were identified, and these strains represent the first report of mutant animals with increased resistance to excess zinc. Consistent with this hypothesis, we demonstrated that dietary histidine promoted tolerance to excess zinc in wild-type worms. Mutations in haly-1 and supplemental dietary histidine also caused resistance to nickel, another metal that can bind histidine. A likely mechanism of protection is chelation of zinc and nickel by histidine. These studies suggest that histidine plays a physiological role in zinc metabolism.

Author Summary

Zinc is an essential nutrient that is critical for human health. However, excess zinc can cause toxicity, indicating that regulatory mechanisms are necessary to maintain homeostasis. The analysis of mechanisms that promote zinc homeostasis can elucidate fundamental regulatory processes and suggest new approaches for treating disorders of zinc metabolism. To discover genes that modulate zinc tolerance, we screened for C. elegans mutants that were resistant to zinc toxicity. Here we demonstrate that mutations of the histidine ammonia lyase (haly-1) gene promote zinc tolerance. haly-1 encodes a protein that is similar to vertebrate HAL, an enzyme that converts histidine to urocanic acid. Mutations in the human HAL gene cause elevated levels of serum histidine and abnormal zinc metabolism. Mutations in C. elegans haly-1 cause elevated levels of histidine, suggesting that histidine causes resistance to excess zinc. Consistent with this hypothesis, we demonstrated that dietary histidine promoted tolerance to excess zinc in wild-type worms. Mutations in haly-1 and supplemental dietary histidine also caused resistance to nickel, another metal that can bind histidine. A likely mechanism of protection is chelation of zinc and nickel by histidine. These studies suggest that histidine plays a physiological role in zinc metabolism.

Results

Identification of the haly-1(am132) mutation

To identify genes that mediate zinc metabolism, we conducted a forward genetic screen for C. elegans mutants that were resistant to toxic levels of dietary zinc [28]. After screening approximately 300,000 mutagenized haploid genomes, nineteen mutations were identified that caused significant resistance to zinc toxicity. These mutations were positioned in the genome by linkage to single nucleotide polymorphism (SNP) markers. Here we focus on two mutations, am130 and am132, that caused strong resistance to dietary zinc (Figure 1). These mutations displayed tightest linkage to the same SNP, pkP6160, positioned at +2.50 map units on chromosome X. Three factor mapping experiments using visible markers indicated that am132 is positioned between unc-115 and egl-15, an interval that contains 863 kb (Figure 2A) [28].

To identify the lesions in these alleles, we refined the interval that contains the am132 mutation using high resolution mapping relative to SNP markers [31]. These experiments established that the am132 mutation is positioned between +2.40 and +2.84 map units, a 270 kb interval that contains 48 predicted open reading frames (Figure 2A) (see Material and Methods). To identify the gene affected by the am132 mutation, we performed whole genome sequencing using DNA from the am132 mutant strain. Candidate mutations were identified by comparing the am132 DNA sequence to the wild-type reference sequence [32]. One candidate mutation caused a predicted nonsense change in the F47B10.2 gene. The presence of this mutation was confirmed using standard DNA sequencing. The mutation is a C to T transition in the wobble position of codon 296 that changes a tryptophan to a stop codon (Figure 3B). F47B10.2 is predicted to encode a histidine ammonia lyase, an evolutionarily conserved enzyme that converts histidine to urocanic acid (Figure 3C) [30]. Therefore, we named the gene haly-1. The mutant allele is predicted to encode a truncated protein lacking a significant

Figure 1. haly-1 mutant animals are resistant to excess dietary zinc. Wild-type, haly-1(am130) and haly-1(am132) hermaphrodites were synchronized at the L1 stage and cultured on NAMM supplemented with zinc (mM). The fraction of worms that grew to adulthood over seven days was monitored; the data were normalized by setting the value at 0 mM supplemental zinc equal to 1.0. Each point indicates mean value ± SE (n = 4 replicates with 50 worms per replicate). The fraction adult for haly-1(am130) and haly-1(am132) was significantly higher than wild type at 0.15 mM and higher concentrations of supplemental zinc (p<0.05).

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portion of the conserved regions. Thus, haly-1(am132) is likely to be a strong loss-of-function mutation.

The am130 mutation also affects haly-1

Similar to am132, the am130 mutation displayed linkage to SNP pkP6160 that is positioned close to haly-1 [28]. To determine if the am130 mutation affects the haly-1 gene, we analyzed the sequence of the haly-1 locus using DNA from am130 mutant animals. A single G to A transition was detected, and the change is a missense mutation predicted to change amino acid 536 from the negatively charged aspartic acid to the polar uncharged asparagine (Figure 3B). The aspartic acid at position 536 is conserved in histidine ammonia lyase found in humans and other vertebrates. The identification of two, independently derived mutations that both affect the haly-1 gene, suggests that mutations in haly-1 cause resistance to dietary zinc toxicity.

