Copper homeostasis in *Drosophila* by complex interplay of import, storage and behavioral avoidance

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Copper is an essential but potentially toxic trace element. In *Drosophila*, the metal-responsive transcription factor (MTF-1) plays a dual role in copper homeostasis: at limiting copper concentrations, it induces the Ctr1B copper importer gene, whereas at high copper concentrations, it mainly induces the metallothionein genes. Here we find that, despite the downregulation of the Ctr1B gene at high copper concentrations, the protein persists on the plasma membrane of intestinal cells for many hours and thereby fills the intracellular copper stores. *Drosophila* may risk excessive copper accumulation for the potential benefit of overcoming a period of copper scarcity. Indeed, we find that copper-enriched flies donate a vital supply to their offspring, allowing the following generation to thrive on low-copper food. We also describe two additional modes of copper handling: behavioral avoidance of food containing high (≥ 0.5 mM) copper levels, as well as the ability of DmATP7, the *Drosophila* homolog of Wilson/Menkes disease copper exporters, to counteract copper toxicity. Regulated import, storage, export, and avoidance of high-copper food establish an adequate copper homeostasis under variable environmental conditions.

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Introduction

Copper is an essential component of several important enzymes involved in respiration, oxidative stress protection, pigmentation and iron homeostasis. Most of these enzymatic reactions rely on the ability of copper to undergo redox transitions between the Cu(I) and Cu(II) state. This important trait of copper is at the same time a threat to the organism, as copper can catalyze the generation of reactive oxygen species (ROS) via the Fenton reaction (Halliwell and Gutteridge,

1990; Puig and Thiele, 2002). Eukaryotic organisms from yeast to humans use elaborate systems to regulate copper homeostasis, consisting of copper importers, copper chaperones, transcription factors, small metal binding proteins called metallothioneins and copper exporters (O'Halloran and Culotta, 2000; Puig and Thiele, 2002; Mercer and Llanos, 2003; Balamurugan and Schaffner, 2006). Studies in yeast have identified the high-affinity copper transporters yCtr1 and yCtr3 (Dancis *et al.*, 1994; Pena *et al.*, 2000). Homologs of these were identified in mammals (Ctr1) and in *Drosophila* (Ctr1A, B and C) (Lee *et al.*, 2000; Zhou *et al.*, 2003). A null mutant of Ctr1 in the mouse is lethal, whereas a mutation of Ctr1B in *Drosophila* results in a pigmentation defect and lethality under conditions of copper scarcity (Kuo *et al.*, 2001; Lee *et al.*, 2001; Zhou *et al.*, 2003).

The low-copper phenotype in the fly is reminiscent of the phenotype of a mutation in the metal-responsive transcription factor-1 (MTF-1) (Egli *et al.*, 2003). We have recently shown that Ctr1B transcription is activated by dMTF-1 under normal conditions and to a greater extent upon copper depletion, but repressed when copper is abundant (Selvaraj *et al.*, 2005). In contrast, when copper is in excess, MTF-1 activates transcription of the genes for metallothioneins, small, cysteine-rich metal scavenger proteins. The genes for Ctr1B and the metallothioneins are therefore both target genes of the same transcription factor but regulated in an opposite manner (Selvaraj *et al.*, 2005). Whereas *Drosophila* Ctr1B is regulated at the transcriptional level, the human and yeast Ctr1 homologs appear to be post-translationally regulated. Copper excess stimulates rapid endocytosis and/or degradation of both yCtr1 and hCtr1 (Ooi *et al.*, 1996; Petris *et al.*, 2003; Guo *et al.*, 2004), apparently to prevent over-accumulation and toxicity of copper. In a different study, hCtr1 remained stable and functional even after copper exposure (Eisses *et al.*, 2005). Another transport system is able to maintain adequate activity under both low and high copper conditions. At normal or low copper, the related mammalian copper exporters ATP7A and ATP7B, also referred to as Menkes and Wilson P-type ATPases, respectively, transfer copper from the cytoplasm to the *trans*-Golgi network (TGN). At high copper, both exporters translocate from the Golgi to the cell surface, where they function to export copper from the cell (Petris *et al.*, 1996). The importance of the latter process is illustrated by mutations in ATP7B, which causes Wilson's disease, where patients accumulate toxic amounts of copper in the liver (Bull *et al.*, 1993; Yamaguchi *et al.*, 1993). In spite of recent progress in our understanding of copper homeostasis, many questions remain concerning how copper levels are sensed and how they feed back to coordinate copper uptake, export or copper sequestration. In the fruit fly *Drosophila*, overall copper levels of the organism can vary at least by 20-fold depending upon the copper concentration in the diet (H Yepiskoposyan, A Simons and W Schaffner, unpublished). Apparently under high copper

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conditions, sequestration is a major mechanism ensuring a remarkable tolerance to copper. This tolerance depends on the copper sequestering ability of metallothioneins and the transcription factor MTF-1, which activates transcription of the metallothionein loci from insects to mammals (Heuchel *et al*, 1994; Zhang *et al*, 2001). Accordingly, metallothionein or MTF-1 mutants are highly sensitive to elevated copper concentrations (Egli *et al*, 2006a). In *Drosophila* larvae, metallothionein expression and the sites of copper accumulation coincide, especially in the cytoplasm of so-called copper cells of the midgut and also in the posterior midgut (Filshie *et al*, 1971; Tapp, 1975; Lauverjat *et al*, 1989; Marchal-Segault *et al*, 1990; Egli *et al*, 2006a). Noteworthy, copper cells display a characteristic orange copper luminescence caused by a copper-metallothionein complex (Egli *et al*, 2006a).

