RESEARCH ARTICLE

Iron depletion in the intestines of Malvolio mutant flies does not occur in the absence of a multicopper oxidase

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SUMMARY

Malvolio (Mvl) encodes the sole Drosophila melanogaster homologue of divalent metal transporter-1 (DMT1). The Drosophila transporter has been implicated in iron, manganese and copper cellular import. Indeed, the extent of metal specificity for this family of transporters is still under investigation in many eukaryotic species. Here, we revisit metal accumulation in Mvl mutants raised under normal and metal-supplemented diets. We found iron deficiency in Mvl mutant flies, whereas whole body copper and manganese concentrations remained unaltered. Iron supplementation restored total body iron concentrations in Mvl mutants, but without replenishing iron stores in the middle midgut, suggesting a role for Mvl in systemic iron trafficking, in addition to a role in intestinal iron absorption. Interestingly, dietary copper sulphate supplementation further exacerbated the iron deficiency. We investigated whether dietary copper affected iron storage through the function of an insect multicopper oxidase (MCO), because the mammalian MCO ceruloplasmin is known to regulate iron storage in the liver. We identified a Drosophila MCO mutant that suppressed aspects of the Mvl mutant phenotype and most notably Mvl, MCO3 double mutants showed normal intestinal iron storage. Therefore, MCO3 may encode an insect ferroxidase. Intriguingly, MCO3 mutants had a mild accumulation of copper, which was suppressed in Mvl mutants, revealing a reciprocal genetic interaction between the two genes.

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INTRODUCTION

All living organisms frequently face changes in the concentration of essential micronutrients, including transition metals, present in their environments. Iron, copper and manganese play crucial roles in bioenergetics as cofactors of proteins mediating electron transfer reactions. A concentration imbalance as a result of genetic disorders, contaminated environments or malnutrition may lead to adverse health consequences in animals and humans (Wright and Baccarelli, 2007). Specialized, potent homeostatic mechanisms regulate the uptake of each metal and its storage and distribution in differentiated tissues during organismal development and adult life (Kambe et al., 2008). Such homeostatic regulation is apparently independent for each metal. Despite each metal having unique biochemical specificities (Waldron et al., 2009), there are notable interdependencies in terms of organismal acquisition, best documented in the case of copper and iron (Fox, 2003). In the absence of adequate dietary copper mammals develop anaemia because of compromised cellular iron mobilization, which depends on the plasma multicopper ferroxidases, ceruloplasmin and hephaestin (Chen et al., 2009; Harris et al., 1999; Hart et al., 1928; Osaki et al., 1966; Vulpe et al., 1999). Furthermore, iron absorption depends on divalent metal transporter-1 (DMT1), lack of which also results in anaemia (Beaumont et al., 2006; Donovan et al., 2002; Fleming et al., 1997; Gunshin et al., 1997; Iolascon et al., 2006; Mims et al., 2005). Iron usage in higher animals is geared towards the continuous generation of red blood cells whose principle role is the exchange of oxygen and carbon dioxide in peripheral tissues. Therefore, the first signs of an iron deficiency almost always manifest in an anaemic phenotype.

Insects, however, possess an open circulatory system fulfilling physiological gas exchanges and do not require significant, constant supplies of iron for haemoglobin biosynthesis (Beitel and Krasnow, 2000). As a consequence, some aspects of systemic iron homeostasis, defined as all movement of iron in the organism, and regulatory mechanisms that control this movement maintaining an overall stable condition (Hentze et al., 2004; Nichol et al., 2002), may differ between invertebrates and vertebrates. Nevertheless, key proteins involved in iron regulation have conserved functions as exemplified by the ferritins (Missirlis et al., 2006; Missirlis et al., 2007; Pham and Winzerling, 2010), the iron regulatory proteins (Lind et al., 2006) and the transferrins (Dunkov and Georgieva, 2006; Tiklović et al., 2010). We became interested in the DMT1 homologue in Drosophila melanogaster, encoded by the Mvl gene, originally identified in a mutagenesis screen designed to identify genes affecting taste behaviour in flies (Rodrigues et al., 1995). A P-element inserted 313bp 5' to the translation initiation site in the MvlP70 strain caused Mvl mutants to lose their preference for sugar (Rodrigues et al., 1995). Mvl was implicated in metal transport when Mvl mutants reared on diets supplemented with iron or manganese recovered their normal taste behaviour (Orgad et al., 1998). The uptake (olfactory) of manganese has also been shown to depend on DMT1 in rats (Thompson et al., 2007). The Drosophila study also demonstrated that rearing Mvl mutants on diets supplemented simultaneously with zinc and manganese did not recover normal taste behaviour, an intriguing observation that is still not understood mechanistically (Orgad et al., 1998). Explaining the impact of dietary metals on the behaviour of Mvl mutants is even more exciting in view of the...
impressive finding that similar dietary manipulations can affect honeybee division of labour, presumably through a Mvl-dependent pathway (Ben-Shahar et al., 2004; Denison and Raymond-Delpech, 2008).