haly-1 genomic DNA can rescue zinc resistance of am132 mutant animals

If the changes in the haly-1 locus detected in the am130 and am132 strains cause zinc resistance, then the introduction of wild-type copies of haly-1 might restore zinc sensitivity. To test this hypothesis, we generated transgenic haly-1(am132) animals containing fosmid WRM0624bE06 that contains the entire haly-1 open reading frame (Figure 2B). Two independently derived transgenic strains were analyzed for zinc sensitivity using noble agar minimal media (NAMM) with a range of supplementary zinc concentrations. Both transgenic strains displayed zinc sensitivity similar to wild-type animals (Figure 2B). To determine if the haly-1 locus is sufficient for the rescue activity, we subcloned a 4318 bp fragment that includes 1567 bp upstream of the haly-1 START codon and 114 bp downstream of the haly-1 STOP codon. Transgenic haly-1(am132) animals containing the haly-1 locus displayed zinc sensitivity similar to wild-type animals (Figure 2B). To determine if an intact haly-1 open reading frame is necessary for the rescue activity, we generated a haly-1 locus with a deletion mutation that removes exons 4-10, haly-1(am132) animals containing or lacking this mutant haly-1 locus displayed similar zinc tolerance, indicating that the rescue activity requires an intact open reading frame that has the capacity to produce HALY-1 protein. To investigate the effect of the am132 mutation, we used site-directed mutagenesis to change the tryptophan located at codon 296 into a stop codon. haly-1(am132) animals containing or lacking this mutant haly-1 locus displayed similar zinc tolerance, indicating that the rescue activity requires an intact open reading frame that has the capacity to produce HALY-1 protein. These results demonstrate that the haly-1 locus is sufficient to rescue the mutant phenotype and the rescue activity requires an intact haly-1 open reading frame, indicating haly-1 is the gene affected by the am132 mutation.

Analysis of the structure and regulation of haly-1 gene products

To determine the products generated from the haly-1 locus, we analyzed haly-1 mRNA. The C. elegans EST project isolated multiple cDNAs corresponding to haly-1, and we determined the complete DNA sequence of six cDNAs. Two cDNAs included the predicted start codon, and five cDNAs included the intact 3' end including the polyA tail. Thus, these data document the complete predicted open reading frame. The analysis indicated

Figure 2. Positional cloning of haly-1. (A) A genetic map of chromosome X. Loci defined by mutations that cause visible phenotypes and SNPs are indicated above, and map units are shown below. Three-factor mapping experiments positioned am132 between unc-115 and egl-15. Multi-factor mapping experiments positioned am132 between the SNPs CE6-177 and CE6-1202, an interval of 270 kb. (B) Lines indicate DNA, boxes represent exons, and shading represents untranslated regions. The fosmid WRM0624bE06 includes eight predicted ORFs shown as arrows indicating orientation of transcription, including haly-1 (F47B10.2). Plasmid pJM1 included only the wild-type haly-1 ORF, whereas pJM2 has a deletion mutation and pJM3 has a nonsense change identical to haly-1(am132). Rescue indicates the number of independently derived transgenic strains that displayed wild-type zinc tolerance over the total number of transgenic strains analyzed.

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that there was only a single spliced form composed of 11 exons (Figure 3A).

To analyze the regulation of haly-1 mRNA, we cultured wild-type animals in *C. elegans* minimal maintenance medium (CeMM), a fully defined, axenic liquid medium [33]. CeMM is formulated from purified vitamins, growth factors, amino acids, nucleic acids, heme, b-sitosterol, sugar, salts, and trace metals. CeMM with no added zinc can be supplemented with a wide range of zinc to analyze both zinc restriction and zinc excess [29]. We cultured wild-type animals in CeMM containing a low concentration of 10 mM zinc or a high concentration of 500 mM zinc, isolated RNA from adult stage animals and measured the level of haly-1 mRNA using quantitative real time PCR (qRT-PCR). The level of haly-1 mRNA varied less than 1.5 fold in animals cultured at 10 mM and 500 mM zinc compared to control genes, indicating that the level of haly-1 mRNA is not regulated by dietary zinc (see Materials and Methods).

Based on the haly-1 mRNA structure, the predicted HALY-1 protein contains 677 amino acids. A BLAST search was used to identify related proteins, and Figure 3B displays an alignment of *C. elegans* HALY-1 with human, zebra fish and bacterial proteins. The amino acid sequence of *C. elegans* HALY-1 is 54% identical to HAL from humans and 37% identical to HAL from *Cupriavidus metallidurans* CH34, a bacteria that has been used to study metal tolerance [34]. Histidine ammonia lyase catalyzes the reductive deamination of L-histidine to urocanic acid (Figure 3C) [30].