Here, we report that in spite of a downregulation of *Ctr1B* transcripts in high copper, the protein persists for many hours in a functional state. In *Drosophila* larvae, *Ctr1B* remains at the plasma membrane of intestinal cells and imports copper to high levels, causing a metal-stress response and the induction of metallothioneins. Taken at face value, this property indicates a flawed regulation of copper homeostasis. However, we provide evidence that transient copper accumulation in larvae can balance fluctuating copper availability in the food and helps to overcome intermittent periods of copper starvation, and is even used to supply copper to the following generation. Thus, the regulation of *Ctr1B* seems to reflect a trade-off between copper excess causing copper toxicity and the prospect of copper starvation, which severely delays development. We also show two additional levels of regulation, which help to limit copper accumulation: copper export via DmATP7, and a behavioral avoidance of food containing 0.5 mM or more copper by larvae.

Results

Metallothionein transcription as a sensitive indicator of heavy metal concentration and distribution

Drosophila larvae that are grown on copper-supplemented food contain high levels of metallothioneins, whereas metallothionein expression is low when larvae are grown on copper-depleted food (Figure 1A). Likewise, larvae that are constantly fed on zinc, cadmium or mercury containing media show a dose-dependent increase in metallothionein expression (Balamurugan *et al*, 2004). The tissue specificity of metal distribution coincides with metallothionein expression, for example, the so-called copper cells of the larval midgut accumulate high levels of copper and strongly express metallothionein genes. The endogenous metallothionein promoters or metallothionein-EYFP (enhanced yellow fluorescent protein) reporter constructs are thus sensitive indicators of metal transport processes within the intact organism.

Ctr1B is the most efficient copper importer in *Drosophila* larvae

The copper transporter *Ctr1B* is expressed during larval stages in the posterior midgut and mediates copper uptake from the food. We determined the induction of the metallothionein A (*MtnA*) promoter by copper and zinc in *Ctr1B* mutants and in wild-type larvae that were grown on normal food (NF) and transferred for 6 h to food supplemented with copper. Whereas wild-type larvae showed a strong induction

Figure 1 A metallotheinein-EYFP reporter as a sensitive indicator of metal content and transport processes. (A) Expression of reporter transgene consisting of the *MtnA* promoter driving expression of EYFP. Shown are third instar *Drosophila* larvae grown on the indicated type of food. *MTF-1* mutant larvae, which lack metallothionein expression, owing to absence of the major transactivator MTF-1 (metal responsive transcription factor 1 or metal regulatory transcription factor 1), also do not show reporter gene expression as measured by fluorescence microscopy. (B) Mutants for the *Ctr1B* copper importer are deficient in copper-mediated induction of the *MtnA* promoter. Larvae at the third instar were transferred from normal to cadmium or copper containing food for 6 h and mRNA expression was determined by S1 nuclease mapping. Bars represent the quantification of *MtnA* mRNA levels normalized with the signal obtained with a probe complementary to *actin* mRNA. Results are the mean $\pm$ s.e.m. of three independent experiments. (C) Mutant and 'wild type' (*y w*) larvae that were continuously raised until third instar on normal food (NF) or copper containing food. Transcripts were quantified as in panel B.

of metallothionein transcripts by copper and cadmium, *Ctr1B* mutant larvae did not display copper-mediated induction, but maintained cadmium-mediated induction of metallothioneins (Figure 1B). This demonstrates that *Ctr1B* is the major intestinal copper importer of larvae growing on normal food. Interestingly, when larvae were constantly raised in high copper, metallothionein levels were as high in *Ctr1B* mutants as in wild type (Figure 1C). At first sight, these results seem to contradict each other. However, metallothionein induction in the first experiment relied on an efficient, rapid import of copper into the cell, whereas in the second, copper could accumulate over a period of several days. Our results suggest that under the latter conditions, copper import occurs independent of *Ctr1B* by less abundant, less efficient or less specific copper importers, possibly *Ctr1A*, *Ctr1C* or Malvolio (a *Drosophila* homolog of Nramp-1) (Rodrigues *et al*, 1995), ultimately leading to equal levels of Mtn induction in both *Ctr1B* mutant and wild-type larvae. Consistent

with this observation is the report that *Ctr1B* mutants contain less copper than wild-type flies under normal conditions, but the same amount under copper load (Zhou *et al*, 2003).

