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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; Tiklová 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 313 bp 5′ to the translation initiation site in the Mvl^{97f} 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, Mvl^{97f} 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 Mvl^{97f} mutant flies subjected systematically to 1 mmol l^{-1} $CuSO_4$, 1 mmol l^{-1} $MnCl_2$, 1 mmol l^{-1} $ZnCl_2$ or 1 mmol l^{-1} ferric ammonium citrate (FAC). Our findings identify that only the accumulation of iron is affected in adult Mvl^{97f} 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 biochemical assays for ferroxidase activity (Dittmer and Kanost, 2010). In the work reported here we used a genetic approach that implicated one of the four *Drosophila* MCOs, MCO3, as a putative ceruloplasmin-like homologue. We determined the metal composition of $MCO3^{C359}$ mutant flies and observed genetic interactions between $MCO3^{C359}$ and Mvl^{97f} mutants.

MATERIALS AND METHODS

Fly maintenance and dietary manipulations

Drosophila melanogaster Meigen 1830 were reared at 25°C on a standard diet containing: agar (6.5%), sucrose (9.7%), glucose (21.3%), yeast (22.6%), maize (9.7%), treacle (19.3%), soya flour (4.6%), propionic acid (0.5%) and nipagin (0.01%). Metals were added to the normal food as indicated in the form of ferric ammonium citrate (FAC), copper sulphate ($CuSO_4$), zinc chloride ($ZnCl_2$) and manganese chloride ($MnCl_2$) to a final concentration of 1 mmol l^{-1}; vials containing the following double metal combinations were also prepared: FAC + $ZnCl_2$, $MnCl_2$ + FAC, $CuSO_4$ + FAC. All chemicals were purchased from Sigma-Aldrich (Gillingham, Dorset, UK).

Three *Mvl* mutant strains were obtained from the Bloomington Drosophila Stock Center: Bl#5151 w^{1118};; $P\{lacW\}Mvl^{97f}$, Bl#14419 y^1w^{67c23};; ry^{506}, $P\{SUPor\text{-}P\}Mvl^{KGO2112}$, and Bl#19886 y^1w^{67c23};; $P\{EPgy2\}Mvl^{EY09165}$, as was the stock carrying a P_{Bac} insertion in the *CG5959* (*MCO3*) gene Bl#16349 y^1w^{1118};; $P_{Bac}\{3HPy^+\}CG5959^{C359}/TM3$, Sb^1 Ser^1 and a deficiency stock with a chromosomal deletion of ~320 kb uncovering the *MCO3* locus Bl#9500 w^{1118};; $Df(3R)BSC140/TM6B$, Tb^+. Classical recombination was employed to generate the y^1w^{1118};; $MCO3^{C359}$, Mvl^{97f} flies.

Flame atomic absorption spectrometry

The metal concentrations of copper, iron, manganese and zinc in flies were determined by flame atomic absorption spectrometry. Both male and females flies were used because of the amount of sample required for each measurement: 100 mg dry mass. The sex ratio was normal in all stocks. 4- to 7-day old flies were collected, fast-frozen 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 (HNO_3) 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 AAnalyst 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 pair-wise 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 measure 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 GCCTTGGTTAAGTCCCG, *Mvl* reverse/antisense GCCATACATGCCATGGGC; *MCO3* forward GCAACAAGAGCTCCCTGGCCG, *MCO3* reverse GCTCCTGAATATTGCGCAAATCG; *Rp49* forward CGATATGCTAAGCTGTCGCACA, *Rp49* reverse CGCTTGTTCGATCCGTAACC.

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% $K_4Fe(CN)_6$ 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% $K_4Fe(CN)_6$ 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; Poulson 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 Mvl^{97f} 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, Mvl^{97f} 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* (Martínez-Barnetche 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} $CuSO_4$ not only led to a dramatic tenfold accumulation of copper (Fig. 1B) but also decreased iron accumulation in *w* control flies (Fig. 1A). The

