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Malvolio is a copper transporter in *Drosophila melanogaster*

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SUMMARY

Divalent metal ion transporter 1 (DMT1; also known as SLC11A2) can transport several metals including Fe and Cu in mammalian systems. We set out to determine whether Malvolio (Mvl), the *Drosophila melanogaster* orthologue of DMT1, can also transport Cu. Overexpression of *Mvl* caused Cu accumulation in *Drosophila* S2 cultured cells and conversely dsRNAi knockdown of endogenous *Mvl* reduced cellular Cu levels. Cell viability under Cu limiting conditions was reduced following dsRNAi knockdown. A homozygous viable *Mvl* loss-of-function mutant (*Mvl*^{97f}) was sensitive to excess Cu and female *Mvl*^{97f} flies were also sensitive to Cu limitation. An MtnA-EYFP reporter was used as a proxy measure of Cu distribution within *Mvl*$^{97f/+}$ larvae. Under basal conditions Cu levels were reduced in the anterior midgut and proventriculus relative to control larvae. These results demonstrate Mvl is a functional Cu transporter and that despite partial functional redundancy with the Ctr1 proteins, Cu uptake through this pathway is necessary for optimal viability at the cellular and organismal levels.

Key words: Malvolio, *Drosophila melanogaster,* S2 cells, copper, DMT1, pigmentation.

INTRODUCTION

The maintenance of copper (Cu) homeostasis at the cellular level is essential for all aerobic organisms and an excess of Cu is toxic as a result of the generation of reactive oxygen species (Linder and Hazegh-Azam, 1996). Mechanisms for uptake, distribution, sequestration and efflux of Cu are well conserved among yeast, insect and mammalian systems (Camakaris et al., 1999). The Ctr family are the best characterised Cu uptake proteins, with *Drosophila melanogaster* having three, designated Ctr1A, B and C. *In vivo* and *in vitro* studies have demonstrated Ctr1A and Ctr1B are functional Cu transporters with different tissue and life stage expression profiles (Southon et al., 2004; Turski and Thiele, 2007; Zhou et al., 2003). Although human CTR1 (also known as SLC31A1 – HUGO) has traditionally been seen as the primary Cu uptake mechanism in gut epithelium, a recent study by Zimnicka et al. (Zimnicka et al., 2007) has found endogenous CTR1 to be localised to the basolateral surface of cultured intestinal and kidney cells, rather than the apical membrane, suggesting CTR1 may not be responsible for dietary Cu absorption. Divalent metal ion transporter 1 (DMT1; also known as SLC11A2 – HUGO, and Nramp2), which is capable of transporting Fe^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} (Gunshin et al., 1997) may also contribute to Cu uptake. DMT1 is expressed in multiple mammalian tissues, whereas its paralogue Nramp1 (SLC11A1) is expressed in macrophages (Gunshin et al., 1997; Vidal et al., 1995) as well as dendrites (Stober et al., 2007) and tertiary granules of neutrophils (Canonne-Hergaux and Gros, 2002). The DMT1 family is evolutionarily conserved, with orthologues in several species including mouse, rat and yeast (Cellier et al., 1995). Although DMT1 is clearly a symporter that transports divalent cations across the plasma or endosomal membranes into the cytoplasm (Gunshin et al., 1997; Touret et al., 2003) the direction of Nramp1 transport is less clear, with some authors suggesting it is an antiporter, exporting divalent cations from the cytoplasm (Eichner Techau et al., 2007; Goswami et al., 2001). Eichner Techau et al. (Eichner Techau et al., 2007) propose that symporter activity was the ancestral function of this family and that following the gene duplication antiporter activity evolved for Nramp1.

Malvolio (*Mvl*) is the only member of the DMT1 family in the *Drosophila* genome and is orthologous to the human proteins NRAMP1 (54.9% identity) and DMT1 (57.4%) (Cellier et al., 1995). *Mvl* was identified in a screen for mutants that affect taste behaviour (Rodrigues et al., 1995). A P-element induced partial loss-of-function mutant (*Mvl*^{97f}) causes flies to lose preference for sugar-supplemented medium. Rearing these flies on medium supplemented with Mn^{2+} or Fe^{2+} corrected this defect, whereas Ca^{2+}, Mg^{2+} or Zn^{2+} did not, suggesting *Mvl*^{97f} flies may have a Mn^{2+} or Fe^{2+} deficiency (Orgad et al., 1998). Recently, Folwell et al. (Folwell et al., 2006) examined Mvl localisation in *Drosophila*. Protein expression was detected in larval Malpighian tubules, testis and brain, in the amnioserosa of embryos and in the larval and adult alimentary canal, with intracellular localisation that was either punctate, cytoplasmic or at the plasma membrane. The authors concluded that Mvl must fulfil the roles of both Nramp1 and DMT1 given its distribution, however, metal homeostasis was not examined. Mvl, like DMT1, was recently shown to be a cation symporter (Eichner Techau et al., 2007), however it is still unclear if Mvl is a Cu transporter.