The Mvl protein has been detected in the gut, Malpighian tubules, brain and testis, a pattern consistent with that of DMT1 homologues in vertebrates, suggesting that components of iron acquisition between flies and mammals might be conserved through evolution (Folwell et al., 2006). Indirect support for this proposition was provided by experiments showing increased iron incorporation in Xenopus oocytes when injected with a mosquito DMT1 homologue (Martínez-Barnetche et al., 2007). More recently, Mvlβ7 mutants were reported to be sensitive to high dietary copper concentrations (Southon et al., 2008). Intracellular copper content correlated with Mvl expression levels in cultured Drosophila cells suggesting that Mvl may also function as a copper transporter (Southon et al., 2008). Given the ambiguity regarding the in vivo role(s) of Mvl in metal trafficking and of DMT1 homologues in other organisms (Au et al., 2008; Garrick et al., 2003), we measured metal accumulation in adult Mvlβ7 mutant flies subjected systematically to 1 mmol l–1 CuSO4, 1 mmol l–1 MnCl2, 1 mmol l–1 ZnCl2 or 1 mmol l–1 ferric ammonium citrate (FAC). Our findings identify that only the accumulation of iron is affected in adult Mvlβ7 mutant flies; they also led us to reconsider the interaction between copper and iron in Drosophila. Drosophila multicopper oxidases (MCOs) have not been tested in vertebrates, suggesting that components of iron acquisition pathway (Ben-Shahar et al., 2004; Denison and Raymond-Delpech, 2008). More recently, Mvl97f mutants were reported to be sensitive to high dietary iron incorporation in liquid nitrogen and stored at ~80°C. Samples were freeze-dried for 24 h and their dry mass was measured. Dried flies (100 mg) were acid digested by adding 1.5 ml of 69% nitric acid (HNO3) and incubating at 50°C for 4 h, then at 100°C for another 4 h, followed by cooling down overnight. Acid-digested samples were diluted with distilled water and the metal content was determined by using an AA Analyst 200 Flame Atomic Absorption Spectrophotometer (Varian Ltd, Yarnton, Oxfordshire, UK). Standards of each metal were used to calibrate the spectrophotometer and calculate metal concentrations in all samples.

Statistical analysis of metal measurements
For paired comparisons, two-tailed, paired student’s t-tests were used. For multiple comparisons, one-way ANOVA was performed. P<0.05 was considered statistically significant.

ANOVA for each metal measurement in all eight treatments indicated, in many instances, a lack of significance between samples, whereas in two-tailed paired t-tests these measurements showed differences with high levels of confidence (P<0.005; supplementary material Table S1), suggesting a source of error in measurements performed at different times. To evaluate this situation, which might mask significant differences of biological origin, we compared the mean values determined for iron, copper, manganese and zinc between treatments that did not interact with the measured values for a given metal. We excluded the treatments where the metal we measured was added to the diet and in the case or iron measurements the diets containing copper, which has an effect on iron concentration. We then employed ANOVA to test for significant differences between genotypes for each metal measured; in this test each average value from non-supplemented diets served as an independent data point. The results showed statistically significant differences and were therefore followed by Tukey’s test to identify which pairwise comparisons were significantly different (Fig. 5 and supplementary material Table S1).

Quantitative reverse transcriptase polymerase chain reaction
5-day-old male flies were collected and frozen in liquid nitrogen and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed as described previously (Gutierrez et al., 2010). The gene-specific primers used were (5’ to 3’ orientation): Mvl forward/sense GCCATTGTTAAGTCCCG, Mvl reverse/ antisense GCCATACATGCATGGGC; MCO3 forward GCAACAAGAGCTCCCTGCGGG, MCO3 reverse GCTCCTGAATTGTGCAAATCG; Rp49 forward CGATATGCTAAGCTTGTCGACA, Rp49 reverse CGCTTTTGGATTGGTAACC.