Rapid copper import by *Ctr1B* causes a metal-stress response

Ctr1B remained active when larvae were transferred from normal food to food containing high copper concentrations, which strongly induced metallothionein gene expression. Thus, we asked whether high expression of *Ctr1B* would cause a copper stress response even with normal food. Larvae that are grown in the presence of the copper chelator bathocuproine disulfonate (BCS) show high levels of *Ctr1B* to ensure sufficient copper uptake (Selvaraj *et al*, 2005). At the same time, transcription of metallothionein genes is low. Indeed, when copper-depleted larvae were transferred to normal food without copper chelator for 6 h, expression of metallothionein genes was strongly induced owing to high *Ctr1B* expression. This indicates that import of copper by *Ctr1B* leads to a copper-mediated stress response characterized by metallothionein gene induction (Figure 2A).

Metallothioneins reduce toxicity of a *Ctr1B*-mediated copper shock

As *Ctr1B* appears to import copper to critical levels, we wished to know whether transfer of larvae from low to high copper results in copper toxicity. To this end, we made use of a copper-sensitive 'quadruple metallothionein' (*qMtn**) mutant strain that lacks all four metallothioneins (Egli *et al*, 2006a). These flies are viable and develop normally in up to 100 μ M copper in the food, that is, they tolerate a certain amount of copper excess (although less than 5% of what wild-type larvae tolerate). We transferred copper-depleted or normally fed third instar larvae to food with high copper and scored for survivors that made it to adulthood. Only one-third of *qMtn* mutant larvae that were copper-depleted and therefore had high starting levels of *Ctr1B* survived the copper shock. *qMtn* mutant larvae that were grown in standard or copper-rich food before the transfer, and therefore had lower starting levels of *Ctr1B*, were more likely to survive (Figure 2B). As copper uptake via importers other than *Ctr1B* (*Ctr1A* and *Ctr1C*) is low under these experimental conditions (Zhou *et al*, 2003), we conclude that *Ctr1B* continues to import copper even after normal levels are reached, resulting in copper excess and, especially in the absence of metallothioneins, imminent toxicity. Wild-type control larvae survived a transfer from low to high copper quite well, indicating that metallothioneins play an important role in buffering fluctuating copper availability and uptake (Figure 1B; see also Egli *et al*, 2006b). We have also measured endogenous *Ctr1B* transcript levels in metallothionein mutants after transferring early third instar larvae from copper-depleted or normal food to high-copper food. We note that upon copper shock, *Ctr1B* transcript levels are similarly reduced in wild-type and metallothionein-knockout larvae, indicating that the copper importing activity of *Ctr1B* is not altered in the latter (Figure 2C). These observations corroborate our previous findings that the copper content of both wild-type and quadruple metallothionein-knockout flies is the same after copper shock (Egli *et al*, 2006a).

Figure 2 Copper shock: a cellular stress response owing to massive copper import. (A) *Drosophila* larvae were copper-starved by growth on food containing 0.5 mM copper chelator (BCS) or on normal food (NF) and transferred to NF for 6 h. Shown are the expression levels of metallothionein A (*MtnA*). Quantification was done as in Figure 1. (B) Wild-type (*y w*) or mutant flies with inactivation of all four metallothionein genes (*qMtn**) were grown for five days on 0.5 mM BCS and 30 third instar larvae were transferred to either normal food or 0.5 mM copper. Shown is the survival to adulthood in percentage. Results are the mean $\pm$ s.e.m. of three different experiments. (C) Wild-type or flies lacking all four metallothioneins (*qMtn**) were first grown in the indicated type of food and allowed to lay eggs. Third instar larvae were transferred to high copper or normal food for 18 h. Total RNA was extracted and transcripts were quantified by S1 nuclease mapping. *Actin5c* RNA was used as a standard for normalization. Results are the mean $\pm$ s.e.m. of three independent experiments.

Ctr1B is mostly, if not exclusively, regulated at the transcriptional level

Copper chelation by metallothioneins reduces copper toxicity following a copper shock. In addition, we considered post-translational regulation of *Ctr1B*, for example, copper-dependent degradation, which was reported for mammalian *Ctr1* (Petris *et al*, 2003). To find whether there was a similar regulation in flies, we transferred larvae from low (250 μ M BCS) to high copper (250 μ M Cu) and tested mRNA and protein levels of *Ctr1B*-EGFP. The transcriptional response was rapid: mRNA levels of *Ctr1B*-EGFP were reduced after merely 2 h, whereas at the same time, metallothionein A mRNA increased in parallel (Figure 3A). In contrast, *Ctr1B*-

EGFP protein levels remained constant even after 18 h of copper load (Figure 3B). In these experiments, we tested the levels of Ctr1B-EGFP fusion construct (AH3) where the EGFP coding region is fused in-frame to the last codon of a complete *Ctr1B* gene as well as endogenous Ctr1B (not shown). The Ctr1B-EGFP fusion construct was previously shown to be regulated by copper at the transcriptional level and to rescue all known aspects of the *Ctr1B* mutant phenotype, namely, sensitivity to both copper scarcity and to very high copper concentration (Selvaraj *et al*, 2005). Nevertheless, AH3 transgenic flies in a Ctr1B wild-type background were not more sensitive to copper shock than wild-

type (*y w*) control larvae, indicating that the fusion construct does not result in a hyperactive allele and excessive copper import (not shown). Taken together, these findings suggest that *Drosophila* Ctr1B is not degraded in a copper-dependent manner; rather, the amount of Ctr1B is mostly, if not entirely, regulated at the transcriptional level.