The high degree of sequence conservation strongly supports the model that *C. elegans* haly-1 is descended from a common ancestral gene that was conserved in humans.
Mutations in *haly-1* promote zinc resistance in multiple genetic backgrounds

Three members of the cation diffusion facilitator family of zinc transporters have been characterized in *C. elegans*, *cdf-1*, *cdf-2* and *sur-7* [22,27,29]. Loss-of-function mutations in these genes cause sensitivity to dietary zinc. To investigate the interactions between *haly-1* and these *cdf* genes, we constructed and analyzed double mutants. Because loss-of-function mutations in *haly-1* and *cdf* genes cause the opposite phenotype, the analysis of double mutant animals can elucidate relationships between these genes. Figure 4A shows that compared to wild-type animals, *haly-1*(am132) mutant animals displayed resistance to dietary zinc, whereas *cdf-1* loss-of-function mutant animals displayed sensitivity. *cdf-1*(lf) *haly-1*(lf) double mutant animals displayed an intermediate phenotype. In particular, in the presence of 0.02 – 0.06 mM supplemental zinc the double mutant animals were significantly more resistant than *cdf-1*(lf) single mutant animals (Figure 4B). At 0.08 mM and higher concentrations of supplemental zinc, the double mutant animals displayed sensitivity similar to the *cdf-1*(lf) single mutant animals. We performed a similar analysis of *haly-1*(lf) *sur-7*(lf) and *haly-1*(lf) *cdf-2*(lf) double mutant animals. In both cases, the double mutant animals displayed zinc sensitivity that was intermediate compared to the single mutant animals (Figure 4C, 4D). These results indicate that the *haly-1*(lf) mutations promote zinc resistance in genetic backgrounds characterized by zinc sensitivity as well as in wild-type animals. Furthermore, these findings suggest that *haly-1* functions in parallel to *cdf* genes to modulate zinc sensitivity.

The zinc content of *haly-1* mutant animals is similar to wild-type worms

The zinc resistance of *haly-1* mutant animals can be explained by two general models. One possibility is that *haly-1* mutant animals have lower levels of zinc, perhaps as a result of reduced uptake or increased excretion. A second possibility is that *haly-1*
mutant animals have the same or higher levels of zinc compared to wild-type animals, but the mutant animals have improved tolerance. To distinguish between these possibilities, we used the method of inductively coupled plasma mass spectrometry (ICP-MS) to measure total zinc content [29]. A mixed-stage population was cultured in CeMM, harvested and analyzed for zinc content. The total zinc content of haly-1(am130) mutant animals was not significantly different from wild-type animals when cultured with optimal or high levels of dietary zinc (Figure 5). ICP-MS was also used to analyze the levels of magnesium, manganese, iron and copper. haly-1(am130) and haly-1(am132) mutants had levels of Mg, Mn, Fe, Cu that were similar to wild-type animals (Table S1). These results suggest that mutations in haly-1 cause zinc resistance by promoting tolerance to zinc rather than reducing the levels of zinc or other metals.

haly-1 mutations cause elevated levels of histidine

HAL is a key enzyme in histidine metabolism, and mutations that diminish HAL activity cause elevated histidine levels in vertebrates [35,36]. To test the hypothesis that haly-1(gf) mutations cause elevated histidine, we developed methods to measure total histidine levels in C. elegans. We cultured animals in CeMM containing 2 mM histidine, harvested a population consisting of mixed developmental stages, and measured the levels of amino acids (Figure 6D). Wild-type animals contained 0.011 ± 0.002 nmoles histidine/µg protein. haly-1(am130) and haly-1(am132) animals displayed significantly higher levels, 0.039 ± 0.006 and 0.066 ± 0.02 nmoles histidine/µg protein, respectively (Figure 6D). The haly-1 mutant animals displayed elevated levels of histidine compared to wild type when cultured at 0 mM, 0.075 mM or 1.5 mM zinc, indicating that the level of dietary zinc has little effect on the level of histidine (data not shown). By contrast, the levels of the other amino acids were not consistently different between haly-1 mutant animals and wild-type animals (data not shown). The levels of urocanic acid, the product of HAL enzymatic activity, have not been determined. These results indicate that haly-1 mutant animals have a specific defect in histidine metabolism that results in elevated levels of histidine and that the am130 and am132 mutations cause a reduction of haly-1 activity.

Dietary histidine promotes zinc tolerance

The observation that haly-1(gf) mutations cause elevated levels of L-histidine led us to hypothesize that the elevated histidine causes zinc resistance. To investigate this hypothesis, we analyzed the effects of feeding animals histidine. If elevated histidine levels promote zinc tolerance, then animals cultured with high levels of dietary histidine are predicted to display zinc tolerance. Wild-type hermaphrodites cultured on NAMM supplemented with 0.1 mM histidine displayed significantly increased tolerance to dietary zinc (Figure 6A). The effects of dietary histidine were dose dependent: weak protection was observed at 0.03 mM, optimal protection was observed from 0.1 to 20 mM, and concentrations greater than 25 mM caused toxicity (data not shown). Dietary histidine further increased the tolerance of haly-1(am130) and haly-1(am132) mutant animals to high levels of dietary zinc (Figure 6B, 6C).

To evaluate the specificity of the protection provided by feeding histidine, we analyzed the remaining amino acids by culturing wild-type hermaphrodites with 0.1 mM amino acid and 0.3 mM zinc. Histidine provided the most dramatic protection: 42% of animals grew to adulthood over 7 days when cultured with histidine compared to 8% when cultured with no amino acid (Table 1). Cysteine (21%) also provided significant, but lower levels of protection, whereas the other 18 amino acids did not provide significant levels of protection (Table 1). These results demonstrate that only a small number of amino acids provide protection against zinc toxicity and histidine is the most effective.