The relative transcript level (RTL) values were calculated relative to the amount of transcript of the constitutively expressed Rp49 gene. Experiments were performed on five biological replicates (three flies per sample).

Iron stain of non-reducing SDS-polyacrylamide gel
For detection of intact iron-loaded ferritin, a non-reducing SDS-polyacrylamide gel was used and subsequently stained by using an acid solution of ferrocyanides. A buffer without reducing agents (0.02 mol l–1 Tris-HCl, 0.137 mol l–1 NaCl, 1% Triton X-100, 1% glycerol) was used to isolate protein from whole fly homogenates. The samples were loaded in a 7% polyacrylamide gel and run overnight (52 mA, 25 W). Following electrophoresis the gel was stained overnight with 2% K3Fe(CN)6 and 2% HCl at room temperature. The iron in the samples was visible as blue bands. Pictures were taken using a Nikon digital camera (20×).
Iron staining and microscopy
The iron staining and microscopy procedures have been described previously in detail (Mehta et al., 2009). Briefly, intestines from third instar larvae were dissected in phosphate-buffered saline (PBS), fixed in 4% formaldehyde in PBS for 30 min and permeabilised with 1% Tween 20 in PBS for 15 min. To detect the ferric iron, the tissues were incubated in the dark with Prussian Blue stain [2% K4Fe(CN)6 and 2% HCl] for 45 min and then washed five times with distilled water. The midguts were mounted in water on slides, visualized and imaged directly by using a Leitz Orthoplan fluorescent microscope coupled to an Olympus DP71 microscope camera. The light exposure was kept constant throughout the imaging process. As a rule we dissected five larvae per genotype per condition and repeated experiments a minimum of three times; variations in staining were small and, overall, the images presented are highly representative. Larvae were used as they have been studied more extensively in the past (Mehta et al., 2009; Poulsone and Bowen, 1952) and because mutants that do not survive to adulthood can be studied (Gutierrez et al., 2010).

RESULTS
Given the controversy surrounding the function of Mvl in metal transport, we compared metal content in the widely used MvlP77 homozygotes [generated on a white (w) mutant background] to w mutant, but otherwise wild-type flies. We reasoned that, unless potent compensatory mechanisms were in place, we would be able to identify deficiencies for those metals that depend primarily on the Mvl protein (Drosophila DMT1) for cellular import. We used adult populations of mixed sex and only collected flies 4–7 days post-eclosion, because, at least in the cases of copper and iron, ageing flies accumulate metals (Massie et al., 1980; Massie et al., 1985). Cohorts of flies were raised to adults on diets including 1 mmol l−1 concentrations of each metal, which they tolerated well (see Materials and methods). Strikingly, MvlP77 loss-of-function mutant flies consistently exhibited an iron-deficient phenotype (Fig. 1A), but were otherwise comparable with control flies with respect to copper, manganese or zinc concentrations (Fig. 1B–D), suggesting a major function for Mvl in iron import. This is consistent with findings in Anopheles albimanus (Martinez-Barrochet et al., 2007) and the anaemic phenotypes in mutants of vertebrate DMT1 homologues (Beaumont et al., 2006; Donovan et al., 2002; Fleming et al., 1997; Iolascon et al., 2006; Mims et al., 2005). Additionally, we found that administration of 1 mmol l−1 CuSO4 not only led to a dramatic tenfold accumulation of copper (Fig. 1B) but also decreased iron accumulation in w control flies (Fig. 1A). The enrichment in ferritin in both control and MvlP77 mutants.
decrease in total body iron was also seen in Mvl^P2/2 mutant flies, but they accumulated iron to similar levels as the controls when the diet was supplemented with 1 mmol l^-1 FAC (Fig. 1A).