The Ctr1B-EGFP fusion protein remains on the plasma membrane upon copper shock

Consistent with a role in copper uptake from the food, the Ctr1B-EGFP fusion protein localized to the apical side of the plasma membrane of larval gut cells (Figure 4A). To find out whether Ctr1B undergoes internalization at elevated copper levels as described for mammalian Ctr1 (Petrus *et al*, 2003), we transferred third instar larvae with the Ctr1B-EGFP transgene from 100 μ M BCS to 250 μ M copper. Even after 24 h, when copper import has led to a strong activation of metallothionein genes, the Ctr1B-EGFP fusion protein persisted at the apical plasma membrane of intestinal cells (Figure 4B). Importantly, these cells also showed the characteristic copper luminescence, indicating that high amounts of intracellular copper were complexed by metallothioneins. Such high amounts of copper are not required for larval development, as larvae that are grown at mild copper depletion (100 μ M BCS) also develop normally and maintain the same copper content as controls owing to the upregulation of Ctr1B (see below). These results show that copper does not alter the cellular localization of Ctr1B and provide cytological evidence that Ctr1B continues to import copper from the food beyond the immediate physiological requirements.

Copper excess is used to cope with periods of nutritional copper scarcity

To test whether stored copper helps to overcome a period of copper scarcity, we shifted larvae from copper-scarce to copper-supplemented food and then back to copper-scarce food. Growth on normal food until pupariation takes about 5 days, but growth on low copper can delay development by twice the normal time period or even more (Figure 5A). The developmental retardation is most pronounced in rapidly growing larvae. High copper concentrations can also delay

Figure 3 Ctr1B protein but not mRNA levels are stable after transfer of larvae from low to high copper. The different time points indicate the time after removing the larvae from low copper (i.e., BCS containing) food. The concentration of copper or BCS was 250 μ M. (A) mRNA levels of MtnA or a Ctr1B reporter construct (Ctr1B-EGFP with the endogenous Ctr1B promoter) determined by S1 nuclease mapping. Note the opposite regulation of the two transcripts by copper availability (see also Selvaraj *et al*, 2005). (B) Western blot of Ctr1B-EGFP and α -tubulin. Unlike mRNA levels, Ctr1B protein levels are hardly affected by copper load during the first 18 h. NF = normal food.

Figure 4 Apical localization of Ctr1B in intestinal cells is not regulated by copper. (A) Localization (apical) of the Ctr1B-EGFP fusion protein on the brush border membrane of an intestinal cell in the posterior midgut. The lumen of the gut is to the right. This larva was raised to the third instar on 100 μ M BCS. Blue: DAPI staining of nucleus. (B) Copper shock does not alter the cellular localization of Ctr1B-EGFP. Third instar larvae were transferred from 100 μ M BCS to 250 μ M copper for 24 h and larval guts were analyzed by confocal microscopy. Copper accumulation is revealed by orange copper luminescence. The lumen of the gut, with some autofluorescent food particles, is to the right.

Figure 5 Copper starvation strongly delays development, but is rescued by a short copper pulse. Shown is the percentage of pupae forming on each day, counted after egg laying. The numbers in parentheses indicate the number of pupae in each experiment. (A) Wild-type flies were raised continuously on the indicated type of food. Note the severe delay in development by copper scarcity and also by copper load. (B–D) Larvae were transferred on the third day after egg laying (ael) to normal or copper-enriched food for further development, or kept in high-copper food for just four hours and then transferred back to the type of food indicated in each panel. (B, C) Wild-type *Ctr1B*^{+/+} flies, (D) *Ctr1B*^{-/-} flies.

growth (see also Egli *et al*, 2006a). Homeostasis of copper is therefore required to minimize the time span of complete development. To investigate the ability of *Drosophila* larvae to cope with nutritional copper fluctuations, we transferred 3-day-old second instar larvae from 750 μ M BCS (wild type) or 15 μ M BCS (*Ctr1B* mutants) to 250 μ M Cu, or to normal food, either for 4 h or for the rest of the larval development, and measured the time needed to reach pupal stage (Figures 5B–D). The short 4 h copper pulse dramatically accelerated

development of wild-type larvae by about 4 days compared to constant growth on 750 μ M BCS. This result is comparable to growth of larvae shifted to normal food for the rest of development. A shift that was followed by constant growth on copper-enriched food however does not accelerate development (Figure 5B and C). Consistent with the role of *Ctr1B* as an intestinal copper importer, *Ctr1B* mutant larvae had great difficulties in exploiting such a transient period of copper abundance and were clearly retarded relative to equally treated wild-type larvae (Figure 5C). These results show that *Ctr1B* mediates efficient copper import within a short time window, ensuring a sufficient copper supply for the rest of development. This confers a substantial growth advantage when copper becomes scarce, but may also increase the risk of copper-mediated oxidative damage.