The model organism *Drosophila melanogaster* has recently been used to enhance our understanding of several aspects of Cu homeostasis including: Cu uptake by the Ctr family (Turski and Thiele, 2007; Zhou et al., 2003); Cu sequestration through the metallothionein (Mtn) system (Balamurugan et al., 2007; Egli et al., 2003; Selvaraj et al., 2005); and Cu efflux *via* ATP7 (Norgate et al., 2006). We now use this system, together with the *Drosophila* embryonic cell line S2, to demonstrate that Mvl is a functional Cu transporter, necessary for optimal Cu uptake and distribution.

MATERIALS AND METHODS

Clones and fly stocks

Full-length *Mvl* cDNA lacking the C-terminal STOP codon (according to FlyBase annotation; The FlyBase Consortium, 2003; http://flybase.org/) was amplified from *Drosophila* S2 cell-derived cDNA using PCR. This was cloned into pAc3.1V5-HisA (Invitrogen, Mount Waverly, Vic, Australia) modified to contain an in-frame C-terminal FLAG epitope tag (MDYKDDDDKA) for expression in S2 cells and cloned into pUAST for expression in *Drosophila* using the UAS-Gal4 system. Pannier-Gal4/Sb, Ser (provided by Prof. E. Hafen, Institute of Molecular Systems Biology, University of Zurich, Zurich, Switzerland) were used to drive the expression of *ATP7* in the *pannier* (*pnr*) domain of cuticular cells down the centre of the adult thorax and abdomen, as described previously (Norgate et al., 2006). To test for genetic interactions with *ATP7*, *UAS-Mvl* and Mvl^{97f} (Bloomington Stock # 5151, a viable P-element insertion mutation of *Mvl*) homozygous flies were crossed to *pnr-Gal, UAS-ATP7/TM3,Sb* flies and progeny examined. All flies were raised on standard *Drosophila* food medium containing approximately 5–10 μmol l^{-1} Cu.

Cell culture

S2 cells were propagated in *Drosophila* serum free medium (SFM, Invitrogen) as previously reported (Southon et al., 2004). Cu was added to SFM as $CuCl_2$ at the concentration specified in the results. 10 μmol l^{-1} Diamsar was added to SFM to deplete intracellular Cu as previously reported (Bellingham et al., 2004; Norgate et al., 2007). Mvl dsRNA was derived from *Mvl* cDNA bases 786–1251 and control dsRNA was derived from EYFP cDNA. dsRNA interference (dsRNAi) knockdown was conducted as previously reported (Southon et al., 2004; Worby et al., 2001). S2 cells maintaining stable overexpression of *Mvl* (55–60% of cells) were generated by co-transfecting pCoHygro with either pAcMvl or pAc empty vector control using Lipofectamine 2000 and cells were propagated in Schneider's Complete Medium (Invitrogen) with 10% foetal calf serum (Trace Scientific, Melbourne, Vic, Australia) supplemented with 300 μg ml^{-1} hygromycin B according to the manufacturer's instructions (Invitrogen). This medium was replaced with SFM for all experiments.

Gene and protein expression analysis

Total RNA for gene expression was extracted using the RNeasy kit (Qiagen, Doncaster, Victoria, Australia) and cDNA was transcribed using AMV reverse transcriptase (Promega, Annandale, NSW, Australia) as previously described (Southon et al., 2004). Primers for real-time PCR were designed using Primer3 software (Rozen and Skaletsky, 2000). *Mvl* forward and reverse primer sequences were: GCACCACCAGCAGATACTCA, and CCACAGCAAGAC-CCACAA, respectively. *Actin 42A* was used as a housekeeping gene and primer sequences have been described elsewhere (Southon et al., 2004). Real-time PCR was performed using the Rotor Gene 3000 (Corbett Research, Mortlake, NSW, Australia) using QuantiTect SYBR Green PCR Master Mix (Qiagen, Doncaster, Vic, Australia) and analysed as previously described (Southon et al., 2004).

