

Transcriptome response to heavy metal stress in *Drosophila* reveals a new zinc transporter that confers resistance to zinc

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ABSTRACT

All organisms are confronted with external variations in trace element abundance. To elucidate the mechanisms that maintain metal homeostasis and protect against heavy metal stress, we have determined the transcriptome responses in *Drosophila* to sublethal doses of cadmium, zinc, copper, as well as to copper depletion. Furthermore, we analyzed the transcriptome of a metal-responsive transcription factor (MTF-1) null mutant. The gene family encoding metallothioneins, and the ABC transporter *CG10505* that encodes a homolog of 'yeast cadmium factor' were induced by all three metals. Zinc and cadmium responses have similar features: genes upregulated by both metals include those for glutathione *S*-transferases *GstD2* and *GstD5*, and for zinc transporter-like proteins designated *ZnT35C* and *ZnT63C*. Several of the metal-induced genes that emerged in our study are regulated by the transcription factor MTF-1. mRNA studies in MTF-1 overexpressing or null mutant flies and *in silico* search for metal response elements (binding sites for MTF-1) confirmed novel MTF-1 regulated genes such as ferritins, the ABC transporter *CG10505* and the zinc transporter *ZnT35C*. The latter was analyzed in most detail; biochemical and genetic approaches, including targeted mutation, indicate that *ZnT35C* is involved in cellular and organismal zinc efflux and plays a major role in zinc detoxification.

INTRODUCTION

Metal ions are vital for many biological processes, such as transcription, respiration and growth. However, overaccumulation of essential metals such as copper and zinc or of

non-essential toxic metals like cadmium and mercury is detrimental. Copper, for instance, can catalyze the generation of aggressive reactive oxygen species (ROS) via the so-called Fenton reaction (1). Zinc is not redox-active under physiological concentrations and is less toxic than copper, but at high concentrations zinc may bind to inappropriate sites in proteins or cofactors and interfere with their functions. Cadmium does not undergo redox changes, nonetheless, it depletes antioxidant components, notably glutathione, which results in enhanced ROS production (2). Genetic disorders or gross environmental fluctuations can distort metal homeostasis and may lead to a number of deficiency/overload diseases. Copper imbalance is the cause of serious diseases, such as Menkes syndrome and Wilson disease, and has also been implicated in common neurologic conditions, like Alzheimer's disease, Parkinson's disease and the prion-type diseases (3,4). Zinc deficiency can lead to severe syndromes, notably acrodermatitis enteropathica, which is characterized by a decreased absorption of ingested zinc, due to a defective zinc importer (5). Zinc toxicity is associated with reduced iron absorption, impaired immune function (6) and neuronal death (7,8). Cadmium, unlike copper or zinc, is a non-essential metal and is toxic even at very low concentrations. A classic example of industrial cadmium pollution is Itai-Itai disease which occurred in Japan several decades ago (9).

As misregulation of copper and zinc homeostasis can lead to disease and cadmium poses a significant health risk to humans and animals, there is considerable interest in understanding how the organism can defend itself against an excess of a particular metal, yet provide all tissues with appropriate amounts of metals under limiting conditions. The fruit fly *Drosophila* is a convenient system to address these questions since many aspects of metal homeostasis are conserved between flies and humans. In recent years numerous studies led to a better understanding of uptake, distribution, detoxification and elimination of metal ions. For copper, the Ctr transporter family mediates cellular uptake and three high-affinity copper importers, Ctr1A, Ctr1B and Ctr1C, are

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known in *Drosophila* (10). Membrane-bound P-type ATPase transporters are responsible for cellular copper efflux. In mammals, two of them, ATP7A and ATP7B, are implicated in Menkes and Wilson diseases and DmATP7, their common ortholog in *Drosophila*, was recently characterized (11–16). Zinc transport is mediated via two families of solute linked carrier (SLC) proteins: SLC39A, also referred to as ZIPs, that function in the uptake of zinc into the cytoplasm, and SLC30A, also called ZnTs, that reduce cytoplasmic zinc concentrations by promoting zinc efflux to the exterior or into intracellular vesicles (17). More than 10 zinc transporter genes are annotated in *Drosophila melanogaster* based on sequence similarities to vertebrate zinc transporters. The ZIP family gene *foi* (fear of intimacy) was characterized in *Drosophila* and shown to be a zinc importer (18,19). Along with metal transport, an important aspect of heavy metal homeostasis is the sequestration of metals by metallothioneins (MTs). MTs are small, cysteine-rich proteins with outstanding metal binding capacity. The fly genome encodes four MTs termed MtnA to -D (20–22).

MT gene expression is dependent on the metal-responsive transcription factor-1 (MTF-1), a key regulator of heavy metal homeostasis and detoxification in higher eukaryotes from insects to mammals (23–25). For transcriptional activation, MTF-1 binds through its zinc fingers to metal response elements (MREs) in the promoter/enhancer regions of the target genes. MRE motifs have a core consensus TGCRCNC (where R stands for A or G and N for any of the bases). In mammals, MTF-1 is essential for liver development as mice lacking MTF-1 die *in utero* due to liver degeneration (26). In contrast, MTF-1 knockout flies (MTF-1^{140-1R}, hereafter termed MTF-1 KO) are viable under laboratory conditions but very sensitive to elevated concentrations of heavy metals, and also to copper scarcity (22). Besides MTs, mammalian MTF-1 contributes to the expression of ZnT1, tear albumin/lipocalin, placenta growth factor, selenoprotein W, N-myc downstream regulated gene 1 (*ndrg1*), cysteine and glycine-rich protein 1 (*csr1*), cEBP alpha, Kruppel-like factor 4 (*klf4*) and hepatitis A virus cellular receptor 1 (27–31). In *Drosophila*, the best characterized MTF-1 target genes are metallothioneins. Recently, the copper importer *Ctr1B* was shown to be induced in an MTF-1-dependent manner upon copper starvation (32). However the knockout of the *Drosophila* MT family or of *Ctr1B* did not reproduce the whole spectrum of the MTF-1 mutant phenotype (10,33). Notably, neither absence of metallothioneins nor *Ctr1B* absence has an appreciable effect on the fly's zinc sensitivity, suggesting the existence of other MTF-1 target genes at least for zinc defense.

Here we present the results of a genome-wide search for *Drosophila* MTF-1 target genes using *in silico* and microarray-based approaches. Furthermore, we present a detailed analysis of transcriptional responses to cadmium, copper and zinc as well as to copper starvation in *Drosophila* larvae. Our study unveiled a great number of genes that respond to environmental metal fluctuations. It also revealed novel MTF-1 regulated genes, such as the ferritin heavy and light chain homologs, the ABC transporter *CG10505* that encodes a homolog of 'yeast cadmium factor' (YCF1) and a zinc transporter which we named *ZnT35C*. In this report, we characterize *ZnT35C*, a member of SLC30A family in

Drosophila and show that it is required for zinc tolerance in the fly.