As an independent readout of iron uptake we analyzed the incorporation of iron into ferritin, the major iron storage protein in Drosophila (Pham and Winzerling, 2010). We have previously demonstrated that iron-loaded ferritin remains stable under non-reducing conditions, in the presence of SDS (Missirlis et al., 2006; Missirlis et al., 2007). To test how much iron was stored in ferritin from Mvl^P2/2 mutant and w control flies raised in the different diets, we stained protein gels, loaded with lysates of homogenized adult males, with Prussian Blue to detect ferric iron (Fig. 1E). Mvl^P2/2 mutants accumulated less iron in ferritin than controls, except when fed on an iron-enriched food. However, we also observed a decrease in iron-loaded ferritin content following dietary copper supplementation, in both control and Mvl^P2/2 mutant flies (Fig. 1E). Therefore, iron loading in ferritin closely mirrored total body iron measurements in all conditions tested.

Drosophila ferritin is predominantly found in the haemolymph, but it is also stored in the iron region of the intestine (Mehta et al., 2009; Poulson and Bowen, 1952). We examined the iron region of wandering third instar larvae to determine tissue iron loading of ferritin in situ (Fig. 2). In this experiment, we included two additional loss-of-function alleles, Mvl^KG02112^ and Mvl^EY09165^. Intestines from w and from Mvl^KG02112^ heterozygous control animals had readily detectable iron in their middle midgut (Fig. 2A,B). Dietary iron accumulates primarily in the anterior midgut (see inset, Fig. 2A) and copper in the diet reduces iron content of the middle midgut (right panels, Fig. 2). Intestines from all three Mvl mutants showed severely reduced iron content in the middle midgut (Fig. 2C–E). Surprisingly, dietary iron failed to rescue iron storage in this region, whereas in the anterior midgut ferritin induction and iron accumulation were normal (Fig. 2E, inset). This result points to a persistent systemic defect in Mvl mutants, even under conditions of apparent iron sufficiency achieved by iron supplementation, as judged by total body iron measurements and iron staining in the anterior midgut and iron loading of ferritin. Finally, addition of copper to the diet reduced iron content in intestines from Mvl mutants below levels of histochemical detection. Thus, independent evidence confirms both the relative iron-deficiency that results as a consequence of Mvl loss of function and the impact of dietary copper on whole fly iron homeostasis.

We next considered how dietary copper caused this reduction in iron accumulation. Reciprocally, mice lacking copper transporter 1 (Ctr1) in intestinal epithelial cells exhibit defects in iron accumulation in peripheral tissues and hepatic iron overload, the latter phenotype is probably related to decreased ceruloplasmin activity due to copper deficiency (Nose et al., 2006). We wondered whether dietary copper could therefore induce activity by protein metallation of a ceruloplasmin-like protein in Drosophila, leading to increased iron mobilization from ferritin stores, thereby signalling a reduction in intestinal absorption. Ceruloplasmin and its close homologue hephaestin belong to a family of MCOs, conserved in fungi and metazoans, that has the ability to oxidize ferrous iron (Kosman, 2010). However, ferroxidase activity has not yet been demonstrated in insects, although MCO homologues have been...
Metal homeostasis in Mvl and MCO3 mutants

We reasoned that should one of the Drosophila MCOs function as a ferroxidase it would conserve features of the iron nucleation site found in close proximity to the MCO T1 copper centre (Kosman, 2010; Taylor et al., 2005). Glutamate at position 291 and aspartate at position 992 in human ceruloplasmin provide ligands for iron, but only the latter is found in suitable proximity to a more complex and sufficiently conserved domain (shown in Fig. 3A). Interestingly, only Drosophila melanogaster MCO3 (CG5959) has a similarly positioned aspartate, a residue conserved in the majority of other Drosophila species so far sequenced. MCO3 has a predicted signal peptide sequence followed directly by a predicted transmembrane helix at the N-terminus (residues 28–48), a feature shared with hephaestin (Vulpe et al., 1999). Consistently, no putative GPI anchor, which would have been evocative of ceruloplasm, was predicted in the protein sequence. The protein also contains a motif thought to be involved in transport from the cell surface to the Golgi (YQRL) at position 123, and a predicted signal peptide cleavage site directly before the transmembrane domain between residues 27 and 28. All these features are consistent with a protein targeted to the secretory pathway.

A transposable element disrupts the open reading frame of MCO3 at amino acid 550, roughly in the middle of the protein sequence, generating a predicted null mutation that we designate MCO3C359 (Bellen et al., 2004) (Fig. 3B). MCO3C359 homozygous flies are viable and fertile. Quantitative RT-PCR of MCO3 mRNA transcripts indicated a clear and significant reduction in their expression in the homozygous MCO3C359 mutants compared with w control flies or Mvl97f mutants (Fig. 3C). We also tested Mvl expression, which was found to be consistently lower in homozygous Mvl97f mutants. The experiment also served as a validation that the recombinant, double-mutant stock y;w; MCO3C359, Mvl97f was generated successfully, as expression of both genes was significantly reduced in these flies (Fig. 3C).