In the feeding experiments described above, histidine and zinc were both placed in the culture media. Therefore, these molecules have the opportunity to interact outside the animal and/or inside the animal after ingestion. To determine if histidine acts externally to the worms to provide zinc protection, we used a modified feeding procedure where worms were first exposed to histidine in the absence of zinc, and then exposed to zinc in the absence of histidine. If histidine acts externally to promote zinc tolerance, then animals subjected to this procedure are predicted to be zinc sensitive. By contrast, if histidine acts internally, then animals that are pre-treated with histidine are predicted to be resistant to a subsequent zinc challenge. Figure 6E shows that pre-treatment with histidine provided significant protection to wild-type animals that were challenged with 0.4 mM zinc. These findings indicate that ingested histidine is sufficient to protect against zinc toxicity.

haly-1 mutant animals and wild-type animals fed a high histidine diet both display enhanced zinc tolerance, indicating that elevated histidine causes zinc tolerance. One possible mechanism is that histidine directly binds zinc and reduces its toxicity to the animal. This possibility is consistent with the fact that free histidine and histidine in proteins display high affinity interactions with zinc [37,38]. A second possible mechanism is that elevated levels of histidine trigger a biological response that promotes zinc tolerance; for example, a transcriptional response. To investigate these possibilities, we analyzed the effects of D-
L- and D-histidine have identical chemical properties, such as pKa and binding affinity for zinc. However, L-histidine is utilized by biological systems for protein synthesis and other enzymatic reactions, whereas the enantiomer D-histidine is not recognized by enzymes or incorporated into proteins. If L-histidine protects against zinc toxicity by directly binding to zinc, then D-histidine is predicted to provide similar protection. By contrast, if L-histidine protects against zinc toxicity by initiating a biological response, then D-histidine is predicted to be inactive in promoting zinc tolerance.

We compared wild-type animals cultured on NAMM plates with L- and D-histidine; both enantiomers caused similar levels of tolerance to zinc toxicity (Table 1). These results indicate that L-histidine promotes zinc tolerance by directly binding zinc.

**haly-1** mutant animals were resistant to nickel toxicity

Our results indicate that **haly-1**(am130) mutant animals have elevated levels of histidine that protect against zinc toxicity. If histidine binding to zinc is the mechanism of protection, then **haly-1** mutant animals might be resistant to additional metals that can bind to histidine. Nickel binds to histidine [39], as demonstrated by the use of nickel affinity chromatography to purify proteins containing a multi-histidine epitope tag [40]. To determine whether **haly-1** mutant animals are resistant to nickel, we established the dose response of wild-type animals cultured in CeMM with either 0, 0.075 or 1.5 mM zinc. Total histidine content was determined by amino acid analyzer and calculated in nmoles L-histidine per µg protein. The histidine values for wild-type and mutant strains were not significantly affected by the level of dietary zinc (data not shown), and bars indicate mean values ± SE of six independent experiments comprised of two samples at each concentration of dietary zinc. Values for wild type were significantly different than values for **haly-1**(am130) (p = 0.0009) and **haly-1**(am132) (p = 0.019), whereas values for the two mutant strains were not significantly different (p = 0.22). (E) Wild-type and **haly-1**(am132) hermaphrodites were synchronized at the L1 stage, cultured on NAMM supplemented with 1 mM L-histidine (+) or 0 mM L-histidine (−) and no supplemental zinc for 24 hours, washed extensively, and then cultured on NAMM supplemented with 0 mM or 0.4 mM zinc and 0 mM L-histidine for six days. The fraction of worms that grew to adulthood over seven days was monitored. Bars indicate mean value ± SE (n = 4 replicates with 50 worms per replicate). At 0.4 mM supplemental zinc, values for wild-type worms pre-treated with L-histidine were significantly higher than values for worms that were not pre-treated (p = 0.022). Values for **haly-1**(am132) animals pre-treated with L-histidine were higher than values for animals that were not pre-treated (p = 0.06). doi:10.1371/journal.pgen.1002013.g006