Protein samples for western blotting were prepared from S2 cells with a lysis buffer containing: 1% Triton X-100, 20 mmol l^{-1} Tris–HCl (pH 7.5), 150 mmol l^{-1} NaCl, 2 mmol l^{-1} EDTA and 10% glycerol (Sigma, Castle Hill, NSW, Australia) with protease inhibitor cocktail added (Roche Diagnostics, Mannheim, Germany). Samples were lysed on ice for 2 h before clearing lysate by centrifugation. Protein (40 μg) was run on NuPAGE 4–12% Bis–Tris gels (Invitrogen), transferred to nitrocellulose membranes and probed with monoclonal mouse anti-FLAG antibody (1:2000, KM5–1C7 Walter and Eliza Hall Institute Biotechnology Centre, Melbourne, Vic, Australia) and polyclonal rabbit anti-mouse horseradish peroxidase coupled secondary antibody (1:7000, Dako, Glostrup, Denmark). Monoclonal mouse anti-α-tubulin was used as a loading control (Sigma).

Microscopy

Immunofluorescent detection of Mvl in S2 cells utilised a monoclonal mouse anti-FLAG antibody (1:200) and Alexa Fluor 488 anti-mouse secondary antibody (1:400, Invitrogen). DAPI (300 nmol l^{-1}; Invitrogen, Mount Waverly, Vic, Australia) was used to detect the nucleus. Images were recorded at 100× magnification using an Olympus FluoView 1000 confocal microscope with Olympus FluoView ver1.6a software (Olympus, Center Valley, PA, USA) and single sections are shown. An MtnA-EYFP reporter (provided by Prof. W. Schaffner, Institute of Molecular Biology, University of Zurich, Zurich, Switzerland) was used as a proxy measure of Cu distribution in dissected third instar larvae as previously described (Norgate et al., 2006; Selvaraj et al., 2005). Mvl^{97f} and w^{1118} larval tissues were examined at 15× magnification (60× for higher magnification images) using the Olympus SZX12 dissecting microscope. Images were captured using the Olympus DP controller software using identical exposure settings (0.1 s exposure time). Twelve larvae (24 for basal food) were examined for each condition. Images of adult *Drosophila* were also recorded using this system. Representative images are shown.

S2 cell copper accumulation and cell viability assays

^{64}Cu accumulation experiments were conducted as previously reported (Camakaris et al., 1995; Southon et al., 2004). Briefly cells were incubated with approximately 0.4 MBq ^{64}Cu (Australian Radioisotopes, Lucas Heights, NSW, Australia) and 2 μmol l^{-1} non-radioactive Cu for 1 or 24 h. Cu accumulation was stopped by washing cells four times with 1 ml cold Hank's balanced salt solution containing 1 mmol l^{-1} histidine (Sigma). Cells were then lysed in 100 μl of 0.1% SDS, containing 2 mmol l^{-1} EDTA. Radioactivity was measured with a γ-counter (1282 CompuGamma, LKB Wallac, Turku, Finland) and Cu levels were standardised to total cellular protein, which was determined using Bio-Rad protein reagent according to the manufacturer's instructions (Bio-Rad, Gladesville, NSW, Australia). Cell viability following Cu chelation was determined by cell counting with a haemocytometer and Trypan Blue (Sigma) staining of dead cells.

Drosophila metal accumulation and survival assays

Five replicates of 50 *Drosophila* where digested in 70% HNO_3 for 3 days at room temperature, with an additional 12 h at 60°C and then diluted to a final concentration of 10% HNO_3 with dH_2O. Metal levels were measured using a Vista-AX inductively coupled plasma atomic emission spectrometer (ICP-AES; Varian, Palo Alto, CA, USA). Five reading at 327.395 and 327.754 nmol l^{-1} were averaged to give one value per biological replicate and expressed relative to the number of flies (ng/fly). A previous experiment showed no difference between normalisation of metal levels to either the number of flies or wet mass (data not shown).

Larval survival to adulthood was determined as reported previously (Norgate et al., 2007). Briefly, five replicates of 50 first instar larvae were scored for survival to adulthood on basal food (5–10 μmol l^{-1} Cu) and 1 and 2 mmol l^{-1} $CuSO_4$ (Sigma) as well as 100 μmol l^{-1} bathocuproinedisulfonic acid (BCS; Sigma) to limit

available Cu. Survival of Mvl^{97f} was compared to that of wild-type Armenia and w^{1118} control *Drosophila*.