MATERIALS AND METHODS

Fly food and RNA extraction

Animals were raised on standard cornmeal molasses-based food. In the third larval instar (fourth day of the development) the animals were transferred from normal to supplemented food containing either 0.05 mM CdCl₂, 0.5 mM CuSO₄, 5 mM ZnCl₂ or 5 mM FeCl₃. After 6 h of feeding on the supplemented food, RNA was extracted. For the treatment with the copper chelator BCS (bathocuproine disulfonic acid), animals were kept continuously in the chelator-containing food, since copper stores of a normally fed animal are not exhausted within a few hours. In BCS larval development is delayed, so the RNA was extracted on the fifth day after egg laying. To control for the handling of the larvae during transfer to supplemented food, the normal food controls and the larvae grown in BCS were also removed at third instar and transferred for the last 6 h to normal or BCS containing food, respectively. Total RNA was extracted using TRIzol reagent (Life Technologies).

S1 nuclease protection assay

Nuclease S1 protection assay was performed as described (34). The dried gels were exposed to storage phosphor screens and analyzed using a PhosphorImager (Molecular Dynamics).

GFP fusion protein expression analysis and microscopy

For ZnT35Ca-GFP fusion protein analysis, flies were allowed to deposit eggs in the food and raised until third instar larvae. Larvae were dissected and analyzed under a Leica DRB fluorescence stereomicroscope (Figure 7A) and a Leica TCS SP spectral confocal microscope (Figure 7B).

Metal measurements

Groups of ~15 adult male flies (2–3 days old, raised on the indicated metal concentrations from egg), were analyzed by inductively coupled plasma mass spectrometry (ICP-MS), as described previously (35). Flies were dissolved in 65% HNO₃ in a microwave oven and then diluted to a 6.5% HNO₃ solution for analysis. ICP-MS was performed using a HP4500 Series 300 ShieldTorch System instrument (Agilent, Waldbronn, Germany) in peak-hopping mode with spacing at 0.05 a.m.u., 3 points/peak, 5 scans/replicate, 2–3 replicates/sample and an integration time of 400 ms/point. The rate of plasma flow was 15 l/min with an auxiliary flow of 1.0 l/min. The RF power was 1.2 kW. The sample was introduced using a cross-flow nebulizer at a flow rate of 1.06 l/min. The apparatus was calibrated using a 6.5% HNO₃ solution containing Cu at 5, 10, 25, 50 and 100 p.p.b. with 103Rh, the internal standard for all isotopes of Cu.

Microarray

Microarray experiments were done in triplicates using a pool of RNA from at least 30 animals for each assay. cDNA was synthesized from larval total RNA with SuperScript reverse

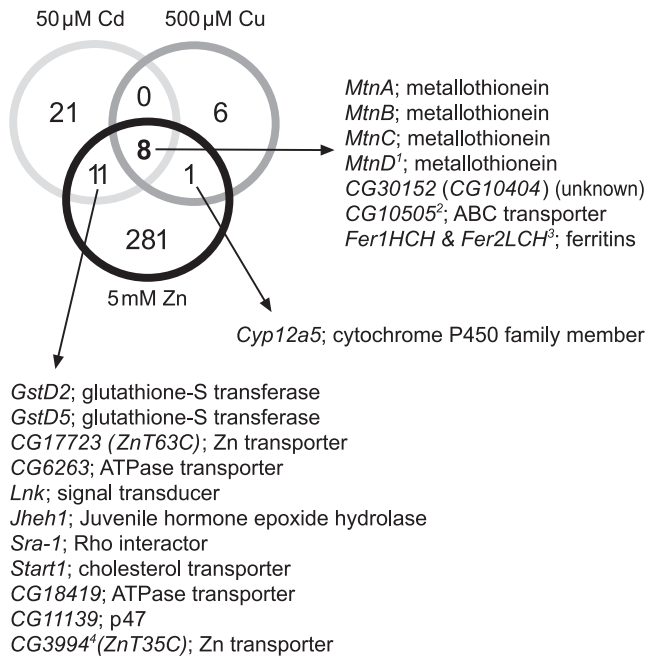


Figure 1. Venn diagram of genes induced at least 1.5-fold by 50 μ M Cd, 500 μ M Cu and 5 mM Zn (P -value \leq 0.05). The genes that are induced similarly in at least two metal treatments are shown. Superscript '1': one of the metallothionein genes, *MtnD*, was not represented in the *Drosophila* genome arrays; it was found to be induced by metals in an S1 nuclease transcript assay. Superscript '2': the ABC transporter *CG10505* was induced both by cadmium and zinc load in the microarray experiment. The S1 nuclease transcript assay shows an induction also in copper load. Superscript '3': ferritin genes *Fer1HCH* and *Fer2LCH* were induced by zinc in the microarray experiment. The S1 nuclease transcript assay revealed an induction also by cadmium and copper. Superscript '4': the significance for zinc induction of *CG3994* (zinc transporter-like gene) in the microarray did not pass our screening criteria; however, *CG3994* was shown to be induced by zinc in an S1 nuclease transcript assay.

subsequent quantification of *ZnT35C* transcripts in the S1 assay clearly showed induction by cadmium and zinc but not by copper treatments (Figure 2C). The genes encoding the ferritin light and heavy chain homologs (*Fer2LCH* and *Fer1HCH*) were 2- to 3-fold induced by zinc. These genes were also almost 2-fold induced by cadmium treatment, an induction that appeared however to be statistically less significant. Transcript quantification revealed a moderate yet significant increase in ferritin mRNAs by cadmium and copper treatments, and more than 2-fold induction by zinc or iron (Figure 2D). Cadmium also elevated the expression of the *Hsp22* gene in *Drosophila* larvae. This is consistent with the observed heat shock response induced by cadmium in yeast (44,45), mice (46), and various human cell lines (47,48) presumably due to protein misfolding caused by ectopic incorporation of cadmium.

In copper, following the metallothioneins, the transcripts coding for proteins involved in immune defense against microbial infection were induced significantly. The high-affinity copper importer *Ctr1B* was strongly downregulated in response to copper treatment [Supplementary Table 2, see also Refs (10,32)]. The other copper transporters *Ctr1A* and *Ctr1C* showed no changes in their mRNA levels. The transcriptome response to the 5-day-exposure to copper

chelator BCS yielded a great number of differentially expressed genes (Supplementary Table 4). Under conditions of copper deprivation, transcripts of the copper importer *Ctr1B* were increased several fold. Also, five cytochrome P450 genes were induced, while another four were down-regulated between 5.3- and 1.6-fold by BCS treatment. It is known that P450 enzymes have a wide spectrum of enzymatic activities; they are able to metabolize many xenobiotic as well as cellular compounds such that they serve as direct GST substrates (49). As reduced copper levels likely affect copper containing proteins, scarcity of copper might result in oxidative stress due to compromised Cu-Zn SOD (superoxide dismutase) activity. In line with this, we observed an \sim 7-fold upregulation in *GstD5* expression. Furthermore, metallothioneins were significantly downregulated by Cu depletion, in agreement with their scavenger function upon metal load (Supplementary Table 4 and Figure 2E).