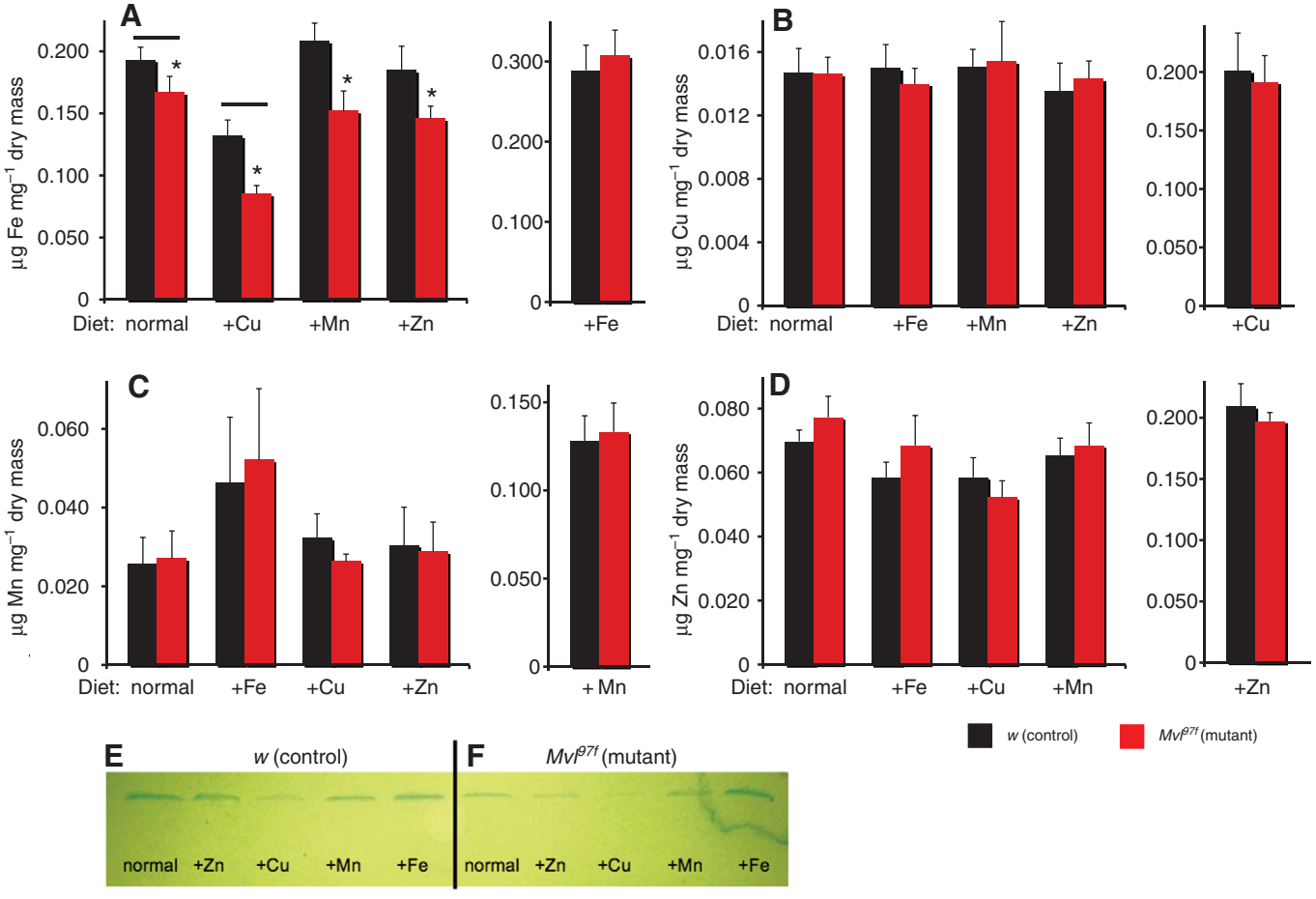

Fig. 1. Comparison of metal accumulation in *Mvl* mutant and *w* control flies raised on different diets. Flame atomic absorption spectroscopy was used for metal analysis; each value is an average of at least five independent measurements. (A) Adult Mvl^{97f} mutant flies are consistently lower in total body iron content when raised on normal diet, or diets supplemented with 1 mmol l^{-1} $CuSO_4$, 1 mmol l^{-1} $MnCl_2$ or 1 mmol l^{-1} $ZnCl_2$. *P<0.05, two-tailed paired Student's *t*-test. Supplementing the diet with 1 mmol l^{-1} ferric ammonium citrate (FAC) restored total body iron content in Mvl^{97f} mutants. Note also that 1 mmol l^{-1} $CuSO_4$ in the diet leads to a reduction in iron accumulation irrespective of genotype (P<0.0001 for both comparisons). Total copper content was no different between *Mvl* mutants and *w* control flies and increased tenfold if added to the diet. (C,D) Total manganese (C) and zinc (D) content is also not altered in Mvl^{97f} mutants. (E) Non-reducing SDS-PAGE of adult fly lysates followed by Prussian Blue staining revealed iron in ferritin. Dietary copper reduced iron accumulation in ferritin in both control and Mvl^{97f} mutants.