**Figure 6. Histidine is elevated in **haly-1** mutant animals, and dietary histidine promotes zinc tolerance.** Wild-type (A), **haly-1**(am130) (B) and **haly-1**(am132) (C) hermaphrodites were synchronized at the L1 stage and cultured on NAMM supplemented with zinc (mM). Closed symbols indicate culture without histidine, and open symbols indicate culture with 0.1 mM histidine. The fraction of worms that grew to adulthood over seven days was monitored; the data were normalized by setting the value at 0 mM supplemental zinc equal to 1.0. Each point indicates mean value ± SE (n = 4 replicates with 50 worms per replicate). The fraction adult for WT + His was significantly higher than WT at 0.15 mM and higher concentrations of supplemental zinc (p < 0.0005). The fraction adult for **haly-1**(am130) + His was significantly higher than **haly-1**(am130) at 0.25 mM and higher concentrations of supplemental zinc (p < 0.03). The fraction adult for **haly-1**(am132) + His was significantly higher than **haly-1**(am132) at 0.25 mM and higher concentrations of supplemental zinc (p < 0.005). (D) Populations of animals consisting of a mixture of developmental stages were cultured in CeMM with either 0, 0.075 or 1.5 mM zinc. Total histidine content was determined by amino acid analyzer and calculated in nmoles L-histidine per µg protein. The histidine values for wild-type and mutant strains were not significantly affected by the level of dietary zinc (data not shown), and bars indicate mean values ± SE of six independent experiments comprised of two samples at each concentration of dietary zinc. Values for wild type were significantly different than values for **haly-1**(am130) (p = 0.0009) and **haly-1**(am132) (p = 0.019), whereas values for the two mutant strains were not significantly different (p = 0.22). (E) Wild-type and **haly-1**(am132) hermaphrodites were synchronized at the L1 stage, cultured on NAMM supplemented with 1 mM L-histidine (+) or 0 mM L-histidine (−) and no supplemental zinc for 24 hours, washed extensively, and then cultured on NAMM supplemented with 0 mM or 0.4 mM zinc and 0 mM L-histidine for six days. The fraction of worms that grew to adulthood over seven days was monitored. Bars indicate mean value ± SE (n = 4 replicates with 50 worms per replicate). At 0.4 mM supplemental zinc, values for wild-type worms pre-treated with L-histidine were significantly higher than values for worms that were not pre-treated (p = 0.022). Values for **haly-1**(am132) animals pre-treated with L-histidine were higher than values for animals that were not pre-treated (p = 0.06). doi:10.1371/journal.pgen.1002013.g006

Histidine Protects Against Excess Zinc and Nickel

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nickel toxicity compared to wild-type animals (Figure 7A). To investigate the specificity of haly-1 resistance to transition metals, we cultured wild-type and haly-1(am132) mutant animals on NAMM supplemented with iron, copper, cobalt, selenium, manganese, or cadmium. haly-1 mutant animals and wild-type animals displayed similar dose responses to selenium and cadmium (Figure S1). haly-1 mutant animals were slightly resistant to copper, slightly sensitive to cobalt and iron, and substantially sensitive to manganese compared to wild-type animals (Figure S1). These results demonstrate that haly-1 mutant animals are specifically resistant to a subset of transition metals including zinc and nickel, supporting the model that elevated histidine binds these metals to promote tolerance.

To test the hypothesis that elevated levels of histidine cause the nickel resistance displayed by haly-1 mutant animals, we analyzed the effect of dietary histidine on nickel toxicity. Wild-type animals cultured with 0.1 mM histidine displayed striking resistance to nickel toxicity (Figure 7B). These findings indicate that elevated levels of histidine promote tolerance to dietary nickel.

Discussion

C. elegans haly-1 has a conserved function in histidine metabolism and modulates zinc tolerance

Histidine levels in animals are regulated by dietary intake and excretion and the activity of catabolic enzymes. Histidine is an essential amino acid in animals that is obtained from the diet [41]. For example, the fully-defined CeMM used to culture C. elegans includes L-histidine [33]. Although animals cannot synthesize histidine, they have catabolic enzymes. Histidine ammonia lyase was identified as an enzyme that converts L-histidine to urocanic acid, and HAL is the first enzyme in the catabolism of L-histidine. Other enzymes that modify L-histidine include histidine decarboxylase, histidyl-tRNA synthetase, and 1-methyl transferase [42]. These findings demonstrate the central role of HAL in histidine metabolism.

We used genetic analysis to characterize the function of the C. elegans haly-1 gene, which has not been previously characterized. C. elegans HALY-1 protein displays a high level of identity with nickel toxicity compared to wild-type animals (Figure 7A). To investigate the specificity of haly-1 resistance to transition metals, we cultured wild-type and haly-1(am132) mutant animals on NAMM supplemented with iron, copper, cobalt, selenium, manganese, or cadmium. haly-1 mutant animals and wild-type animals displayed similar dose responses to selenium and cadmium (Figure S1). haly-1 mutant animals were slightly resistant to copper, slightly sensitive to cobalt and iron, and substantially sensitive to manganese compared to wild-type animals (Figure S1). These results demonstrate that haly-1 mutant animals are specifically resistant to a subset of transition metals including zinc and nickel, supporting the model that elevated histidine binds these metals to promote tolerance.

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Table 1. Supplementation with amino acids.

| Amino Acid | Fraction Adult (%) |
|------------|-------------------|
| None       | 8 ± 0.2           |
| L-histidine| 42 ± 11           |
| D-histidine| 40 ± 3            |
| Cysteine   | 21 ± 2            |
| Threonine  | 19 ± 1.0          |
| Tryptophan | 17 ± 4            |
| Proline    | 13 ± 4            |
| Aspartic Acid | 8 ± 0.2   |
| Tyrosine   | 8 ± 6             |
| Asparagine | 6 ± 0             |
| Methionine | 6 ± 4             |
| Serine     | 6 ± 6             |
| Valine     | 4 ± 2             |
| Glutamic Acid | 2 ± 2        |
| Glutamine  | 2 ± 2             |
| Lysine     | 2 ± 2             |
| Alanine    | 0 ± 0             |
| Arginine   | 0 ± 0             |
| Glycine    | 0 ± 0             |
| Isoleucine | 0 ± 0             |
| Leucine    | 0 ± 0             |
| Phenylalanine | 0 ± 0     |

1 Amino Acid. Wild-type hermaphrodites were synchronized at the L1 stage and cultured on NAMM supplemented with 0.3 mM zinc and 0.1 mM amino acid or no amino acid as a control (none).