Transcriptional profile of MTF-1 mutants

Since the MRE-binding transcription factor MTF-1 is a major player in the transcriptional response to heavy metals in mammals, we reasoned that at least some differentially expressed genes as determined by our microarray experiments are regulated by the *Drosophila* homolog of MTF-1. Moreover, several genes that emerged in our study do harbor MREs in their promoter regions, indicating a possible role for MTF-1 in their regulation, as was recently shown for the copper transporter *Ctr1B* (32). To find the genes regulated by MTF-1 we have conducted microarray experiments with RNA from MTF-1 KO and wt larvae kept in NF or transferred to 50 μ M cadmium or 500 μ M copper containing food for 6 h during the third instar. MTF-1 target genes are expected to lose their metal regulation in the MTF-1 mutants or also to be downregulated in the mutants compared to the wt in the non-supplemented food.

In MTF-1 KO *Drosophila*, 43 genes were downregulated >2 -fold (P -value cutoff 0.05) (Table 1 and Supplementary Table 5). As expected, the transcripts of metallothioneins were absent in the knockout animal and remained undetectable after metal treatment. *CG4716*, a gene of predicted methylene-tetrahydrofolate dehydrogenase activity, was 8.7-fold downregulated in the MTF-1 mutant. This gene is also zinc-inducible (Table 1) and was found to be upregulated with increasing age in *Drosophila* (50). It harbors a single MRE 63 bp upstream of the transcription start. Both the copper importer *Ctr1B* and zinc exporter-like *ZnT35C* were strongly downregulated in the MTF-1 KO. Two triacylglycerol lipases, *CG5966* and *CG6283*, were downregulated at least 4-fold in MTF-1 mutants, and another lipase-like gene, *CG17191*, was more than 2-fold downregulated (Supplementary Table 5). Interestingly, the transcripts of the heat shock proteins 23 and 67Bb were also reduced in the KO, in line with the role of MTF-1 in cell stress response. Furthermore, mRNA levels of several structural proteins, TFs and carrier proteins were downregulated, however only 2- to 3-fold. Not unexpectedly, several genes, such as metallothioneins and the zinc transporter-like *ZnT35C* that are affected by the absence of MTF-1, are also induced by metals in MTF-1 wt larvae (Table 1). In line with MTF-1 being the main regulator of these genes, their metal induction was

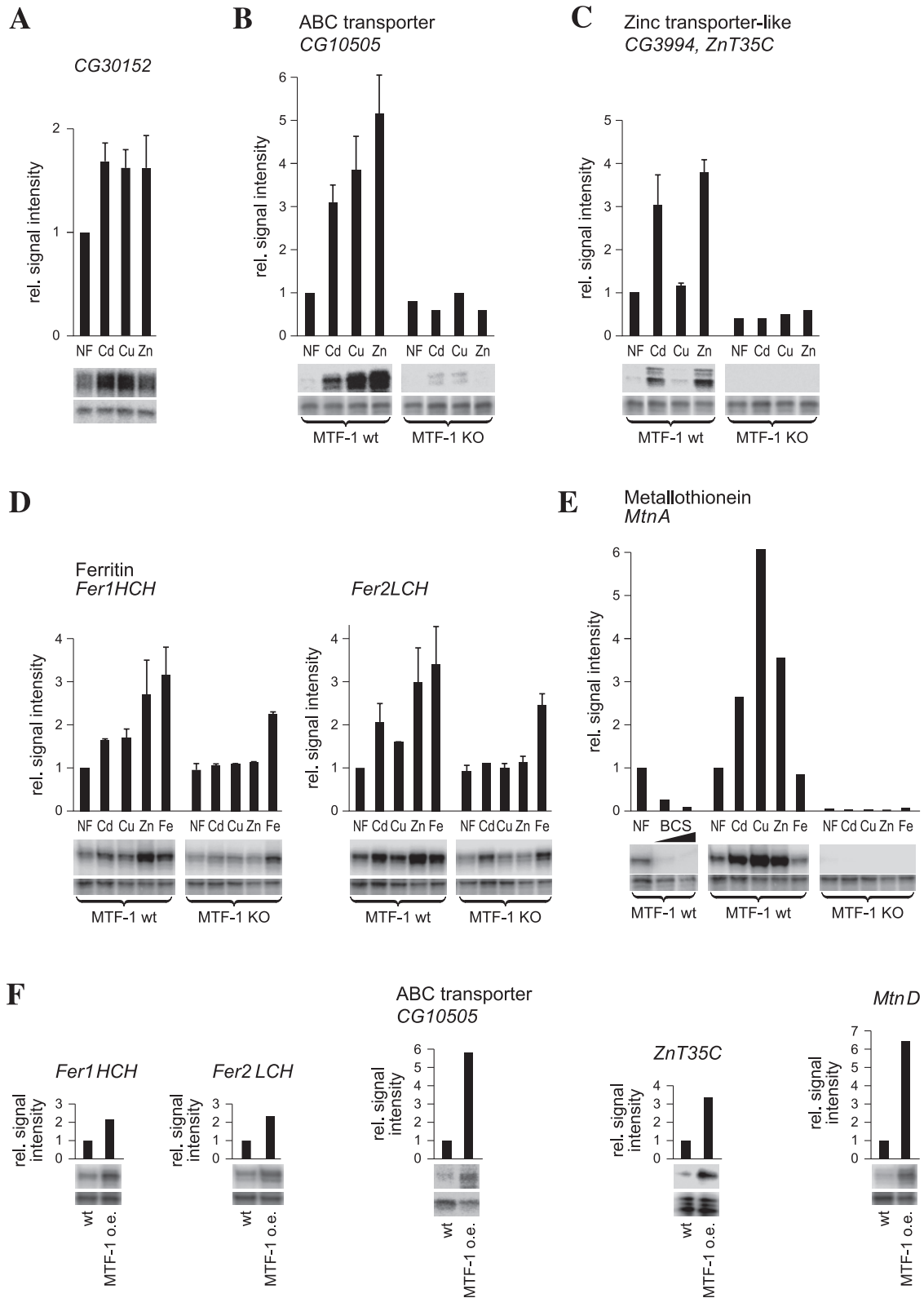


Figure 2. S1 nuclease protection assay for metal-induced genes. Transcripts of the indicated genes were determined at different food conditions: NF, normal food; Cd, 50 μ M CdCl₂; Cu, 0.5 mM CuSO₄; Zn, 5 mM ZnCl₂; Fe, 5 mM FeCl₃; and BCS, 0.3 mM (lower concentration) and 0.5 mM (higher concentration). Representative gels are shown below the bars. The lower panel of the gel indicates the expression of the reference gene: *tubulin85* for *CG30152* measurement and *actin5* for the rest. (A) Metal induction of *CG30152* (triplicate experiment) (B) ABC transporter *CG10505*, (C) zinc transporter *ZnT35C*, (D) ferritin genes *Fer1HCH* and *Fer2LCH* and (E) metallothionein (*MtnA*) gene transcripts from MTF-1 wt larvae (MTF-1 wt) or MTF-1 knockout larvae (MTF-1 KO). (F) Transcript response of ferritin genes, *CG10505*, *ZnT35C* and *MtnD* (positive control) to overexpression of MTF-1. MTF-1 o.e., overexpression of MTF-1 under the *tubulin* promoter.