Statistics

Statistical analysis was conducted using SPSS v11 (SPSS, Chicago, IL, USA). A one-sample Kolomogorov-Smirinov test was used to assess whether data was normally distributed. Statistical analyses are described in figure legends. $P<0.05$ was deemed statistically significant.

RESULTS AND DISCUSSION

Malvolio transports Cu in *Drosophila* S2 cells

We utilised *in vitro* overexpression of *Mvl* as well as dsRNAi knockdown of endogenous *Mvl* in cultured S2 cells to demonstrate this gene encodes a functional Cu transporter. *Mvl* containing a C-terminal FLAG epitope tag (pAcMvl) was stably overexpressed in S2 cells. Western blotting using anti-FLAG detected a band of approximately 52 kDa corresponding to Mvl in lysate from pAcMvl cells (Fig. 1A), comparable to that seen with *Mvl* overexpression in *Drosophila* (Folwell et al., 2006). No significant signal was visible in lysate from cells expressing an empty vector control (pAc). Immunofluorescent confocal microscopy clearly demonstrated Mvl was localised to the plasma membrane of pAcMvl cells, with additional large punctate staining (Fig. 1B), whereas no significant staining was detected in pAc cells (Fig. 1C). Mvl localisation was not affected by either 24 h Cu limitation or 2.5 h exposure to 0.8 mmol l^{-1} Cu (data not shown). The cellular localisation of Mvl is consistent with that seen *in vivo* with larval and adult *Drosophila* (Folwell et al., 2006) and is also similar to that of DMT1 in mammalian cells. DMT1 is localised to the apical membrane of mammalian intestinal and kidney cells where it is involved in Fe uptake and reabsorption, respectively (Canonne-Hergaux and Gros, 2002; Knopfel et al., 2005), as well as in recycling endosomes to mediate Fe transport into the cytosol (Gruenheid et al., 1999; Lam-Yuk-Tseung et al., 2005). The punctate localisation of Mvl is likely to be a similar endosomal compartment.

We next measured Cu uptake using the radioisotope ^{64}Cu. S2 cells overexpressing *Mvl* or an empty vector control were exposed to 2 μmol l^{-1} Cu. Relative to control, overexpression of *Mvl* increased Cu levels by 9% and 35% after 1 h and 24 h exposure times, respectively (Fig. 2A). Conversely, dsRNAi knockdown of endogenous *Mvl* reduced Cu accumulation by 8% and 28% when

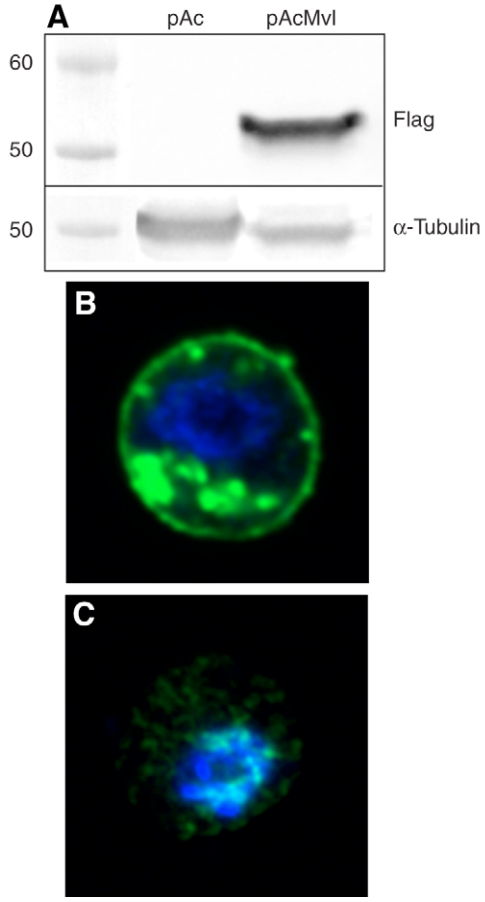

Fig. 1. Mvl expression and localisation in S2 cells. (A) Western blotting using anti-FLAG antibody detected a 52 kDa band corresponding to Mvl in lysate from pAcMvl cells. No significant signal was visible in lysate from empty vector control cells (pAc). α-Tubulin was used as a loading control. (B,C) Mvl was detected in S2 cells by immunofluorescence using anti-FLAG and Alexa Fluor 488 anti-mouse antibodies (green) and the nucleus was stained with DAPI (blue). Mvl was detected at the plasma membrane as well as in punctate structures in pAcMvl cells (B), but no significant staining was visible in pAc cells (C).