Accumulation of copper in the body: another key component in *Drosophila* copper homeostasis

Intestinal cells have a remarkable ability to induce transcription of metallothionein genes and to accumulate and detoxify high amounts of copper. In *Drosophila* larvae, *Ctr1B* is expressed in the intestinal cells of the posterior midgut, where metallothionein induction is high upon copper load (this study). To determine the effect of ectopic *Ctr1B* expression, we have tested a transgene where *Ctr1B* is under the control of an eye-specific *GMR* promoter. Such an ectopic (over)expression of *Ctr1B* resulted in distorted eye development, manifesting itself as ‘rough eyes’. This phenotype critically depends on copper availability: copper scarcity alleviated the rough eye phenotype, whereas copper supplementation of the food aggravated it in a dose-dependent manner (Figure 6A). We also observed that the rough eye phenotype is more severe in flies that were grown in food containing both 20 μ M copper and 0.02% hydrogen peroxide as compared to 20 μ M copper or 0.02% hydrogen peroxide alone (Figure 6B), probably as a result of radical generation via the Fenton reaction (Halliwell and Gutteridge, 1990; Puig and Thiele, 2002).

To find out whether overexpression of MTF-1 and metallothioneins affected the rough eye phenotype, we coexpressed *Ctr1B* with transgenes encoding either metallothionein A or dMTF-1, using the eye-specific *GMR* promoter. Consistent with their role in copper detoxification, overexpression of metallothionein or dMTF-1 rescued the toxic effects of excessive copper import in the eye, whereas a dominant-negative version of dMTF-1 that maintains DNA binding but lacks activator function yielded a more severe phenotype (Figure 6A). Metallothioneins show their highest expression levels in the gut and are also expressed in the fat body upon copper load, but their expression is below detectable levels in imaginal discs and the brain. In line with this finding, imaginal discs do not accumulate metal under normal conditions (Ballan-Dufrançais, 2002), and may lack an efficient defense system. Therefore, copper distribution as well as the expression of proteins involved in copper homeostasis is tissue-specific. Some tissues, especially larval tissues that do not develop into the adult organism, tolerate high levels of copper and are apparently used to keep copper from the more sensitive tissues. Copper-accumulating tissues probably donate regulated amounts of the stored copper to copper-sensitive tissues during copper scarcity. Indeed, we observed that copper luminescence in the larval midgut that

Figure 6 Ectopic copper import interferes with development. (A) Phenotype of flies with ectopic copper import into developing eye imaginal cells. Ctr1B is expressed under the control of the eye-specific GMR promoter in all panels. Shown are the scanning electron micrographs of the *Drosophila* eye. Flies were grown either in NF or 20 μM copper-supplemented food. 5 day-old flies were fixed in 2% osmium tetroxide for 1 h and then subjected to scanning electron microscopy. MTF-1, MTF-1^{dn} and MtnA designate fly lines with the co-overexpression of additional transgenes (dn = dominant negative form). (B) Synergistic effect of copper and hydrogen peroxide on rough eye phenotype. Flies were grown in food containing either 20 μM copper or 0.02% hydrogen peroxide (H₂O₂) or both. (C) Decreased copper luminescence in the intestine of larvae following transfer from food containing 250 μM copper to normal food. Shown is the confocal microscopic picture of the *Drosophila* larval midgut. (D) The phenotype caused by the expression of Ctr1B in the eye is rescued by co-expression of the *Drosophila* copper exporter DmATP7. Scanning electron micrographs of the eye of a Ctr1B overexpressing fly (left panel) is compared to the one of a Ctr1B and DmATP7 co-overexpressing fly (right panel).

marks sites of copper accumulation disappears within about 10 h upon withdrawal of copper from the food, showing that copper is not irreversibly trapped in copper-binding proteins in intestinal cells (Figure 6C). It remains to be seen what fraction of the copper is transported to other parts of the body and what, if any, is excreted, but it seems safe to state that copper storage is a dynamic process.

Role of copper transporter DmATP7 (*Drosophila* Menkes/Wilson's disease homolog) in counteracting copper toxicity

Besides the regulation of copper import, copper sequestration by metallothioneins and copper avoidance, copper export also plays a role to defend against copper toxicity. The *Drosophila* genome encodes a single homolog of the two related mammalian copper exporters ATP7A and ATP7B, also referred to as Menkes and Wilson's disease proteins, respectively (Southon *et al*, 2004; Norgate *et al*, 2006). We over-expressed the *Drosophila* homolog DmATP7 in the *Drosophila* eye by the UAS-Gal4 system. Overexpression of DmATP7 does not cause an eye phenotype *per se* but alleviates the toxicity of excessive copper import mediated by Ctr1B (Figure 6D). Detoxification most likely occurs via removal of copper from affected cells, indicating that copper export is yet another defense mechanism against copper excess, similar to the excretion of copper in the liver by the related mammalian Wilson's copper transporter ATP7B. While these experiments were underway, a detailed study on the role of DmATP7 in *Drosophila* copper homeostasis came to the same conclusions (Norgate *et al*, 2006).