Table 1. Genes at least 2.5-fold downregulated in MTF-1 KO (P -value ≤ 0.05)

Genes (functions) ^a	Gene expression in MTF-1 KO versus wt	MREs (1 kb upstream of transcription start)	Fold changes in the gene expression compared to normal food in wt flies fed with			
			500 μ M copper	50 μ M cadmium	5 mM zinc	500 μ M BCS (copper deficiency)
<i>MtnA</i> , metallothionein	-500	2	4	2.7	3	-25
<i>MtnC</i> , metallothionein	-143	5	6	13.2	16.6	-28
<i>CG4716</i>	-8.7	1	1.7	1.7	2.45	-1.4
<i>ZnT35C</i> (<i>CG3994</i>) zinc transporter	-6.4	2	-1.2	1.7	1.7	-1.7
<i>Ctr1B</i> , copper transporter	-5.7	4	-3.85	1.2	1	4.3
<i>CG6283</i> , triacylglycerol lipase	-4.9	—	-1.4	1.2	1.35	-14
<i>CG5966</i> , triacylglycerol lipase	-4	1	-1.1	-1.2	1.75	-1.2
<i>CG6910</i>	-4	1	-1.2	1	-1.1	-1.1
<i>CG18576</i>	-3.7	—	-1.2	-1.1	-1.2	-1.7
<i>kuzbanian</i> , metalloendopeptidase	-3.4	—	-1.7	1	-1.8	-2
<i>CG4840</i>	-3.2	2	-1.7	-1.4	-1.4	-3.3
<i>CG3264</i> , alkaline phosphatase	-3.15	—	-1.4	1	-1.1	-1.3
<i>Hsp67Bb</i> , heat shock protein	-2.9	6	1.1	1.2	1	-1.7
<i>Hsp23</i> , heat shock protein	-2.9	1	1.2	1.4	1.4	-4
<i>Mlp84B</i> , Muscle LIM protein	-2.8	1	-1.2	1	-1.1	-1.3
<i>CG3672</i> , structural protein	-2.75	1	-1.2	-1.4	1	-2
<i>CG17752</i> , organic cation transporter	-2.7	—	-1.1	-1.1	-2.5	1.2
<i>ultraspiracle</i> , transcription factor	-2.7	1	-3.3	-1.7	-1.7	-2.3
<i>CG11916</i> , transcription factor	-2.6	2	1	1.1	-1.35	-1.25

The expression in different metal concentrations is shown in positive (fold-upregulation) and negative (fold-downregulation) numbers (i.e. -2 means reduction to half).

^a*MtnB* is not shown, since the downregulation in the MTF-1 KO larvae in the microarray was considered not significant. The *MtnD* gene was not included in the Affymetrix arrays. However, in a previous study using MTF-1 KO flies we have demonstrated the MTF-1 dependence of all metallothioneins including *MtnB* and *D(22)*.

abolished in the MTF-1 KO (Figure 2C and E and data not shown).

The expression of the other metal inducible genes such as ferritins, ABC transporter *CG10505*, zinc transporter-like *ZnT63C*, *CG30152* or *Hsp22* was not significantly changed in the MTF-1 mutant larvae compared to the wt when both genotypes were raised on NF. However, in MTF-1 KO larvae we did not detect the metal-induced expression of ferritins, *CG30152*, the ABC and zinc transporters as we observed in wt larvae. Thus MTF-1 is required in the presence of excess metal to induce the expression of these genes but does not seem to be involved in their basal expression. However, unlike ferritins and the ABC transporter, *CG30152* and *ZnT63C* do not contain classical MREs in their putative gene-regulatory regions, suggesting rather an indirect regulation by MTF-1.

A peculiar group of genes comprise glutathione *S*-transferases D2 and D5. As noted before, these genes are strongly induced by cadmium and zinc. Curiously, when MTF-1 KO larvae were challenged with cadmium, the induction of both genes became more pronounced. Notably, in MTF-1 mutant animals, *GstD5* was 5-fold induced already in the normal food (Supplementary Table 5) suggesting a compensatory role of this gene for the loss of MTF-1 and an important function in anti-cadmium (and zinc) defense [see also Ref. (30)].

MREs in the regulatory regions of MTF-1 target genes

As mentioned earlier, MTF-1 activates transcription via binding to the MREs invariably present in the promoter regions of metallothionein genes. A closer look at the 18 MREs from the genomic region of *Drosophila* metallothioneins revealed

a striking conservation of thymidine nucleotides 5' to the consensus (Supplementary Figure 2A). Also, the MRE core sequences are frequently followed by a guanine and position 6 in the consensus is predominantly adenine. This 'refined' *Drosophila* MRE sequence is very similar to the mammalian extended MRE sequence (51). We attempted to find MTF-1 target genes by searching through the *Drosophila* genome for clusters of extended MREs using the 'Fly Enhancer' search engine developed by Markstein *et al.* (52). Twelve regions in the genome contained two or more extended MRE sites within a span of 300 bp or less. Three of them are associated with *MtnB*, *MtnC* and *MtnD*. One cluster is in the upstream region of *Ctr1B*, a target gene of MTF-1 under conditions of copper scarcity. Another cluster containing four MREs is in the locus of divergently transcribed ferritin genes, namely in the first intron of the *Fer1HCH* and 0.7 kb upstream of the *Fer2LCH* transcription start (Supplementary Figure 2B). A closer look at the ferritin region revealed two more clusters of MRE core sequences located in the 5'-untranslated region (5'-UTR) and in the coding region of *Fer2LCH* gene (Supplementary Figure 2B). The comparison of these sequences between seven *Drosophila* species revealed 100% conservation of the three *Fer1HCH* intronic MREs (Supplementary Figure 2C). The MREs in the two exonic clusters are conserved mainly between phylogenetically closer species (*D.melanogaster*, *Drosophila erecta*, *Drosophila yakuba* and *Drosophila pseudoobscura*). Our genome-wide search located another MRE cluster in the fifth exon of glutamate receptor gene *Glu-RI*, a gene involved in synaptic transmission and cation transport. The other six clusters of MREs are either at least 1.5 kb away from a start of any transcription unit or in the intergenic regions of unknown genes. Since ferritin genes are induced

by metals, we investigated the potential role of MTF-1 in their inducibility.