decrease in total body iron was also seen in Mvl^{97f} 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^{97f} 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^{97f} 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^{97f} 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 copper 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 belongs 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

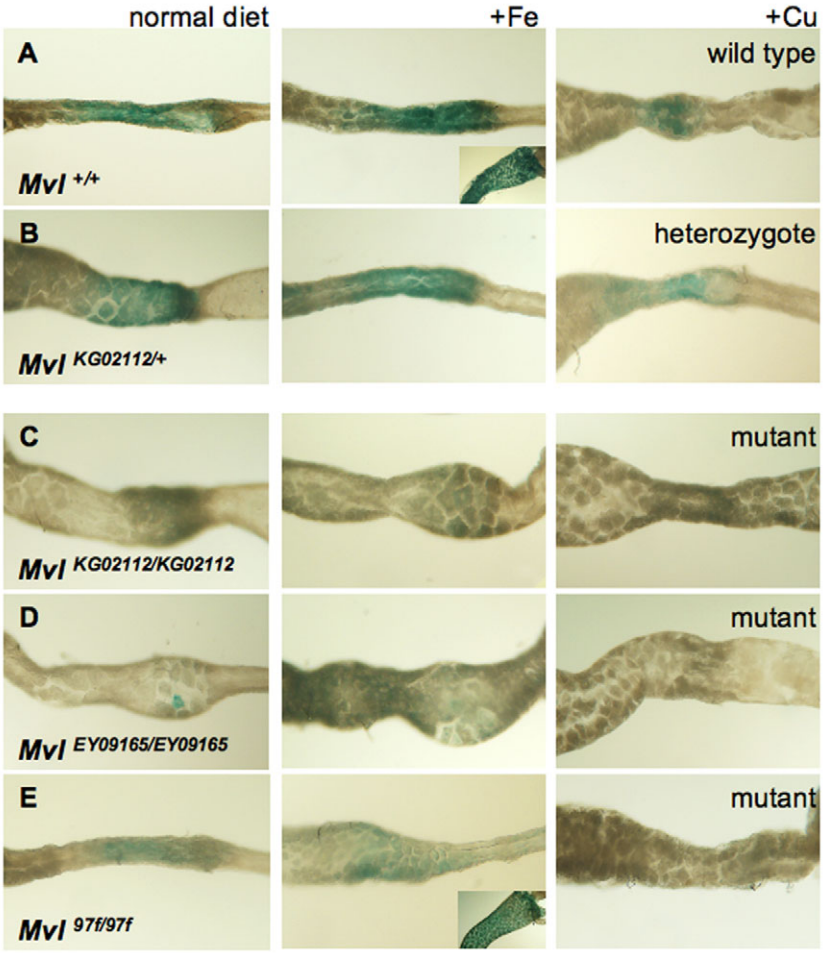

Fig. 2. *Mvl* mutants have iron-deficient intestinal stores even under conditions of dietary iron overload. Prussian Blue staining was performed on dissected intestines of wandering third instar larvae; the iron region is shown, except for the insets in the middle panels of A and E that show anterior midgut staining to demonstrate iron overload. (A) In control animals the iron region is easily identifiable under standard laboratory conditions; iron supplementation leads to accumulation of iron in the anterior midgut, whereas copper supplementation leads to a reduction of iron in the middle midgut (left panel). (B) A heterozygous strain of the $Mvl^{KG02112}$ mutant shows similar iron staining patterns as controls. (C–E) Three independent transposon-induced mutations in the *Mvl* locus result in decreased iron stores, a phenotype unaffected by dietary iron supplementation (middle panels) and enhanced by dietary copper (left panels). Importantly, dietary iron accumulates in the anterior midgut of *Mvl* mutants (shown in inset of E).

identified and in some cases partially characterized (Dittmer and Kanost, 2010). Most research on insect MCOs has focused on their role in sclerotization and pigmentation of the cuticle, a function typically carried out by laccases. The *Drosophila melanogaster* genome contains four MCO genes, which we have designated *MCO1–4* (Fig. 3A) based on phylogenetic conservation (MCO2 is predicted to be a laccase, but the substrate(s) of MCO1, MCO3 and MCO4 remain unclear) (Dittmer and Kanost, 2010).