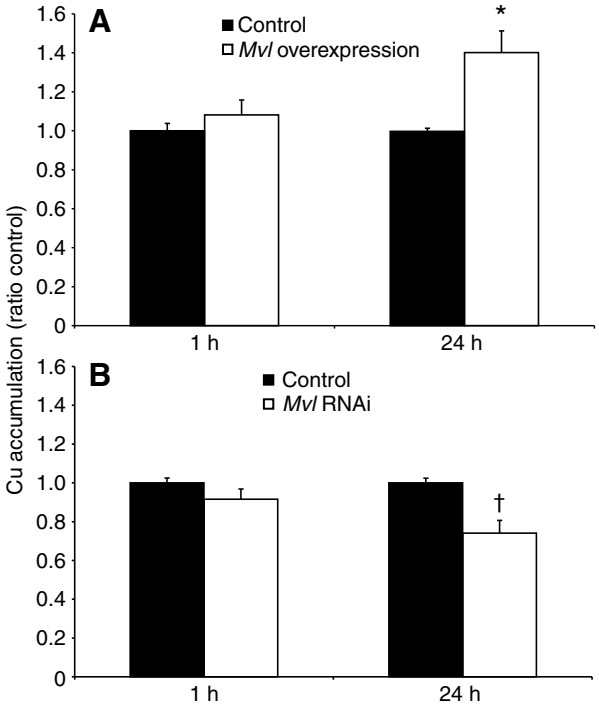

Fig. 2. Cu accumulation in S2 cells. Cu accumulation was measured in S2 cells exposed to 2 μmol l^{-1} Cu. Intracellular levels were measured after 1 h and 24 h and normalised to total cellular protein. An independent samples *t*-test was used to determine significant differences. (A) Cu accumulation in S2 cells stably overexpressing *Mvl*, expressed relative to an empty vector control. Values are mean ± s.e.m. of 15 replicates from four independent experiments. *$P<0.0005$ compared to control. (B) Cu accumulation following dsRNAi knockdown of *Mvl*, expressed relative to control. Cells were pre-treated with *Mvl* or control dsRNA 48 h prior to Cu exposure. Real-time PCR demonstrated *Mvl* expression was reduced to 18.1±4.6% (mean ± s.e.m.) of control after 48 h. Values are mean ± s.e.m. of 21 replicates from seven independent experiments. †$P<0.0001$ compared to control.

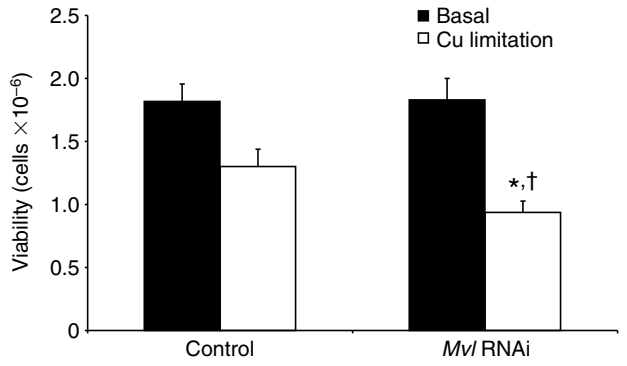

Fig. 3. S2 cell viability following Cu limitation. S2 cells were pre-treated with *Mvl* or control dsRNA for 6 days. Real-time PCR demonstrated *Mvl* was suppressed to 47.3±16.1% (mean ± s.e.m.) of control after 6 days. Cells were exposed to basal medium or Cu-limited medium using 10 μmol l^{-1} Diamsar for 6 days and viable cells were counted. Values are mean ± s.e.m. of nine replicates from three independent experiments. An independent samples *t*-test was used to determine significant differences. **P*<0.05 compared to basal medium, †*P*<0.05 compared to control cells exposed to Cu-limited medium.