A new role for ferritins

Ferritin is well characterized as an iron binding protein. There are also reports that it binds to a variety of other metals such as beryllium, copper, zinc, cadmium, lead and aluminium (53). Transcripts and protein levels for both *Drosophila* ferritin subunits have been shown to strongly increase when larvae are fed a diet rich in iron (54,55). We confirmed the iron inducibility of *Fer1HCH* and *Fer2LCH* transcript levels (Figure 2D). Furthermore, both genes were considerably upregulated by zinc and to a less extent by cadmium and copper. We tested the possible role of MTF-1 in the basal and metal-induced expression of *Drosophila* ferritins. Indeed, cadmium, copper and zinc induction was abolished in the MTF-1 KO, whereas the iron induction remained (Figure 2D). Moreover, there is also a significant upregulation of both ferritin transcripts in larvae overexpressing *Drosophila* MTF-1 compared to the ones with only endogenous MTF-1 (Figure 2F). These data imply that ferritins are MTF-1 target genes under metal, notably zinc (but not iron) stress. A direct transcriptional regulation by MTF-1 is suggested by the presence of evolutionarily conserved MRE motifs, which strongly bind MTF-1 in EMSA (Supplementary Figure 2D).

CG10505, a homolog of yeast cadmium factor, contributes to metal homeostasis in the fly

CG10505 is a member of the ABC transporter family. Members include YCF1 found in *Saccharomyces cerevisiae*, and multidrug resistance associated proteins (MRP/CFTR family) found in humans, that confer resistance to cadmium or to various cytotoxic drugs (56,57). The promoter/enhancer region of *CG10505* contains one MRE 0.8 kb upstream of the annotated translation start. We tested the expression of *CG10505* by the S1 nuclease protection assay. Here also we saw a loss of metal induction in the MTF-1 KO (Figure 2B). Moreover, overexpression of *Drosophila* MTF-1 increased transcript levels of *CG10505* (Figure 2F). For the indicated concentrations of metals used zinc is the best inducer of the gene. To test the function of this transporter *in vivo* we expressed the *CG10505* coding sequence under the control of synthetic, Gal4-inducible promoter. The cross of transgenic flies with a fly strain that expresses Gal4 transactivator under the actin promoter (*actin-Gal4*) did not produce any obvious phenotype in normal food or when challenged with different concentrations of zinc, cadmium or copper. We then tested if the overexpression of *CG10505* could rescue the metal sensitivity phenotype of MTF-1 mutants. MTF-1 mutants do not survive in food containing either 5 mM zinc, 100 μ M cadmium or 500 μ M copper (22). In a competitive situation where both MTF-1 KO and wt flies are raised in the same tube, the KO genotype suffers even more and fails to survive in 2 mM zinc or 40 μ M cadmium. To test for rescue of the MTF-1 KO by CG10505 we brought *UAS-CG10505* and *actin-Gal4* driver transgenes into the MTF-1 mutant background and let the offspring of crosses grow up in different concentrations of metals. Under these conditions, we observe a partial rescue of the

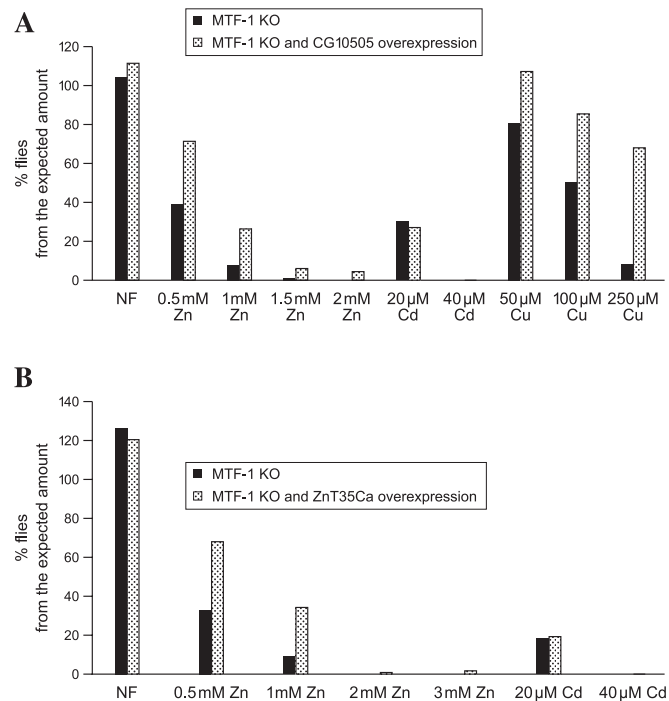


Figure 3. Overexpression of ABC transporter *CG10505* (A) and zinc transporter *ZnT35Ca* (B) in MTF-1 KO background. Shown is the percentage of flies from the expected mendelian ratio numbers in average (4–8 tubes for each condition). NF, normal, non-supplemented food.

zinc and copper sensitivity phenotype (Figure 3A). However, somewhat unexpectedly, considering the relation of this ABC transporter to YCF1, the MTF-1 KO did not benefit from CG10505 overexpression in cadmium containing food. Based on the transcript quantification and genetic rescue results, we propose that *CG10505* is a target gene of MTF-1 with a role in zinc and copper homeostasis.

ZnT35C zinc transporter is required for zinc tolerance

Next, we examined *ZnT35C*, a gene that was >6-fold down-regulated in the MTF-1 KO larvae. In agreement with the role of MTF-1, *ZnT35C* transcripts remained at background level in metal-treated MTF-1 KO larvae (Figure 2C). Furthermore, expressing MTF-1 under the tubulin promoter in addition to the wt MTF-1, caused the transcripts of *ZnT35C* to markedly increase (Figure 2F). In *D.melanogaster*, two alternative transcripts of the gene, *ZnT35Ca* and *ZnT35Cb*, are annotated. They differ in the first few exons and share the 5th to 10th exons (Figure 4A). The TMpred software, that predicts membrane spanning regions of proteins, identified six strong transmembrane helices in both variants of the *ZnT35C* protein. A search for putative MTF-1 binding site revealed two MREs in the promoter/enhancer region of *ZnT35Ca* variant and a third one in the 5'-UTR of *ZnT35Ca*. Two more MREs are found further downstream before the *ZnT35Cb* transcription start.