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 ceruloplasmin, was predicted in the protein sequence. The protein

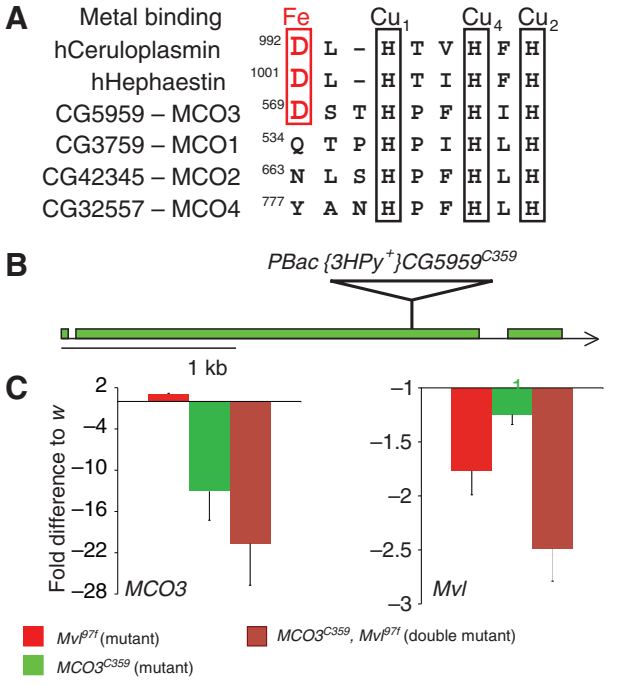

Fig. 3. Partial alignment of the four MCOs conserved in *Drosophila melanogaster* and validation of the $MCO3^{C359}$ allele. (A) The motif shown provides crucial ligands to all three copper binding sites, mononuclear T1, T2 (Cu_1, Cu_2) and binuclear T3 (Cu_4). In all known ferroxidases an aspartic acid is conserved in the vicinity and provides one of the binding sites for ferrous iron, adjacent to the T1 centre (shown here for the human ceruloplasmin and hephaestin proteins). Only one of the four *Drosophila* MCOs, encoded by *CG5959*, has a conserved aspartic acid in a relevant position, which was why this gene was selected for further study. (B) *MCO3* has two small introns and a transposable element disrupting the open reading frame of the gene. (C) qRT-PCR results from male adult *w* and Mvl^{97f} homozygous mutants (in red), $MCO3^{C359}$ homozygous mutants (green) and Mvl^{97f}, $MCO3^{C359}$ homozygous double mutants (brown). *MCO3* mRNA levels are dramatically reduced in the presence of the $MCO3^{C359}$ allele (left panel), whereas *Mvl* mRNA is moderately reduced in Mvl^{97f} mutants (right panel).

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 $MCO3^{C359}$ (Bellen et al., 2004) (Fig. 3B). $MCO3^{C359}$ 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 $MCO3^{C359}$ mutants compared with *w* control flies or Mvl^{97f} mutants (Fig. 3C). We also tested *Mvl* expression, which was found to be consistently lower in homozygous Mvl^{97f} mutants. The experiment also served as a validation that the recombinant, double-mutant stock *y,w;;* $MCO3^{C359}$, Mvl^{97f} 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 $MCO3^{C359}$ 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 $MCO3^{C359}$ homozygous and transheterozygous $MCO3^{C359}$ 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, $MCO3^{C359}$, Mvl^{97f} double mutants were markedly different from Mvl^{97f} single mutants (compare Fig. 4B with E), indicating that lack of MCO3 could rescue the iron depletion observed in the middle midgut of Mvl^{97f} 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 ceruloplasmin-like homologue.