A key prediction of the hypothesis that MCO3 encodes an insect multicopper ferroxidase responsible for iron mobilization from tissue stores is that MCO3C359 mutants should have increased iron storage in the iron region, mimicking the phenotype in mice lacking ceruloplasmin (Chen et al., 2009; Harris et al., 1999; Nose et al., 2006). Our results are consistent with such an interpretation, and further demonstrated in larvae grown on copper-enriched medium (Fig. 4). Iron regions from control larvae show very weak staining when raised on dietary copper, but staining is substantial in iron regions from MCO3C359 homozygous and transheterozygous MCO3C359 over Df(3R)BSC140 animals (the latter is a large deficiency that uncovers MCO3 along with 55 unrelated genes; see Materials and methods) raised on the same high-copper diet (Fig. 4C, D). Therefore, it seems probable that MCO3 influences iron storage in the middle midgut. More importantly, MCO3C359, Mvl97f double mutants were markedly different from Mvl97f single mutants (compare Fig. 4B with E), indicating that lack of MCO3 could rescue the iron depletion observed in the middle midgut of Mvl97f mutants. This result identifies the first genetic suppressor of Mvl and strongly implicates MCO3 in systemic iron homeostasis, making it a good candidate for a ceruloplasm-in-like homologue.

Next, we determined the metal content in MCO3C359 single mutants and in MCO3C359 Mvl97f double mutants and compared it with that of w controls and Mvl97f mutants (supplementary material Table S1 and Fig. 5). MCO3C359 mutants did not differ in their total iron content when compared with w control flies, but had higher copper and lower manganese accumulation compared with either w or Mvl97f flies. Zinc levels were the same in all conditions tested in this experiment. Strikingly, feeding MCO3C359 mutants on high copper led to significantly lower copper accumulation than in controls (supplementary material Table S1), suggesting that MCO3 mutations affect copper homeostasis differentially under opposing dietary copper conditions, a feature of other copper regulatory genes (Balamurugan et al., 2007).

The metal content of MCO3C359, Mvl97f double mutants was also examined (Fig. 5, brown bars). MCO3C359, Mvl97f double mutants did not show statistically significant differences in iron content when compared with either Mvl97f single mutants (which are low in iron) or w controls (see also supplementary material Table S1). In contrast, MCO3C359, Mvl97f double mutants had normal copper content, suggesting in this instance that Mvl loss of function could suppress the MCO3-related copper accumulation phenotype. Finally, MCO3C359, Mvl97f double mutants had low manganese content, similar to the MCO3C359 single mutant. The experiments with the
iron, copper and manganese. Interacting genes in the homeostasis of all three transition metals, raised in the different metal-supplemented diets implicate the two stores. Copper supplementation reduced iron content below the detection level of the Mvl mutant raised under different metal-supplemented diets that strongly affected body iron content to levels seen in control flies (Fig. 1A), suggesting that iron can also be absorbed through other routes. Nonetheless, alternative iron transporters are inadequate to compensate for the loss of Mvl under standard dietary conditions. More intriguingly, iron supplementation failed to restore iron levels in the iron region of the Mvl mutant (Fig. 2C–E). This result suggests that the transporters that mediate iron absorption under high dietary iron cannot equally well traffic iron in the systemic circulation, leading to decreased iron storage in the middle midgut, despite normal induction of ferritin in the anterior midgut seen in the mutants. That Mvl functions in more than one tissue was expected on the basis of its complex expression pattern, previously described (Folwell et al., 2006; Rodrigues et al., 1995). Expression in the nervous system is likely to be important for behaviour (see below), expression in the testes may be linked to a recently described role of iron in spermatogenesis (Metzendorf and Lind, 2010), and expression in the Malpighian tubules may reveal that function in iron homeostasis, in addition to osmoregulation and other metabolic functions (Dow, 2009).