cells were exposed to 2 μmol l^{-1} Cu for 1 and 24 h, respectively (Fig. 2B). These results demonstrate Mvl is a functional Cu uptake protein *in vitro*, able to transport Cu across the plasma membrane under physiological conditions. To determine if Mvl-mediated Cu uptake is important for Cu homeostasis, we examined the viability of S2 cells following dsRNAi knockdown of *Mvl*. Cell viability was determined after 6 days following exposure to basal medium or Cu-limited medium using the Cu chelator Diamsar (Fig. 3). Viability under basal conditions was not affected by dsRNAi knockdown of *Mvl*. Control cells were sensitive to Cu limitation, with the number of viable cells reduced by 28% compared with cells maintained on basal medium. Cells were significantly more susceptible to Cu limitation following knockdown of *Mvl*, with viability reduced by 49%. Under Cu limiting conditions, relative to control cells, significantly fewer cells were viable when *Mvl* was knocked down. By contrast, dsRNAi knockdown of *Mvl* did not affect cell viability, relative to control, when cells were challenged with excess Cu for 48 h (data not shown).

The overexpression of *Mvl* in S2 cells significantly increased Cu levels, however, the effect was not as dramatic as that previously seen with *Ctr1A* and *Ctr1B* (Zhou et al., 2003), supporting the established view that the Cu-specific Ctr1 system is the primary Cu uptake mechanism. Nevertheless, impairment of *Mvl* function significantly decreases Cu uptake and reduces cell viability when Cu availability is low, whereas cell viability is unaffected when Cu levels are either adequate or elevated. This reduced Mvl-mediated Cu uptake suggests Ctr1A and Ctr1B are unable to elevate Cu uptake to compensate for reduction in Mvl activity. Similar results were seen when either *Ctr1A* or *Ctr1B* were suppressed in these cells (Southon et al., 2004) suggesting the Ctr1 and Mvl systems are independently regulated. Although speculative, the two systems may transport Cu to different cellular compartments. It is unclear if DMT1 can transport Cu to Atox1 as occurs with Ctr1 (Xiao and Wedd, 2002), or whether an alternative Cu chaperone is used for this non-Cu-specific transport system in mammals.

Malvolio transports Cu in *Drosophila melanogaster*

We sought to explore Mvl-mediated Cu transport *in vivo* utilising the homozygous viable, partial loss-of-function, Mvl^{97f} mutant flies (Rodrigues et al., 1995) as well as overexpression of *Mvl* with the UAS-Gal4 system. We examined viability, Cu uptake and distribution and markers of Cu-dependent enzyme activity.

Hypopigmentation seen with impairment of *Ctr1B* (Zhou et al., 2003) or with overexpression of *ATP7* (Norgate et al., 2006) is indicative of a functional Cu deficiency and has previously been used to demonstrate *in vivo* Cu uptake and efflux, respectively, as tyrosinase is a Cu-dependent phenol oxidase involved in the production of biogenic amines needed for pigmentation (Wright, 1987). *ATP7* overexpression in the adult thorax also results in developmental disruption of the thorax, including absence of scutelar bristles and reduced thorax width (Norgate et al., 2006), presumably due to impaired activity of lysyl oxidase, the Cu-dependent enzyme involved the biogenesis of connective tissue matrices (Smith-Mungo and Kagan, 1998).

We examined adult cuticle in homozygous Mvl^{97f} mutant flies under basal conditions, Cu-limited and Cu-excess conditions, but did not observe any significant change to pigmentation or cuticle morphology (data not shown). Similarly overexpression of *Mvl* in the *pannier* domain under the control of the *pnr*-Gal4 driver did not have any observable effect (data not shown). These results demonstrate that impaired Mvl function does not cause a functional Cu deficiency and suggests other transporters, presumably Ctr1A or Ctr1B, are primarily responsible for Cu uptake into the developing epidermis.

To determine if any Mvl-mediated Cu transport occurs in the thorax, we next examined the effect of impaired *Mvl* function and *Mvl* overexpression when cuticle Cu levels are depleted by *ATP7* overexpression. Compared with w^{1118} control flies (Fig. 4A), overexpression of *ATP7* in the *pannier* domain (Fig. 4B) caused a severe Cu depletion, including hypopigmentation and reduced thoracic width (indicated by the distance between the two major posterior sensory bristles) as previously reported (Norgate et al., 2006). Overexpression of *ATP7* in the *pannier* domain in $Mvl^{97f/+}$ flies did not significantly affect pigmentation or thoracic development, relative to *ATP7* overexpression in a wild-type background (Fig. 4C). By contrast, simultaneous overexpression of *Mvl* and *ATP7* caused a more severe hypopigmentation phenotype and further reduction in thorax width, than *ATP7* overexpression alone, indicative of a more severe functional Cu deficiency (Fig. 4D). These phenotypes were not affected by raising these flies on Cu-limited or Cu-excess food (data not shown).