Next, we determined the metal content in $MCO3^{C359}$ single mutants and in $MCO3^{C359}$, Mvl^{97f} double mutants and compared it with that of *w* controls and Mvl^{97f} mutants (supplementary material Table S1 and Fig. 5). $MCO3^{C359}$ 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 Mvl^{97f} flies. Zinc levels were the same in all conditions tested in this experiment. Strikingly, feeding $MCO3^{C359}$ 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 $MCO3^{C359}$, Mvl^{97f} double mutants was also examined (Fig. 5, brown bars). $MCO3^{C359}$, Mvl^{97f} double mutants did not show statistically significant differences in iron content when compared with either Mvl^{97f} single mutants (which are low in iron) or *w* controls (see also supplementary material Table S1). In contrast, $MCO3^{C359}$, Mvl^{97f} double mutants had normal copper content, suggesting in this instance that *Mvl* loss of function could suppress the *MCO3*-related copper accumulation phenotype. Finally, $MCO3^{C359}$, Mvl^{97f} double mutants had low manganese content, similar to the $MCO3^{C359}$ single mutant. The experiments with the

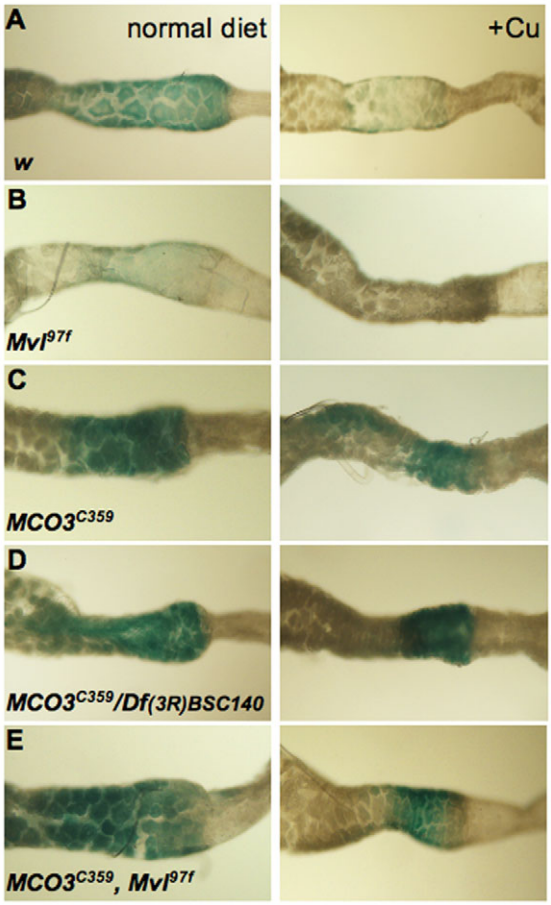

Fig. 4. *MCO3* prevents iron depletion in intestines of *Mvl* mutant flies. Prussian Blue staining was performed on dissected intestines of wandering third instar larvae; the iron region is shown. (A) Dietary copper reduced intestinal iron storage in control flies. (B) Mvl^{97f} mutants had depleted 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).

single mutants, Mvl^{97f} and $MCO3^{C359}$, and the double mutant flies raised in the different metal-supplemented diets implicate the two interacting genes in the homeostasis of all three transition metals, iron, copper and manganese.

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 *w* control 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 albimanus* 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 testis may be linked to a recently described role of iron in spermatogenesis (Metzendorf and Lind, 2010), and expression in the Malpighian tubules may reveal they 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.

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^{3+} 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