Copper can be transferred to the next generation

In spite of the mechanisms used by *Drosophila* to alleviate copper toxicity, in balance it seems best to maximally fill the copper stores, because even the following generation can profit from the supply. To test this hypothesis, *Drosophila* larvae were raised in copper-supplemented food and the resulting flies were transferred to copper-depleted food for egg deposition. Such offspring grew faster (not shown) and survived better than the offspring of control flies (Figure 7). Strikingly, this beneficial effect extended to a further generation: offspring of second-generation low-copper flies raised again in low copper fared much better if their grandparents had been grown up in copper-supplemented food (Figure 7, lanes G3).

Drosophila larvae avoid potentially toxic levels of copper

The ability of Ctr1B to excessively import copper suggests the presence of other mechanisms to protect the organism from copper toxicity. We therefore tested the behavioral response of wild-type larvae to copper. Third instar larvae raised in normal food were placed in a Petri dish and given the choice between two types of semi-solid food (Figure 8A). One-half of the dish contained normal food, whereas food on the other half was supplemented with different copper concentrations or other transition metals. Irrespective of whether larvae were initially placed on the half containing normal food or copper-enriched food, they rapidly redistributed over the whole plate in an initial phase. However, after prolonged crawling, they obviously sensed and avoided the copper-containing half, such that only 10–20% localized to that

half after 60 min. While a copper concentration of 0.25 mM copper elicits at most a mild avoidance, a clear avoidance was observed at 0.5 or 1 mM copper. Different anions, either CuCl_2 or CuSO_4 , yielded very similar results (not shown).

Figure 7 Copper supply to following generation. *Drosophila* larvae of generation 2 (G_2) thrive on copper-starved food if their parents had been raised during larval stage in copper-supplemented food. Controls whose parents grew up in standard food are severely delayed (not shown), and many fail to develop to adulthood, as illustrated by the ratio of flies that eclosed from originally laid eggs on various concentrations of copper chelator (BCS). The beneficial effect of copper transfer to G_2 even extends to a further generation (G_3) raised again in low-copper food.

Drosophila larvae also avoided food with 1 mM silver, but were indifferent to 4 mM zinc, 0.5 mM cadmium or 5 mM iron (Fe^{3+}) (Figure 8B and C). These controls demonstrate that the avoidance reaction is specific to copper, or in general to elements of group Ib (which includes silver) in the periodic table of elements and not due to a difference of osmolarity between the metal-containing half and the normal half. None of the metal concentrations tested affected viability during the 2 h interval of the food-choice test; 4 mM zinc, 0.5 mM copper or 5 mM iron was well tolerated by wild-type larvae even upon constant exposure to these metals, whereas 0.5 mM cadmium or silver was toxic to larvae upon prolonged exposure (not shown). We also tested whether the avoidance of copper and silver depends on the function of either the regulator MTF-1 or the copper importer Ctr1B. However, both *MTF-1* and *Ctr1B* mutant larvae displayed the same copper avoidance as wild type, suggesting that another copper importer or some kind of a copper receptor on the cell surface triggers this behavioral response (data not shown).

Discussion

Our results imply that copper levels in the food or in the cell do not alter the localization of the Ctr1B copper importer protein in *Drosophila*. Instead, copper import by Ctr1B is mostly, if not exclusively, regulated at the transcriptional level by copper availability in an MTF-1-dependent manner. This form of regulation is in apparent contrast to yeast Ctr1, which is regulated post-translationally (Ooi *et al*, 1996). Concerning human Ctr1, one study has shown that it under-

Figure 8 Behavioral avoidance of copper. (A) Groups of 30 larvae were placed on the metal-containing half food in a Petri dish, from where they crawled on the surface of solid food in search of a hole to enter the 'food cake', thereby given the choice between metal containing and normal food. At the time points indicated, larvae were counted in each half. The colors of the two halves differ in this Figure for didactical purposes only. (B, C) Shown is the percentage of larvae crawling on the metal-containing food as a function of time.

goes degradation in response to copper (Petrís *et al*, 2003), whereas another reports that it stably resides on the plasma membrane (Eisses *et al*, 2005). Interestingly, some other Ctr family members, including hCtr1 and yCtr1, possess 'Mets motifs' (MxxM or MxM) that are not present in *Drosophila* Ctr1B and yCtr3, or in paCtr3 of the filamentous fungus *Podospora anserina* (Ooi *et al*, 1996; Koch *et al*, 1997; Pena *et al*, 2000; Guo *et al*, 2004). Several studies have shown that these 'Mets motifs' are pivotal for copper-dependent endocytosis (Guo *et al*, 2004; Jiang *et al*, 2005). Consistent with the idea that the 'Mets motifs' are necessary for copper-stimulated endocytosis and degradation, yCtr3, in contrast to yCtr1, does not undergo copper-dependent endocytosis (Pena *et al*, 2000). Indeed yCtr3, Ctr1B and paCtr3 are all transcriptionally activated under conditions of copper scarcity by their respective transcription factors Mac1 (which also activates yCtr1), MTF-1 and Grisea (Graden and Winge, 1997; Labbe *et al*, 1997; Borghouts *et al*, 2002; Selvaraj *et al*, 2005). The two other *Drosophila* copper importers Ctr1A and Ctr1C, whose role in copper homeostasis remains to be elucidated, are not regulated at the transcriptional level but contain Mets-like motifs and may thus be regulated at the protein level by copper (Zhou *et al*, 2003). In the present study, we show that copper does not trigger endocytosis of *Drosophila* Ctr1B, and that Ctr1B continues to import copper irrespective of the ambient copper concentration. In agreement with such a differential mode of regulation, Ctr1B is the closest *Drosophila* homolog of yCtr3, whereas Ctr1A and Ctr1C are more closely related to yCtr1 (Ooi *et al*, 1996; Koch *et al*, 1997; Pena *et al*, 2000; Zhou *et al*, 2003).