The exacerbation of the functional Cu deficiency phenotype by co-overexpression of *Mvl* and *ATP7* was unexpected, given that our previous studies found co-overexpression of *Ctr1A* and *ATP7* almost completely rescued the hypopigmentation phenotype, despite no obvious pigmentation phenotype when *Ctr1A* was overexpressed alone (Norgate et al., 2006). These results are inconsistent with a simple model of Mvl acting at the plasma membrane to transport Cu into a tissue, and may be due to a more complex level of intracellular Cu regulation. In mammals, DMT1 not only functions at the plasma membrane but also transports Fe (Gruenheid et al., 1999; Lam-Yuk-Tseung et al., 2005) and Cd (Abouhamed et al., 2007) from an endosomal compartment into the cytosol. Therefore, it is reasonable to speculate that the punctate intracellular localisation of Mvl in S2 cells (Fig. 1) and *Drosophila* tissues (Folwell et al., 2006) represents an endosomal compartment and that Mvl can transport Cu from this organelle into the cytosol. It is possible that the overexpression of *Mvl* may worsen the hypopigmentation and cuticle phenotype through a redistribution of intracellular Cu, increasing transport of Cu out of an endosomal compartment and further restricting Cu delivery to Cu-dependent enzymes within the

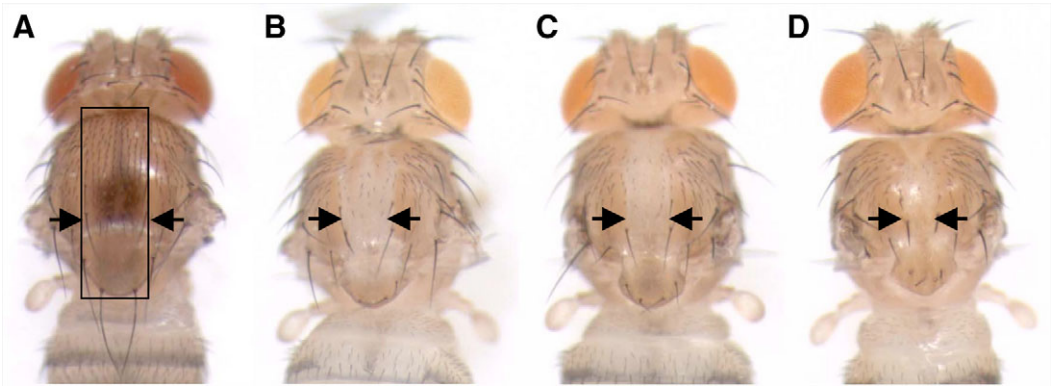

Fig. 4. Hypopigmentation and thorax development in *Drosophila.* Hypopigmentation and thorax development were used as markers of Cu deficiency in adult female *Drosophila* that overexpress *ATP7* in the *pannier* region (boxed in A) using a *pnr*-Gal4 driver. Overexpression of *ATP7* results in a hypopigmentation and a loss of sensory bristles in the *pannier* region. In addition the width of the thorax is reduced, as indicated by the arrows that represent the distance between the two major posterior sensory bristles. (A) w^{1118} control flies. (B) Overexpression of *ATP7*. (C) Overexpression of *ATP7* in the $Mvl^{97f/+}$ background. (D) Co-overexpression of *ATP7* and *Mvl* significantly and reliably further reduced thoracic width and pigmentation.

secretory pathway. The hypothesis that there are distinct pathways for Cu uptake and delivery to specific subcellular compartments and Cu-dependent proteins is supported by our *in vitro* results, which show Ctr1A and Ctr1B are unable to maintain optimal Cu levels and prevent susceptibility to Cu limitation when Mvl is impaired (Fig. 3).