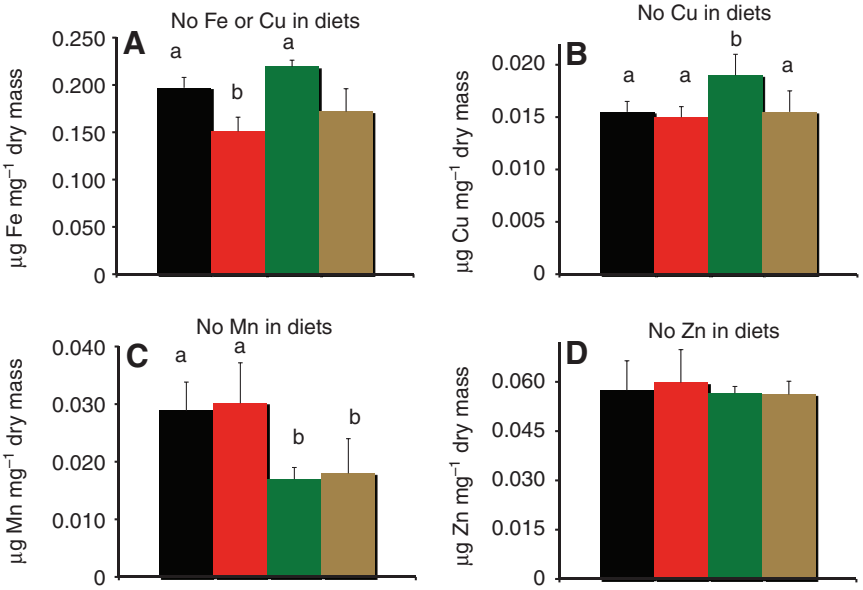

Fig. 5. Comparison of metal accumulation in *w* (black bars), Mvl^{97f} (red bars), $MCO3^{C359}$ (green bars) and Mvl^{97f}, $MCO3^{C359}$ (brown bars) adult flies raised on different diets. Values are the average of pooled metal determinations from independent dietary treatments (see Materials and methods). Statistically significant differences ($P<0.05$) following one-way ANOVA and Tukey's test are indicated by different letters. (A) Mvl^{97f} mutants show a statistically significantly reduction in iron concentration when compared with *w* and $MCO3^{C359}$ flies, but the iron content of *w* and Mvl^{97f} is not statistically significantly different from that of the double mutant. (B) $MCO3^{C359}$ mutants show statistically significant higher copper concentration compared with *w* and Mvl^{97f} flies; Mvl^{97f} loss of function rescues this phenotype. (C) $MCO3^{C359}$ and $MCO3^{C359}$, Mvl^{97f} flies show statistically significant lower manganese concentration compared with *w* and Mvl^{97f} flies. (D) There are no differences with respect to zinc concentrations in the four genotypes.

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 Mvl^{97f} mutant, thus ruling out this mechanism.

Is MCO3 a *Drosophila* ferroxidase?

Alternatively, a copper-containing protein might mediate the effect of copper on iron homeostasis. An obvious candidate would be a multicopper ferroxidase, such as the yeast homologue involved in cellular iron import (Askwith et al., 1994; Dancis et al., 1994) and mammalian homologues functioning in cellular iron export (Chen et al., 2009; Harris et al., 1999; Osaki et al., 1966; Vulpe et al., 1999). We have identified a candidate protein that may act as a *Drosophila* ferroxidase, but as it is not uncommon for enzymes to defy homology-based predictions of their activity (Kanzok et al., 2001; Missirlis et al., 2003), a biochemical proof remains necessary. Nonetheless, MCO3 is a good candidate for a ferroxidase based on, (1) the conservation of a strategically positioned aspartate residue that could potentially provide a ligand to bind iron near the T1 copper centre, (2) the tendency of the *MCO3* mutant to accumulate high levels of ferric iron in the middle midgut and (3) its unequivocal rescue of the iron deficiency, characteristic of the Mvl^{97f} mutant, in the same tissue. However, despite the genetic interaction between *MCO3* and *Mvl*, we show quite clearly that the effect of dietary copper on iron accumulation is not *MCO3* dependent. Indeed, total body iron content drops to a similar extent in *MCO3* mutants and *MCO3, Mvl* double mutants as it does in *w* control flies, following copper administration (supplementary material Table S1). Furthermore, despite enhanced accumulation of ferric iron in the iron region of the middle midgut in *MCO3* mutants compared with controls, we have also repeatedly noted a reduction of this iron upon feeding on copper (Fig. 4C,D). Therefore, neither *MCO3* nor *Mvl* participate in the downstream signalling events that reduce iron accumulation when dietary copper is high. Yet, this interaction should be of high physiological significance given the role of iron in aerobic metabolism and could underlie the relatively low dose at which copper toxicity is manifest (Balamurugan et al., 2007; Massie et al., 1984), the avoidance of this element by flies (Balamurugan et al., 2007), and the sensitivity to dietary copper observed in *Mvl* mutants (Southon et al., 2008), which we show survive on very low levels of iron when grown on 1 mmol l^{-1} $CuSO_4$. Finally, it will be interesting to turn our attention to mitochondria, because a recent study indirectly highlights mitochondria as a putative regulator of iron accumulation when *Drosophila* were subjected to high levels of aluminium (Wu et al., 2010).