Drosophila has a remarkable ability to accumulate copper. This accumulation is mediated by the major larval copper importer Ctr1B upon an increase of copper levels in the diet, as Ctr1B remains on the brush border membrane of gut cells. Copper accumulation strongly induces transcription of metallothioneins, with subsequent copper sequestration and storage primarily in intestinal cells. The stored copper can serve as a source in periods of copper scarcity, whether in the same or, surprisingly, even in the following generation(s) (Figure 7). Intestinal accumulation of copper also helps to protect other tissues such as brain and imaginal discs, whose development or function might be disrupted by copper load (Rodrigues *et al*, 1995; Folwell *et al*, 2006; Egli *et al*, 2006a). Indeed an increased copper import by ectopic expression of Ctr1B in the eye interferes with its development, resulting in a characteristic 'rough eye' phenotype.

An important question is whether the seemingly sluggish regulation of Ctr1B that can result in a copper-stress response serves a purpose under natural conditions or whether a more precise regulation is simply not necessary. A sufficient uptake and the ability to store copper may be particularly important in geographical regions where copper is scarce. Copper scarcity results in a strong delay of larval development, a condition that may be of selective disadvantage in a rapidly changing environment. Fluctuations of copper content in the nutrients may be quite common, at least locally: *Drosophila* had been exposed to high copper levels over the course of the last century as copper-containing sprays are used in vineyards and orchards to combat bacterial and fungal infections (Maroni *et al*, 1987). A downregulation of Ctr1B upon a change from untreated to copper-treated fruits appears necessary but copper shock can also be controlled by the copper

avoidance reaction. Therefore, *Drosophila* may get by with a sluggish downregulation under most circumstances.

Copper, like other essential trace elements or nutrients such as amino acids or lipids, is a limiting factor for development even when all other nutrients are present in abundance. To avoid such a situation, most organisms have the ability to store nutrients, for example, lipids in fat tissue, sugars as glycogen and iron in ferritins, iron-binding proteins (Harrison and Arosio, 1996). Similarly, metallothioneins can be considered as stores of essential transition metals, notably copper. Ctr1B would simply fill these stores as long as copper is abundant. Because of this potential benefit, we propose that *Drosophila* allows copper accumulation, which is accompanied by a copper-mediated physiological stress response. The accumulated copper, in spite of its tight binding to metallothioneins, could be made available in periods of copper scarcity by oxidation of metallothioneins in a manner akin to that described for copper-thioneins in mammals (Feldman and Cousins, 1976; Bremner *et al*, 1978; Liu *et al*, 2000). The released copper may be distributed to other parts of the body via DmATP7 analogous to mammalian ATP7A, which exports copper from intestinal cells through the basolateral membrane to the bloodstream (Petrís *et al*, 2003; Southon *et al*, 2004; Norgate *et al*, 2006). DmATP7, like mammalian ATP7A, is required for proper pigment formation by the copper-containing enzyme tyrosinase (Petrís *et al*, 2003; Norgate *et al*, 2006). The importance of copper accumulation and distribution is illustrated by the amazing ability of *Drosophila* to thrive on low-copper food if the parents had been raised during their larval development in copper-supplemented food. We propose that copper accumulation and transfer to the next generation ensures a sufficient copper supply and is of primary importance for the organism, whereas the threat of copper toxicity may be secondary. It remains to be determined which component(s) of the copper homeostasis system is responsible for this trans-generation effect.

The behavioral aspect of copper homeostasis is also intriguing. In *Drosophila*, copper avoidance, which was also observed in other organisms (Rabin *et al*, 1985; Sambongi *et al*, 1999; Lopes *et al*, 2004; Van Zwieten *et al*, 2004), occurs at relatively high levels of copper that would lead to a copper-mediated developmental delay of wild-type flies. The threshold of about 0.5 mM copper to trigger the avoidance probably reflects a tradeoff between accumulation to overcome periods of copper scarcity and the prospect of copper-mediated damage. Taken together, regulated copper import with specific chelation and storage, export, and also copper avoidance, establish an adequate homeostasis of this essential but potentially toxic trace metal.