2 Fraction Adult. The fraction of worms that grew to adulthood over seven days was monitored. Numbers indicate mean value ± SE (n = 3 replicates with 50 worms per replicate).

*, values greater than none (p<0.05).

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Figure 7. haly-1 mutations and dietary histidine cause resistance to dietary nickel. (A) Wild-type, haly-1(am130), and haly-1(am132) hermaphrodites were synchronized at the L1 stage and cultured on NAMM supplemented with nickel (mM). The fraction of worms that grew to adulthood over seven days was monitored; the data were normalized by setting the value at 0 mM supplemental nickel equal to 1.0. Each point indicates mean value ± SE (n = 6 replicates with 50 worms per replicate). The fraction adult for haly-1(am130) and haly-1(am132) was significantly higher than wild type at 0.02–0.06 mM supplemental nickel (p<0.01). (B) Wild-type animals were cultured with no histidine (closed circles) or with 0.1 mM L-histidine (open circles). Each point indicates mean value ± SE (n = 6 replicates with 50 worms per replicate). The fraction adult for WT + His was significantly higher than WT at 0.02 mM and higher concentrations of supplemental nickel (p<0.01).

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Histidine Protects Against Excess Zinc and Nickel

Histidine chelates zinc and protects against zinc toxicity

The analysis of haly-1 suggests a model for the role of histidine in zinc biology in animals. We propose that elevated levels of histidine in haly-1 mutant animals chelate zinc and protect against zinc toxicity. This model predicts that dietary administration of histidine to wild-type animals can phenocopy the haly-1(mu) mutant and protect against zinc toxicity. Our results confirmed this prediction and demonstrated that dietary histidine acts inside the animals to promote zinc tolerance. Histidine has been demonstrated to bind zinc, suggesting that elevated histidine acts by direct chelation [50]. An alternative possibility is that elevated histidine triggers a biological response that promotes zinc tolerance. We used two approaches to test these possibilities. First, we demonstrated that dietary supplementation with D- and L-histidine protected against zinc toxicity. Since D-histidine has the same chemical properties as L-histidine, but lacks biological activity, these findings suggest that L- and D-histidine act by directly chelating zinc. Second, we analyzed the specificity of the protective effects and demonstrated that haly-1 mutant animals are strongly resistant to zinc and nickel, but not other metals. Like zinc, nickel binds histidine with high affinity [39], and these results suggest that elevated histidine in haly-1 mutant animals directly chelates nickel to protect against nickel toxicity. Furthermore, dietary histidine protected against nickel toxicity, consistent with the chelation model. haly-1 mutant animals were sensitive to excess dietary manganese, indicating that abnormalities in histidine metabolism can be deleterious and result in susceptibility to some stresses. The response of haly-1 mutant animals to other metals such as copper might be a combination of protection mediated by histidine chelation of the metal and susceptibility caused by abnormal histidine metabolism.

The effects of dietary histidine supplementation have been analyzed in vertebrates. In humans and rats, dietary supplementation with histidine increases urinary excretion of histidine and zinc, and in some cases is associated with symptoms of zinc deficiency [51,52,53]. These results are consistent with the model that elevated levels of histidine promote chelation of zinc and document the relevance of the studies of C. elegans to vertebrate biology.

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Materials and Methods

General methods and strains

*C. elegans* strains were cultured at 20°C on nematode growth medium (NGM) seeded with *E. coli* OP50 unless otherwise noted [66]. The wild-type *C. elegans* and parent of all mutant strains was Bristol N2. The following mutations were used: *haly-1(am130)* and *haly-1(am132)* [28], *cdf-1(n2527)* [22], *cdf-2(tm228)* [29], *sur-7(ku119)* [27], *dpy-6(e14)* [67] and *egl-15(n484)* [67]. Double mutant animals were generated by standard methods, and genotypes were confirmed by PCR or DNA sequencing.

Determining the effects of metals and amino acids on hermaphrodites cultured on NAMM

To make NAMM, we prepared a solution with 1.7% Noble agar (U.S. Biological, Swampscott, MA) and a final concentration of 5 mg/liter cholesterol using a stock solution of 5 mg/ml cholesterol in 100% ethanol using water from a Milli-Q synthesis A10 machine (Millipore, Billerica, MA). The solution was autoclaved for 30 minutes – autoclave times greater than 45 minutes impaired solidification. Metals such as zinc chloride, nickel chloride, sodium selenite, cadmium chloride, cobalt (II) sulfite hexahydrate, copper chloride, ammonium iron (II) sulfate hexahydrate or manganese chloride tetrahydrate (Sigma-Aldrich, St. Louis, MO) were added to yield the desired final concentrations, and 7 ml of molten agar was immediately dispensed to 6 cm Petri dishes. NAMM was allowed to harden overnight at room temperature. *E. coli* OP50 was grown overnight in LB, concentrated ten-fold in Milli-Q water, and 100 μl was dispensed to each dish. To make NAMM supplemented with L- and D-amino acids (Sigma-Aldrich, St. Louis, MO), amino acids were added to the molten agar and dispensed to Petri dishes.