MCO3 and *Mvl* interact genetically

Whatever the biochemical function of MCO3 will prove to be, the protein has an impact on metal homeostasis in *Drosophila*. $MCO3^{C359}$ mutant flies accumulate more copper than control flies (20% increase; Fig. 5). Copper accumulation was normal in the $MCO3^{C359}$, Mvl^{97f} double mutant, exposing a condition where Mvl^{97f} loss of function affects copper homeostasis *in vivo*. However, under high dietary copper, $MCO3^{C359}$ mutants accumulated approximately 35% less copper than *w* control flies, independently of *Mvl*, suggesting that MCO3 can act as a potent protein acceptor of copper under conditions of overload.

The $MCO3^{C359}$ mutation strongly suppressed the depletion of intestinal iron stores seen in Mvl^{97f} mutants (Fig. 4E). Therefore, the two genes interact in a reciprocal manner, each contributing to some extent to the metal misregulation seen when the other is mutated (increased copper in $MCO3^{C359}$ mutants, decreased iron in Mvl^{97f} mutants). $MCO3^{C359}$ mutants also accumulated less manganese than control flies (Fig. 5). The physiological significance of these findings and the molecular events that mediate the differential accumulation of metals in the mutant flies await future investigations, which are likely to reveal novel aspects of metal crosstalk in the systemic regulation of insect metal homeostasis.

LIST OF ABBREVIATIONS

DMT1	divalent metal transporter-1
FAC	ferric ammonium citrate
MCO	multicopper oxidase
Mvl	*Malvolio*
w	*white*

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Table S1. Summary of all metal measurements conducted in this study

			[Fe] mg/g		[Cu] mg/g		[Mn] mg/g		[Zn] mg/g	
DIET	GENOTYPE	N	VALUE	STDEV	VALUE	STDEV	VALUE	STDEV	VALUE	STDEV
Normal	*white*	16	**0.203**	0.044	**0.014**	0.005	**0.027**	0.019	**0.066**	0.012
	Mvl	17	**0.170**	0.044	**0.014**	0.004	**0.028**	0.019	**0.070**	0.021
	MCO3	9	**0.214**	0.033	**0.019**	0.007	**0.016**	0.005	**0.059**	0.011
	MCO3, Mvl	9	**0.197**	0.048	**0.014**	0.005	**0.016**	0.007	**0.062**	0.010
1mM Fe	*white*	14	**0.325**	0.095	**0.014**	0.004	**0.037**	0.037	**0.057**	0.012
	Mvl	13	**0.335**	0.119	**0.015**	0.007	**0.042**	0.036	**0.063**	0.020
	MCO3	6	**0.346**	0.055	**0.016**	0.006	**0.016**	0.007	**0.055**	0.015
	MCO3, Mvl	6	**0.318**	0.037	**0.013**	0.005	**0.015**	0.006	**0.057**	0.012
1mM Cu	*white*	13	**0.144**	0.029	**0.194**	0.048	**0.030**	0.013	**0.055**	0.014
	Mvl	13	**0.092**[a]	0.023	**0.183**	0.036	**0.029**	0.008	**0.050**	0.012
	MCO3	6	**0.132**	0.027	**0.127**[b]	0.021	**0.021**	0.009	**0.054**	0.014
	MCO3, Mvl	6	**0.120**	0.047	**0.118**[b]	0.017	**0.027**	0.012	**0.056**	0.010
1mM Mn	*white*	8	**0.209**	0.043	**0.015**	0.002	**0.128**	0.043	**0.065**	0.015
	Mvl	7	**0.152**	0.049	**0.015**	0.005	**0.133**	0.049	**0.068**	0.021
	MCO3	3	**0.214**	0.075	**0.020**	0.002	**0.153**	0.042	**0.056**	0.000
	MCO3, Mvl	3	**0.150**	0.013	**0.017**	0.000	**0.133**	0.023	**0.052**	0.001
1mM Zn	*white*	7	**0.185**	0.051	**0.017**	0.009	**0.030**	0.026	**0.210**	0.048
	Mvl	7	**0.146**[a]	0.026	**0.014**	0.002	**0.029**	0.020	**0.197**	0.019
	MCO3	3	**0.220**	0.011	**0.018**	0.001	**0.016**	0.006	**0.172**	0.037
	MCO3, Mvl	3	**0.189**	0.021	**0.016**	0.001	**0.012**	0.001	**0.186**	0.042
1mM Fe+Cu	*white*	6	**0.262**	0.031	**0.157**	0.038	**0.024**	0.010	**0.044**	0.016
	Mvl	6	**0.282**	0.062	**0.162**	0.018	**0.031**	0.008	**0.048**	0.013
	MCO3	5	**0.289**	0.069	**0.118**[b]	0.036	**0.018**	0.007	**0.059**	0.034
	MCO3, Mvl	5	**0.229**	0.038	**0.104**[b]	0.015	**0.024**	0.009	**0.054**	0.012
1mM Fe+Zn	*white*	9	**0.390**	0.101	**0.016**	0.005	**0.025**	0.016	**0.168**	0.064
	Mvl	9	**0.289**	0.071	**0.016**	0.005	**0.022**	0.009	**0.177**	0.048
	MCO3	5	**0.327**	0.067	**0.021**	0.009	**0.017**	0.007	**0.197**	0.108
	MCO3, Mvl	5	**0.349**	0.068	**0.015**	0.006	**0.016**	0.007	**0.161**	0.057
1mM Mn+Zn	*white*	3	**0.191**	0.011	**0.017**	0.003	**0.169**	0.031	**0.182**	0.028
	Mvl	3	**0.134**	0.032	**0.016**	0.002	**0.156**	0.053	**0.160**	0.043
	MCO3	3	**0.229**	0.060	**0.017**	0.001	**0.160**	0.031	**0.169**	0.038
	MCO3, Mvl	3	**0.152**	0.028	**0.018**	0.001	**0.124**	0.025	**0.139**	0.030
pooled average	*white*	-	**0.197**	0.011	**0.016**	0.001	**0.029**	0.005	**0.057**	0.009
	Mvl	-	**0.151**[c]	0.015	**0.015**	0.001	**0.030**	0.007	**0.060**	0.010
	MCO3	-	**0.219**	0.007	**0.019**[d]	0.002	**0.017**[e]	0.002	**0.057**	0.002
	MCO3, Mvl	-	**0.172**	0.024	**0.016**	0.002	**0.018**[e]	0.006	**0.056**	0.004