Materials and methods

Fly stocks and genetics

The dMTF-1 (*dMTF-1^{140-1R}*) and *Ctr1B* (*Ctr1B³⁻⁴*) mutant alleles, the Ctr1B reporter transgenes and the *UAS-Ctr1B* transgene used for ectopic expression of Ctr1B were described in previous studies (Egli *et al*, 2003; Zhou *et al*, 2003; Selvaraj *et al*, 2005). As all experiments were carried out in a *y w* background that is often used for *Drosophila* experiments, the term 'wild type' is meant to indicate wild-type status of the relevant genes of copper homeostasis. We used Oregon R *y w* strain in all our experiments. The full-length as well as the dominant-negative MTF-1 lacking the transcriptional activation domains C-terminal to the zinc fingers

was cloned into the widely used pUAST vector. The EP insertion into the *DmATP* locus was kindly provided by Marcel Zarske and Ernst Hafen. Overexpression of these transgene in the eye was performed by crossing to a GMR-Gal4 transgenic fly. Experimental procedures of copper shock and transfer experiments are indicated in the figure legends.

MtnA-EYFP reporter construct

The *MtnA* promoter (−446 to +74) was PCR amplified from genomic DNA using the primer pair 5′-CGG GAT CCA GGT ATG GGC TAT TTA GGC C-3′ and 5′-GGG ATG GCC CCA AAG GAT CTG-3′. The PCR product was cloned in pCasper4 vector containing the coding region of EYFP (Thummel and Pirrotta, 1992).

Quantification of metallothionein and Ctr1B transcripts, and metal measurements

Larvae were either continuously raised on the indicated type of food or transferred for 6 h to normal food or metal-supplemented food (for simplicity, the terms or symbols copper, cadmium, silver, zinc and iron are meant to refer to Cu^{2+} , Cd^{2+} , Ag^+ , Zn^{2+} and Fe^{3+} , respectively). Only third instar feeding larvae were used for analysis. Total RNA was extracted using the TRIzol reagent (Life Technologies). S1 nuclease mapping of transcripts with 50 µg of total RNA was performed as described previously (Weaver and Weissmann, 1979). The gels were developed using PhosphorImager (Molecular Dynamics) and bands were quantified. The signal from the endogenous *actin5c* gene was used for normalization of metallothionein transcript levels. Metal measurements were made as described elsewhere (Egli *et al*, 2006b).

Drosophila protein extracts and Western blot analysis

Transgenic flies harboring the full-length Ctr1B ORF fused to the EGFP-coding sequence (designated as AH3; Selvaraj *et al*, 2005) were allowed to grow on NF or food supplemented with 250 µM BCS or 250 µM copper. About 30–50 third instar larvae were taken for homogenization in a buffer containing 20 mM Hepes pH 7.5, 100 mM KCl, 5% glycerol, 100 mM EDTA, 0.1% Triton X-100, aprotinin (5 µg/ml), leupeptin (5 µg/ml), 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 2.5 mM dithiothreitol (DTT). The mixture was spun, the supernatant was collected, 50 µg of the extracts was resolved by 11% SDS-PAGE and Western blotting was performed according to standard procedures. The mouse anti-GFP was used at 1:2500 dilution (BD Biosciences, USA). For the GFP control, *Drosophila* S2 cells were transfected with pAc-GFP expression vector and whole-cell extracts were prepared 72 h after transfection. Blots were visualized using the ECL kit (Amersham Biosciences, Sweden).

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Imaging and microscopy

Images were taken with a Leica MZ FLIII fluorescence stereomicroscope and a Nikon COOLPIX950 digital camera for whole larvae. Eyes were photographed using a Leica MZ16 stereomicroscope equipped with a Leica DFC280 camera and scanning electron microscopy images were taken with a JEOL JSM-6360LV scanning electron microscope. Copper cell luminescence and fluorescent protein expression in dissected larval guts were analyzed with a Leica DMRB fluorescence stereomicroscope equipped with the filters A for DAPI and copper cell luminescence and I3 for EGFP and EYFP, respectively. Pictures were taken with a Zeiss Axiocam. Confocal images were taken with a Leica SP1 UV CLSM.

Food choice and copper donation experiments

About 25–40 third instar larvae were placed on 5 cm Petri dishes. These contained two halves consisting of normal food or metal-supplemented food (CuSO_4 , CuCl_2 , AgNO_3 , ZnCl_2 , FeCl_3 , CdSO_4). One liter of normal food consisted of 15 g agar, 16.6 g sugar (D-glucose), 10 g wheat, 25 g yeast and 330 ml fruit juice (90% apple juice and 10% pear juice). The agar concentration was chosen relatively high to prevent the rapid disappearance of larvae in the substrate.

For copper transfer studies, in each plastic vial, 20–30 wild-type adult flies were placed on either normal food or food containing 1 mM CuSO_4 and allowed to lay eggs. The resulting progeny that grew up in normal food or copper-supplemented food was designated as G1_{NF} and G1_{Cu} , respectively. A total of 20–30 adult flies of each G1_{NF} and G1_{Cu} were placed on food containing copper chelator BCS for egg deposition, and were removed from the vials after 12 h. The number of eggs in each vial was determined and 10 days later the eclosed flies in that vial were counted. Similarly, G2 flies raised in BCS were transferred to a new vial with food, allowed to lay eggs and removed after 12 h. Again, the ratio of flies (G3) eclosing per originally deposited eggs was determined.

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