We also raised Mvl mutants and w control flies on a variety of transition metal-supplemented diets, hoping to discover why zinc suppresses the rescue of Mvl mutant behavioural phenotypes (Orgad et al., 1998). However, Mvl mutants reacted identically to w controls to the addition of manganese, and manganese and zinc in the diet (supplementary material Table S1). Furthermore, manganese and iron content did not differ in Mvl mutants raised on high manganese with or without added zinc. From this analysis, we could not identify a reason for the alterations in behaviour based on total body metal accumulations. Study of local interactions of metals in neurons of the peripheral nervous system would require higher resolution techniques. Somewhat more interestingly, dietary iron brought total body iron content of Mvl to the level of w controls (a rescue), but this increase was suppressed in the presence of zinc (P<0.05 in paired \( t \)-test; supplementary material Table S1). Hence, iron accumulation in the absence of Mvl can be inhibited by zinc. Competition with zinc for cellular entry through zinc transporters (Wang and Zhou, 2010) might explain this observation.

**DISCUSSION**

**Mvl functions in Drosophila iron import**

Mvl has been suggested to transport nitrate (Rodrigues et al., 1995), manganese (Orgad et al., 1998), iron (Folwell et al., 2006; Orgad et al., 1998) and copper (Southon et al., 2008) across the plasma membrane. We undertook a systematic metal analysis of the Mvl\(^{97f} \) mutant raised under different metal-supplemented diets that strongly suggested Mvl participates in Drosophila iron homeostasis (Figs 1 and 5 and supplementary material Table S1). Total body iron content of the Mvl\(^{97f} \) mutant was approximately 75% that of normal flies, causing reduced iron loading in circulating and intestinal ferritin (Fig. 1E and Fig. 2). The conclusion that Mvl primarily functions in iron transport is consistent with the established function of DMT1 in higher animals (Beaumont et al., 2006; Donovan et al., 2002; Fleming et al., 1997; Iolascon et al., 2006; Mims et al., 2005) and with a study using the mosquito Anopheles albinanus DMT1 homologue in a metal transport assay (Martínez-Barnetche et al., 2007). Our data provide further experimental evidence that components of the iron acquisition process are evolutionarily conserved between flies and mammals.

We saw no differences in manganese or copper concentrations in Mvl mutant flies compared with w control flies (Fig. 1B,C). However, our results do not exclude a role of Mvl in the trafficking of these metals, because other proteins may compensate for the loss of Mvl. For copper, these could be the Ctr1 proteins (Steiger et al., 2010; Turski and Thiele, 2007; Zhou et al., 2003), whereas specific manganese transporters have not yet been identified. Iron supplementation restored total body iron content to levels seen in control flies (Fig. 1A), suggesting that iron can also be absorbed through other routes. Nonetheless, alternative iron transporters are inadequate to compensate for the loss of Mvl under standard dietary conditions. More intriguingly, iron supplementation failed to restore iron levels in the iron region of the Mvl mutant (Fig. 2C–E). This result suggests that the transporters that mediate iron absorption under high dietary iron cannot equally well traffic iron in the systemic circulation, leading to decreased iron storage in the middle midgut, despite normal induction of ferritin in the anterior midgut seen in the mutants. That Mvl functions in more than one tissue was expected on the basis of its complex expression pattern, previously described (Folwell et al., 2006; Rodrigues et al., 1995). Expression in the nervous system is likely to be important for behaviour (see below), expression in the testes may be linked to a recently described role of iron in spermatogenesis (Metzendorf and Lind, 2010), and expression in the Malpighian tubules may reveal that function in iron homeostasis, in addition to osmoregulation and other metabolic functions (Dow, 2009).

Dietary copper reduces Drosophila iron stores

Another interesting observation relates to the observed loss of iron accumulation upon feeding on a copper-enriched diet (Figs 1, 2, 4, 5 and supplementary material Table S1). This phenomenon was described almost 60 years ago: “In Drosophila melanogaster the amount of cytoplasmic iron demonstrable in midgut cells is profoundly affected by the copper concentrations of the medium, even if it does not seem to be influenced by other heavy metals. With increasing copper concentrations, less Fe\(^{2+} \) is found in the cytoplasm” (Poulson and Bowen, 1952). At the time, the finding was solely based on the decrease seen in ferric iron stored in the middle midgut. We showed that copper exerts a more profound, quantifiable effect on total body iron. We are keen to understand the mechanism underlying this interaction and in this study we have excluded some of the possible