The number of biological replicates (*N*), average values (VALUE) and standard deviations (STDEV) are indicated for each condition. One-way ANOVA was performed for each metal in all eight treatments and for the pooled data. Superscript letters denote statistically significant results.

[a] In two (out of five non-iron supplemented) treatments Mvl^{97f} mutants show statistically significantly lower iron concentration than *w* and $MCO3^{C359}$ flies.

[b] In both treatments where copper was added to the diet, $MCO3^{C359}$ and $MCO3^{C359}$, Mvl^{97f} flies accumulated less copper than *w* and Mvl^{97f} mutants.

[c] Mvl^{97f} mutants had a statistically significantly reduction in iron concentration compared with *w* and $MCO3^{C359}$ flies but the value was not significantly different from that of the double mutant.

[d] $MCO3^{C359}$ mutants had a statistically significant higher copper concentration than *w* and Mvl^{97f} flies; Mvl^{97f} loss of function rescues this phenotype.

[e] $MCO3^{C359}$ and $MCO3^{C359}$, Mvl^{97f} flies had a statistically significant lower manganese concentration than *w* and Mvl^{97f} flies.

Different coloured text indicates the key findings:

Red: the average concentration of iron in Mvl^{97f} mutants was lower than in *w* and $MCO3^{C359}$ mutants when no iron was added to the diet.The iron concentration was the same in Mvl^{97f} and *w* mutants when the diet was supplemented with iron.

Orange: When compared with *w* or Mvl^{97f} flies, the concentration of copper in $MCO3^{C359}$ mutants was higher, provided no copper was added to the diet. Mvl^{97f} loss of function rescued the aforementioned effect (i.e. $MCO3^{C359}$, Mvl^{97f} double mutants accumulated less copper than $MCO3^{C359}$ single mutants).

Brown: $MCO3^{C359}$ and $MCO3^{C359}$, Mvl^{97f} mutants fed on copper accumulated less total copper than *w* flies.

Blue: $MCO3^{C359}$ mutants had a lower manganese concentration than *w* or Mvl^{97f} flies. Manganese concentration was comparable between all genotypes if the diet was supplemented with manganese.

Green: dietary copper reduced iron concentrations in all genotypes.

Background colours indicate that the measured metal was also supplemented in the diet.