To analyze the response of worms to dietary metals and/or amino acids, we generated a population of hermaphrodites cultured on NGM plates, treated the animals with alkaline hypochlorite to obtain eggs, and cultured the eggs in M9 media cultured on NGM plates, treated the animals with alkaline hypochlorite to obtain eggs, and cultured the eggs in M9 media cultured on NGM plates, treated the animals with alkaline hypochlorite to obtain eggs, and cultured the eggs in M9. To obtain arrested first larval stage (L1) animals, L1 animals were pipetted onto each NAMM dish, counted and cultured overnight to obtain arrested first larval stage (L1) animals. L1 animals were placed on NAMM with or without supplemental amino acids for 24 hours, washed, then transferred to NAMM supplemented with zinc. To analyze amino acids in the intestinal lumen, we washed the worms three times in M9, incubated the worms for thirty minutes in M9 with 1 mM serotonin to stimulate pharyngeal pumping and defecation, then washed the worms two additional times in M9.

Positioning *am132* using a high-resolution local SNP map

*dpy-6(e14) haly-1(am132)* hermaphrodites were crossed to males of the wild isolate CB4856 that contains multiple polymorphisms compared to N2, F1 cross progeny were selected, and 18 F2 self-progeny were selected as non-Dpy hermaphrodites that displayed zinc resistance. Similarly, *haly-1(am132) egl-15(n484)* hermaphrodites were crossed to CB4856 males, F1 cross progeny were selected, and one F2 self-progeny was selected as a non-Egl hermaphrodite that displayed zinc resistance. Hermaphrodites homozygous for the recombinant chromosomes were selected and used to prepare genomic DNA. Nine SNP markers positioned on chromosome X between *dpy-6* at position 0.0 and *egl-15* at position +2.86 were analyzed. The most informative non-Dpy zinc resistant recombinant contained the CB4856 SNP marker *CE6-177* at position +2.40, indicating that *am132* is positioned to the right of this marker. The non-Egl zinc resistant recombinant contained the CB4856 SNP marker *CE6-1202* at position +2.94, indicating that *am132* is positioned to the left of this marker.

Determining the genomic sequence of *am132* mutant animals

DNA was isolated from mixed stage animals grown on NGM plates using the Purification of Total DNA from Animal Tissues Spin Column Protocol from the DNeasy Blood and Tissue Kit (Qiagen) with minor modifications. The gDNA was fragmented by sonication and used to generate Illumina random whole genome sequencing libraries consisting of two size fractions, 250-300 bp and 350–400 bp. The libraries were amplified *in situ* on Illumina flow cells according to the manufacturer’s protocol, and sequence data consisting of 50 bp reads were obtained using the Solexa/Illumina platform [32]. The number of short DNA sequences that were determined corresponds to approximately 30-fold coverage of the *C. elegans* genome. Sequences that corresponded to the 270 kb mapping interval for the *am132* mutation were aligned to the reference wild-type *C. elegans* sequence using the maq utility [http://maq.sourceforge.net/]; read-depth $> = 3$; mapping quality $> 40$, consensus quality $> = 15$. 251 candidate base changes were identified in the 270 kb interval. The majority of these candidate base changes were identified with low confidence, indicating they were likely to be sequencing errors. All candidate base changes were analyzed by determining the effect on predicted open reading frames. Only one candidate base change was predicted to cause a nonsense mutation, and this candidate base change was identified with high confidence. The presence of this candidate base change in the *am132* strain was confirmed by conventional DNA sequencing, and other candidate base changes were not further analyzed.

DNA sequencing

We prepared DNA using standard methods, determined the sequence using Applied Biosystems 3730 and/or 3130xl DNA sequencers, and analyzed data using Sequencher (Gene Codes Corporation, Ann Arbor, MI). To determine the DNA sequence of the *haly-1* locus, we analyzed from 75 bp upstream of the predicted START codon to 100 bp downstream of the predicted STOP codon.

Plasmid construction and generation of transgenic animals

Plasmid pJM1 is pBlueScript SK+ (Stratagene) containing a 4,318 bp fragment of *C. elegans* genomic DNA from fosmid WRM0624bE06. The fragment extends from 1,567 bp upstream of the predicted *haly-1* START codon to 114 bp downstream of the predicted STOP codon. To generate pJM2, we modified pJM1 by digestion with Ncol (New England Biolabs) and religation, resulting in the deletion of 1542 bp that removes *haly-1* exons four through ten. To generate pJM3, we performed site-directed mutagenesis using PCR-mediated overlap extension [68] to generate a mutation in exon six that changes codon 296 from translational STOP to STOP.