**Fig. 4. MCO3 prevents iron depletion in intestines of Mvl mutant flies.**

Prussian Blue staining was performed on dissected intestines of wandering third instar larvae; the iron region is shown. (A) Dietary copper reduced intestinal iron storage in control flies. (B) Mvl\(^{97f} \) mutants had depleted iron stores. Copper supplementation reduced iron content below the detection limit. (C,D) MCO3\(^{C359} \) homozygous larvae and MCO3\(^{C359}/Df(3R)BSC140 \) transheterozygous larvae had intense iron staining, which was reduced by dietary copper. (E) The MCO3\(^{C359}, Mvl^{97f} \) double mutant larvae accumulated iron in contrast to the Mvl\(^{97f} \) single mutant (shown in B).
explanations. As Mvl can transport excess copper into cells (Southon et al., 2008), one might expect negative feedback regulation that would lower levels of the functional transporter and lead to iron deficiency. However, we demonstrated that dietary copper significantly decreased the total iron content of the body in the 

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Mvl and MCO3 mutants participate in the downstream signalling events that reduce iron accumulation in vertebrate and invertebrate models. In vertebrates, the iron regulatory proteins (IRPs) play a key role in iron homeostasis by regulating the translation of ferritin mRNAs. However, in Drosophila, the IRPs are not involved in iron homeostasis, suggesting that other mechanisms are used to regulate iron metabolism.

**List of abbreviations**

- DMT1: divalent metal transporter-1
- FAC: ferric ammonium citrate
- MCO: mult铜opper oxidase
- Mvl: Malvolio
- w: white

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REFERENCES

Askwith, C., Eide, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M., Brownlie, A., Dorschner, M. O., Zhou, Y., Pratt, S. J., Paw, B. H., Iolascon, A., d’Apolito, M., Servedio, V., Cimmino, F., Piga, A. and Camaschella, C. (2004). Balancing acts: division of labor. J. Exp. Biol. 276, 2381-2389.

Askwith, C., Eide, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M., Brownlie, A., Dorschner, M. O., Zhou, Y., Pratt, S. J., Paw, B. H., Iolascon, A., d’Apolito, M., Servedio, V., Cimmino, F., Piga, A. and Camaschella, C. (2004). Balancing acts: division of labor. J. Exp. Biol. 276, 2381-2389.

Beitel, G. J. and Krasnow, M. A. (2008). The genetics of essential metal homeostasis during development. Genesis 46, 180-193.

Ben-Shahar, Y., Dudek, N. L. and Robinson, G. E. (2007). Copper homeostasis in Drosophila through cooperation of the ICHS and SMCYR1 genes. J. Exp. Biol. 210, 3271-3282.

Bell, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, R. P., Schulze, K. L., Rubin, G. M. et al. (2004). The BDGP gene dissection project: single transposon insertions associated with 40% of Drosophila melanogaster genes. Genetics 167, 761-781.

Ben-Shahar, Y., Dudek, N. L. and Robinson, G. E. (2007). Phenotypic deconstruction reveals involvement of manganese transporter malvolio in honey bee division of labor. J. Exp. Biol. 207, 3281-3288.

Ben-Shahar, Y., Dudek, N. L. and Robinson, G. E. (2007). Phenotypic deconstruction reveals involvement of manganese transporter malvolio in honey bee division of labor. J. Exp. Biol. 207, 3281-3288.

Beitel, G. J. and Krasnow, M. A. (2006). The genetics of essential metal homeostasis during development. Genesis 46, 180-193.

Bellen, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, R. P., Schulze, K. L., Rubin, G. M. et al. (2004). The BDGP gene dissection project: single transposon insertions associated with 40% of Drosophila melanogaster genes. Genetics 167, 761-781.

Ben-Shahar, Y., Dudek, N. L. and Robinson, G. E. (2007). Phenotypic deconstruction reveals involvement of manganese transporter malvolio in honey bee division of labor. J. Exp. Biol. 207, 3281-3288.

Bell, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, R. P., Schulze, K. L., Rubin, G. M. et al. (2004). The BDGP gene dissection project: single transposon insertions associated with 40% of Drosophila melanogaster genes. Genetics 167, 761-781.

Ben-Shahar, Y., Dudek, N. L. and Robinson, G. E. (2007). Phenotypic deconstruction reveals involvement of manganese transporter malvolio in honey bee division of labor. J. Exp. Biol. 207, 3281-3288.