The *ZnT35Ca* variant predominates over the *b* form at all developmental stages, with a particularly high expression in adult females (Figure 4B). Neither variant could be detected in cultured S2 cells. In larvae, the expression of the gene was

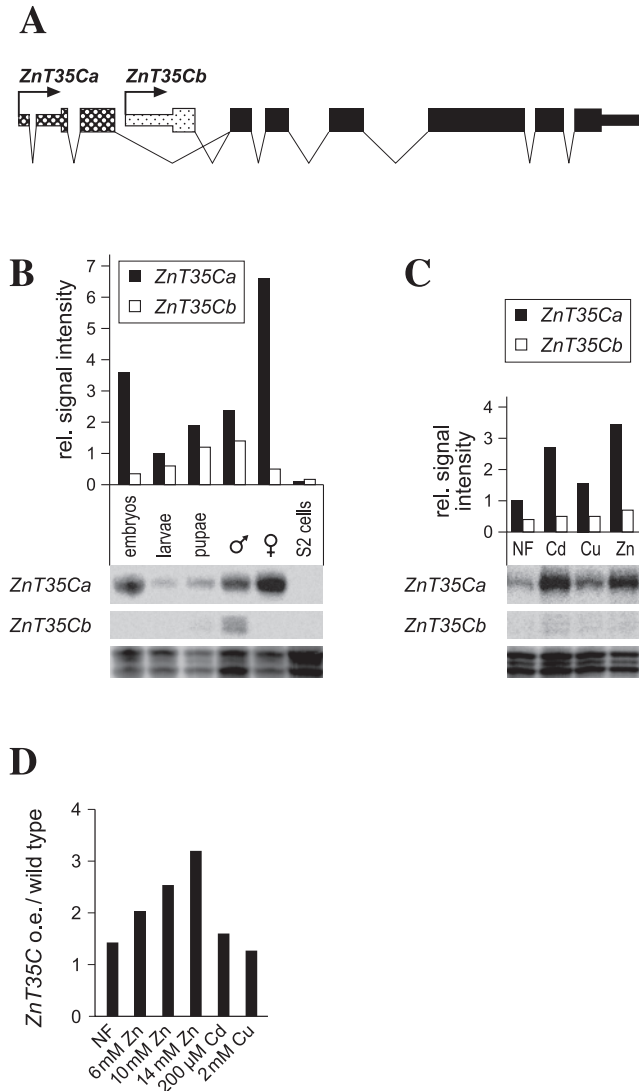


Figure 4. Zinc transporter ZnT35C: the genomic organization and expression. (A) Schematic view of the *ZnT35C* gene structure. The arrows show the transcription starts. The narrow rectangles correspond to the UTRs, the large ones show translated regions, black rectangles represent the shared exons for both variants. A gene of unknown function (*CG33911*) is located in the antisense strand of *ZnT35C* spanning introns 5 and 6. (B) S1 nuclease transcript assay for the *ZnT35Ca* and *ZnT35Cb* variants with RNA from different developmental stages and S2 Schneider cells. The lower panel of the gel shows *actin5* reference transcripts. (C) Response of the *ZnT35C a* and *b* variants in third instar larvae to cadmium, copper and zinc. NF, normal food; Cd, 50 μM CdCl₂; Cu, 500 μM CuSO₄; and Zn, 5 mM ZnCl₂. The lower panel of the gel shows *actin5* reference transcripts. (D) Overexpression of ZnT35C (both variants simultaneously) by actin-Gal4. The graph shows the ratio of flies overexpressing ZnT35C to wt flies.

very low; it is tempting to speculate that at this stage of rapid growth zinc is probably of great need, and that the low zinc exporter expression would help to maintain zinc supply. Next, we checked the metal induction of each transcript variant in the S1 nuclease protection assay. Here also, the *ZnT35Ca* was induced upon cadmium and zinc challenge, whereas we could hardly detect any mRNA from *ZnT35Cb* (Figure 4C). To test the functions of the gene *in vivo*, we made Gal4-inducible expression constructs by fusing the

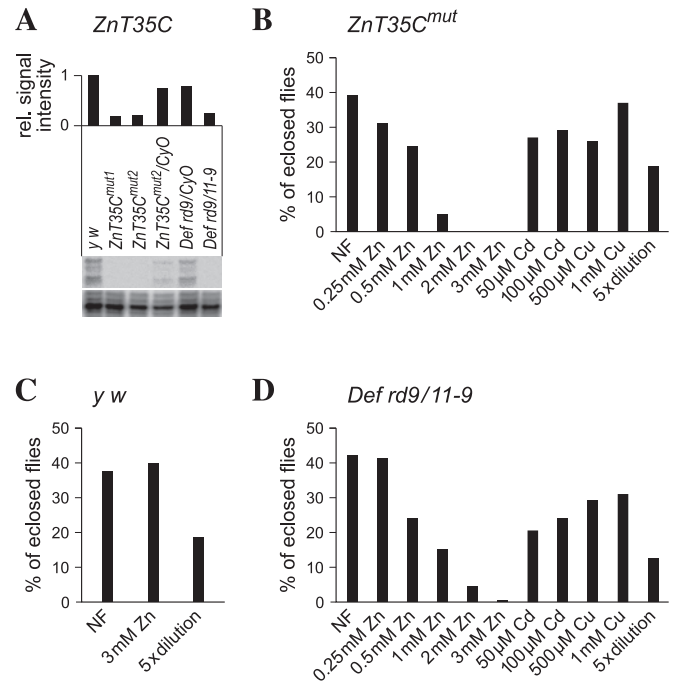


Figure 5. ZnT35C mutants; viability on different food conditions. (A) S1 nuclease transcript assay using an oligonucleotide that is complementary to the common region of *ZnT35Ca* and *b* variants. Note that two lines of *ZnT35C^{mut}* and the deficiency of the region over the deletion allele of *ZnT35Ca* (*Def rd9/11-9*) do not yield any detectable signal. (B) Percentage of *ZnT35C^{mut}* flies eclosing from a certain number of eggs laid in the indicated metal concentrations of the food or 5x dilution of NF (normal food). (C) Eclosure percentage of *y w* and (D) *Def rd9/11-9* flies.

coding sequences of both isoforms to the UAS promoter. The overexpression of ZnT35C yielded zinc-super-resistant flies; at least 3-fold more ZnT-overexpressing flies eclosed at 14 mM Zn compared to wt flies (Figure 4D). However, in cadmium and not unexpectedly also in copper, ZnT35C overexpression did not confer any advantage.

Keeping in mind the MTF-1-dependent expression of *ZnT35C* on the one hand, and the sensitivity of MTF-1 KO to metal load on the other, we attempted to rescue the metal sensitivity of MTF-1 KO flies by overexpressing *ZnT35C*. The actin-driven overexpression of *ZnT35Cb* isoform did not show any difference in viability; however, *ZnT35Ca* overexpression gave an advantage to MTF-1 KO *Drosophila* in zinc, but not in cadmium food (Figure 3B). Rescue was not complete, presumably because of unspecific general expression.