Transgenic animals were generated by co-injecting fosmids with the transformation marker pEL123 that has homology to the fosmid backbone and expresses GFP or co-injecting plasmids with the dominant transformation marker pRF4 [69]. *haly-1(am132)* hermaphrodites were injected, and we selected Rol or GFP self-progeny and then selected strains that transmitted the marker. For
each of these strains, the Rol or GFP phenotype was transmitted to only a sub-set of self-progeny, indicating that these transgenes were extrachromosomal. We defined rescue as a significant difference in zinc sensitivity between F1 progeny that displayed the marker phenotype compared to F1 progeny that did not display the marker phenotype and were presumed to lack the extrachromosomal array (P<0.05 Student T-Test).

haly-1 RNA analysis

We analyzed six haly-1 ESTs obtained from the National Institutes of Genetics, Japan (yk1370x8, yk1228g10, yk1325f01, yk1035h03, yk1086a05 and yk1286b1). We determined the complete DNA sequence of the inserts using standard techniques. A poly A tail began 104 bp from the stop codon in three cDNAs and 125 bp from the stop codon in two cDNAs. None of the cDNAs contained a spliced leader sequence, indicating that they did not include the 5’ end.

haly-1 mRNA levels were analyzed as described by Davis et al. with minor modifications [29]. Briefly, wild-type worms were cultured in CeMM containing 10 μM or 500 μM zinc chloride for six days. The COPAS Biosort was used to collect 1000 adult animals for RNA preparation. Quantitative real-time PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System and the Applied Biosystems SYBR Green Master Mix. Forward and reverse amplification primers for haly-1 were cttattcagcttgtggccaag and caacgcttgcagcgacaatgatg, respectively.

Metal content analysis by inductively coupled plasma-mass spectrometry (ICP-MS)

We obtained a large population of animals by culturing worms in CeMM with 0.075 mM zinc. The worms were placed in 75 cm² flasks containing 7.5 mL CeMM at a concentration of 10,000 worms per mL. Then, 7.5 mL of CeMM containing zinc was added to make a final volume of 15 mL. The worms were cultured at 20°C for 18 days. Worms were washed three times in magnesium-free M9 solution, incubated in 1 mM serotonin in Mg-free M9 solution for 30 minutes, washed twice in Mg-free M9 solution, transferred to pre-weighed tubes (Stockwell Scientific, part #3220N) and immediately frozen at −80°C. Serotonin stimulates pharyngeal pumping and defecation, and the incubation with serotonin improves the accuracy of the measurement of zinc content by promoting exchange of zinc-containing culture medium in the intestinal lumen with Mg-free M9 solution [29]. The metal content was determined using ICP-MS [29]. Samples were freeze-dried, reweighed to obtain the dry pellet weight, and digested by heating in a hot block digester at 90°C for 1.5 h with concentrated nitric acid (HNO₃) and hydrogen peroxide (H₂O₂) solution. The solution was diluted to a volume of approximately 10 mL with deionized water, and internal standards were added to correct for matrix effects. Instrument calibration standards were prepared from multi-element stock solutions (High-Purity Standards, Charleston, SC) to generate a linear calibration curve, and samples were analyzed using a VG Axiom high-resolution ICP-MS (Thermo Fisher Scientific). Blank tubes were included in all processes as a control. The content of zinc, iron, copper, magnesium and manganese was determined as a value in parts-per-million (ppm or μg/g) by dividing measured metal content by the dry pellet weight.

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We obtained a large population of animals as described above by culturing worms in CeMM with 0.075 mM zinc and then transferring worms to CeMM containing 0 mM, 0.075 mM or 1.5 mM zinc. The worms were cultured at 20°C for 7 days, then prepared for analysis. A whole worm extract was made by sonicating worms in a 20 mM HEPES buffer pH 7.55 using a Branson Digital Sonifier with a Model 102G CE Converter (Danbury, CT). The extract was placed on ice and frozen at −80°C. The protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions and calibrated to a standard curve using bovine serum albumin. Total amino acid content was measured using a Hewlett Packard Amino Quant II System by the Protein Chemistry Laboratory at Texas A&M University.

Statistical analysis

To compare two sets of values, we used the Student’s T Test when the number of samples was greater than two and the Fisher’s Exact T Test when there were two samples (Microsoft Excel, Seattle WA).

Supporting Information

Figure S1  haly-1(0f) causes resistance to specific metals. Wild-type (blue circles) and haly-1(am132) (red diamonds) hermaphrodites were synchronized at the L1 stage and cultured on NAMM supplemented with (A) cadmium (mM), (B) iron (mM), (C) cobalt (mM), (D) manganese (mM), (E) copper (mM) or (F) selenium (mM). The fraction of worms that grew to adulthood over seven days was monitored; the data were normalized by setting the value at 0 mM supplemental metal equal to 1.0. Each point indicates mean value ± SE (n = 4 replicates with 50 worms per replicate). The fraction adult for haly-1(am132) was significantly different than wild type at the following concentrations (mM) (p<0.05): cadmium (none), iron (0.53, 0.60), cobalt (0.09, 0.46), manganese (0.05, 0.05) and selenium (none).

Table S1  Total content of zinc, magnesium, manganese, iron and copper was determined by ICP-MS for wild-type, haly-1(lf), haly-1(am130) and haly-1(am132) animals cultured in CeMM with 0.075 or 1.0 mM zinc.

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Author Contributions

Conceived and designed the experiments: JTM JJB DLS SC JG. Analyzed the data: JTM JJB AC KK. Wrote the paper: JTM KK.
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