Next, we generated a mutant of *ZnT35C* by gene targeting based on homologous recombination (22,58). The targeting in the *ZnT35C* locus resulted in a truncation of the gene that eliminated 60% of the C-terminal coding sequence including the part coding for three transmembrane domains. The S1 probe designed to hybridize to the exons that are removed in our truncation mutant did not reveal any signal in the homozygous mutant flies, confirming successful targeting in the *ZnT35C* locus (Figure 5A). The mutant of *ZnT35C* (*ZnT35C^{mut}*) is viable with no apparent phenotype under normal laboratory conditions. However, the mutant animals are extremely sensitive to zinc load in that no mutant larvae

develop in 2 mM zinc (Figure 5B) whereas wt larvae develop to adulthood in as high as 14 mM zinc concentrations. Even adult *ZnT35C^{mut}* flies die within a few days when transferred to 3 mM zinc food while wt flies are not affected by this condition. *ZnT35C^{mut}* animals did not suffer from high cadmium or copper concentrations, and were similarly affected as wt by starvation in 5-fold diluted standard food, thus excluding a generally reduced viability (Figure 5B and C). We also generated a genomic deletion of the *ZnT35C* locus by imprecise excision of an adjacent P-element that removes the transcription start of the *ZnT35Ca* isoform (allele 11-9). The flies that carry 11-9 over a deficiency of the *ZnT35C* region (*Def rd9*) are almost as sensitive to zinc load as *ZnT35C^{mut}* (Figure 5D). Interestingly, as revealed by an S1 nuclease protection assay, the *ZnT35C* promoter is hyperactivated in *ZnT35C^{mut}*, suggesting that the absence of functional protein compromises zinc efflux, leading to metal accumulation and induction of metal defense mechanisms (Figure 6A). In agreement with such a scenario, metallothionein (*MtnA*) expression was noticeably elevated in *ZnT35C^{mut}* (Figure 6B). Furthermore, flies lacking *ZnT35C* have more than 2-fold elevated total body zinc, and the animals heterozygous for *ZnT35C^{mut}* contain at least 50% more zinc (Figure 6C). Manganese or copper amounts remained unchanged in all tested genotypes.

Finally, we also checked the expression pattern and cellular localization of *ZnT35C*, using a transgene where the *ZnT35C* promoter drives a fusion construct of *ZnT35Ca-GFP*. The malpighian tubules (the insect analog of a vertebrate kidney) of third instar larvae grown on normal food displayed a faint green fluorescence, which was strongly induced by zinc treatment (Figure 7A). In tubular cells, expression was primarily confined to the apical membrane (Figure 7).

DISCUSSION

The imbalance of metals due to genetic disorders or environmental pollution is a worldwide problem associated with adverse health effects. Our study unveils a number of genes and regulatory mechanisms involved in maintaining heavy metal homeostasis. The metals tested in our study, cadmium, copper and zinc, induce a wide range of responses, including induction of genes coding for metallothioneins, transporters, components of the glutathione-mediated detoxification pathway, antimicrobial peptides, ubiquitin-conjugating enzymes, heat shock proteins and cytochrome P450 enzymes (Supplementary Tables 1–4). The universal induction of metallothioneins by all three metals and the downregulation by copper depletion is in line with their metal scavenger function and protective role against metal toxicity. However, there are differences in the strength of the response of individual metallothioneins: *MtnA* induction is more prominent in copper (Figure 2E and Supplementary Tables 1–3), whereas *MtnB* and *MtnC* are more strongly induced by zinc and cadmium (Supplementary Tables 1–3). Indeed, a recent study suggests that individual metallothioneins preferentially defend against specific metals, with *MtnA* playing a major role in handling copper excess, *MtnB* during cadmium and zinc excess, while *MtnC* and

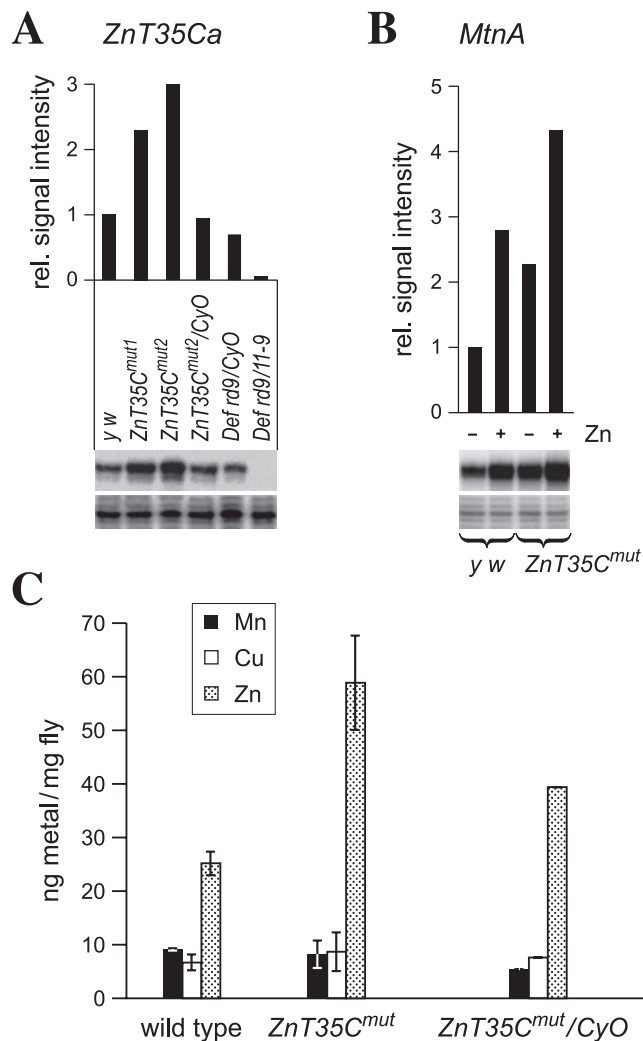


Figure 6. Promoter activities of *ZnT35C* and *MtnA* genes and metal contents of the *ZnT35C^{mut}* flies. (A) The *ZnT35C* promoter is induced in the *ZnT35C^{mut}*, as detected by an S1 nuclease protection probe that hybridizes to the intact 5' region of the truncated gene. (B) *MtnA* is induced in *ZnT35C^{mut}*. Zn, 5 mM ZnCl₂ (C) Mn, Cu and Zn content (ng metal per mg of wet weight) in flies wt, mutant or heterozygous for *ZnT35C*.

MtnD play only minor roles in defending against these three metals (59). In absolute terms, expression of *MtnA* is higher than that of other metallothioneins, with an impressive basal level and consequently a lesser fold-induction, suggesting *MtnA* to be the major metal scavenger at *Drosophila* larval stage.

Another essential system for metal ion homeostasis is metal export and import. Although transcriptional control is only one of several regulatory processes, we could follow numerous changes in characterized, as well as putative, *Drosophila* metal transporters in the microarray experiment. To provide adequate intracellular copper concentrations, the transcripts of the high-affinity copper importer *Ctr1B* are strongly induced by copper depletion; conversely, they are downregulated at high Cu concentration in order to reduce copper uptake. Two zinc transporter genes (*ZnT35C* and *ZnT63C*), both encoding ZnT family members involved in zinc efflux, as well as two ATPase transporters (*CG6263*

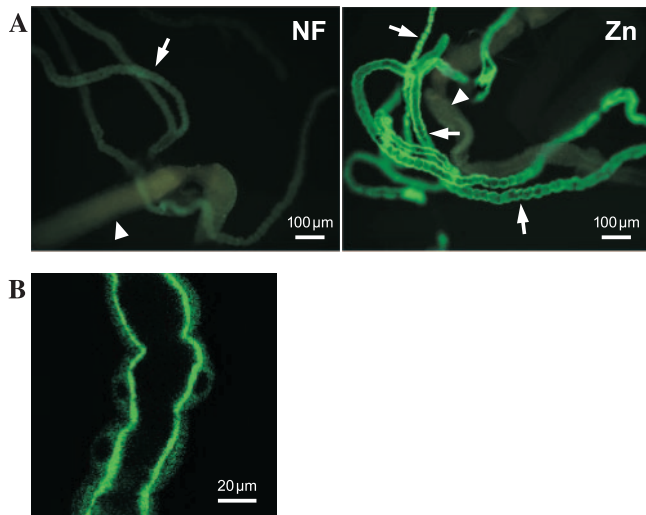


Figure 7. Expression pattern of the ZnT35Ca-GFP fusion protein driven by *ZnT35C* promoter in *Drosophila* larvae. (A) *ZnT35C* is expressed predominantly in malpighian tubules (arrows), rather than midgut (arrowheads). Zn, 5 mM ZnCl₂ treatment for 5 h. (B) Segment of malpighian tubule from an animal fed with zinc; cells show strong green fluorescence on the apical membrane.

and *CG18419*) presumably involved in cation and lipid transport, were upregulated by Zn and Cd treatments. Of note, several ubiquitin-conjugating enzymes (Ubc) were induced by zinc. In this context it is worth mentioning that the yeast zinc importer *Zrt1p* is ubiquitinated and subsequently degraded upon zinc load (60). The upregulation of Ubc might also help to degrade misfolded proteins which are probably formed upon metal load.

Our results indicate that the larval transcriptome responses to zinc and cadmium share several features. Even though they exert different biological effects, the chemical properties of Zn and Cd are similar, thus part of the regulatory mechanisms to cope with excess zinc are also functional in cadmium stress. Another example of the similarity between the zinc and cadmium responses is the specific and strong induction of two members of the glutathione *S*-transferase Delta class genes. There are more than 40 GST genes in *Drosophila* grouped into six classes (61). Three GSTs, *CG17524*, *CG6776* and *CG1681* with homology to epsilon, omega and theta class GSTs, respectively, are preferentially upregulated by zinc. GSTs catalyze the conjugation of glutathione (GSH) to a variety of harmful compounds. The resulting glutathione conjugates (GS-X) can then be sequestered by the vacuole or excreted from the cell by ATP-dependent GS-X pumps (62). The details of this process vary with different compounds and in different species. In *S.cerevisiae*, e.g. cadmium ions are conjugated to GSH and the Cd-GS₂ conjugate is transported to the vacuole by YCF1, an ATP-binding cassette transporter (56). It is plausible that the GSTs induced in our studies catalyze the conjugation of Cd and/or Zn to GSH, which eventually will be removed from the cytoplasm. Alternatively, or in addition to conjugating metals, they might conjugate ROS which can occur as byproducts of heavy metal metabolism. Interestingly, a specific ATP-binding cassette transporter *CG10505*, that shares 26% identity to YCF1, is strongly upregulated by all tested metals. It is

tempting to speculate that *CG10505* acts in a similar fashion to its yeast counterpart in exporting the metal-GSH conjugates. Nevertheless, the metal specificity of *Drosophila* *CG10505* differs from that of YCF1 because the former contributes to elevated resistance to zinc and copper, rather than to cadmium. Another enzyme involved in GSH conjugate metabolism and excretion, gamma-glutamyl transferase, which is an integral part of the gamma-glutamyl cycle involving the degradation and neo-synthesis of GSH (63) was also induced by zinc in *Drosophila*. Taken together, these data suggest that GSH conjugation plays a substantial role in the response of *Drosophila* to metal stress. Support for such a scenario comes from a recent study in the mouse showing the existence of two branches of cellular anti-cadmium defense, one via MTF-1 and its target genes, notably metallothioneins, and the other via glutathione (30). In agreement with this concept our data indicate that when the MTF-1-dependent response is abrogated, the glutathione-associated pathway is upregulated to compensate for this compromised genetic background.

The knockout of the *Drosophila* *MTF-1* gene altered the expression of more than 50 genes in our transcriptome assay. However, there is no obvious phenotype unless the animal is subjected to metal stress: copper, zinc, cadmium and mercury load, or copper starvation (22,64). The copper importer *Ctr1B* and metallothionein genes are well-established targets of *Drosophila* MTF-1. The *Ctr1B* mutant is sensitive to changes in copper concentration and the KO of the *Drosophila* metallothionein family is sensitive to cadmium and copper load; however, these mutants hardly show any phenotype upon zinc challenge (33). Here we suggest a major role in zinc detoxification for several other genes.

Next to the aforementioned ABC transporter *CG10505*, zinc exporter *Zn35C* contributes to zinc homeostasis in that the truncation mutant *ZnT35C^{mut}* is extremely sensitive even to a mild zinc load of 2 mM, while wt tolerates up to 14 mM zinc excess. Also, an overexpression of this transporter confers resistance to zinc and can partially rescue the zinc sensitivity of MTF-1 knockout flies. Given the strong zinc sensitivity phenotype of the mutant, we propose that *ZnT35C* is the major transporter of cellular zinc, similar to the mammalian zinc exporter *ZnT1* (65). It remains to be seen whether the other zinc exporter-like genes annotated in the *Drosophila* genome transport zinc from the cytoplasm to vesicular compartments, as do several of the mouse zinc transporters, such as *ZnT2*, 3, 5 and 7 (66–69). *Drosophila* *ZnT35C* localizes to the plasma membrane both at normal and elevated zinc concentrations; besides, mutant flies have a high zinc content, in support of a role in zinc excretion. We note that the mouse zinc exporter *ZnT1* was also found to be induced both by cadmium and zinc treatment in an MTF-1-dependent fashion similar to *ZnT35C* (27). Thus not only the regulation of metallothioneins but also that of zinc transporters is conserved between insects and mammals. In other cases, metal responses of *Drosophila* and mammals diverge: the fly orthologs of the mouse *cEBP alpha*, *ndrg1* or *klf4* were unchanged in our microarray experiments.

An unexpected result of the present study is the induction of ferritin heavy and light chain homologue genes by heavy metals. Ferritin is well known to detoxify, store and transport iron. It was also shown to bind other metal ions

and has been speculated to function as a general metal detoxicant in mammals (53). Our results suggest an induction of *Drosophila* ferritins by metals other than iron, notably zinc. The latter induction depends on MTF-1, based on the following findings: (i) the presence of multiple MREs, (ii) the loss of zinc induction in the MTF-1 mutant, and (iii) elevated ferritin transcripts upon MTF-1 overexpression. In contrast, iron inducibility must be regulated independently since it is not altered by the absence of MTF-1. In line with different signaling pathways responding to iron load versus excess of zinc, copper or cadmium, we note that the classical MTF-1 dependent *MtnA* gene is not inducible by iron (Figure 2E). Yet these pathways apparently overlap in the ferritin genes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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