To assess the importance of Mvl to Cu homeostasis in live *Drosophila* we examined the viability of Mvl^{97f} flies in response to Cu limitation as well as Cu excess. First instar larvae were raised on basal food, on food containing the Cu chelator BCS, or on food with excess Cu, and survival to adulthood was determined for female (Fig. 5A) and male (Fig. 5B) flies separately. Compared to the control strains Armenia (wild-type) and w^{1118}, Mvl^{97f} males and females showed a strong sensitivity to excess Cu, with a significant reduction in viability at 1 mmol l^{-1} Cu and essentially no survival at 2 mmol l^{-1} Cu. Under limiting Cu conditions Mvl^{97f} females again demonstrated reduced viability but, interestingly, Mvl^{97f} males were unaffected. Thus reduced *Mvl* function confers sensitivity to Cu excess and, in female flies, Cu limitation as well. It is unclear why male flies were unaffected by Cu limitation, but it is possible that *Mvl* expression levels are gender specific. SEBIDA, the *Drosophila* sex bias database, cites seven different cDNA microarray studies that have compared male and female *Drosophila melanogaster* gene expression profiles and all have shown greater *Mvl* expression in male flies, with this difference statistically significant in four of the arrays (Gnad and Parsch, 2006). Given the Mvl^{97f} mutation is only a partial loss of function it is possible that male flies are able to produce sufficient Mvl protein for Cu uptake, whereas female flies are not.

The increased sensitivity of Mvl^{97f} flies to altered Cu conditions clearly demonstrates Mvl is an essential component of the Cu regulatory system, and is strikingly similar to that seen with *Ctr1B* null flies (Zhou et al., 2003). Absence of *Ctr1B* causes sensitivity to Cu limitation, suggestive of reduced transport from the midgut, as well as sensitivity to excess Cu due to reduced transport into a detoxification tissues such as the fat body or Malpighian tubules [analogous to the mammalian liver and kidneys, respectively (Cagan, 2003; Sondergaard, 1993)].

To explore why Mvl^{97f} flies are sensitive to Cu manipulation we used an MtnA-EYFP reporter as a marker for Cu distribution within third instar larvae. Excess Cu induces expression of the EYFP marker from the promoter of *MtnA*, and this technique has previously been used as a proxy measure of Cu distribution (Norgate et al., 2006; Selvaraj et al., 2005). We examined male and female larvae separately, but did not detect any differences between the sexes (data not shown). $Mvl^{97f/+}$/MtnA-EYFP larvae were dissected and

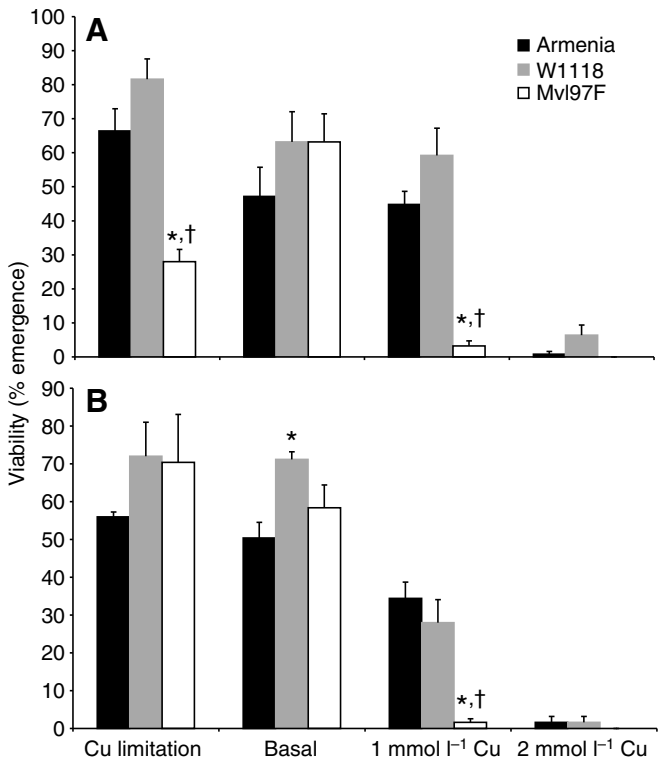

Fig. 5. *Drosophila* viability following Cu limitation or Cu excess. Larval survival to adulthood was determined for Mvl^{97f} and w^{1118} *Drosophila* when raised on Cu-limited medium using 100 μmol l^{-1} BCS, basal food, or 1 or 2 mmol l^{-1} Cu. (A) Female survival. (B) Male survival. Values are expressed as percentage emergence and are mean ± s.e.m. of five replicates of 50 larvae. A one-way ANOVA with a Games Howell *post-hoc* test was used to determine significant differences. $^{*}P<0.05$ compared to Armenia and $^{\dagger}P<0.05$ compared to w^{1118} control flies.

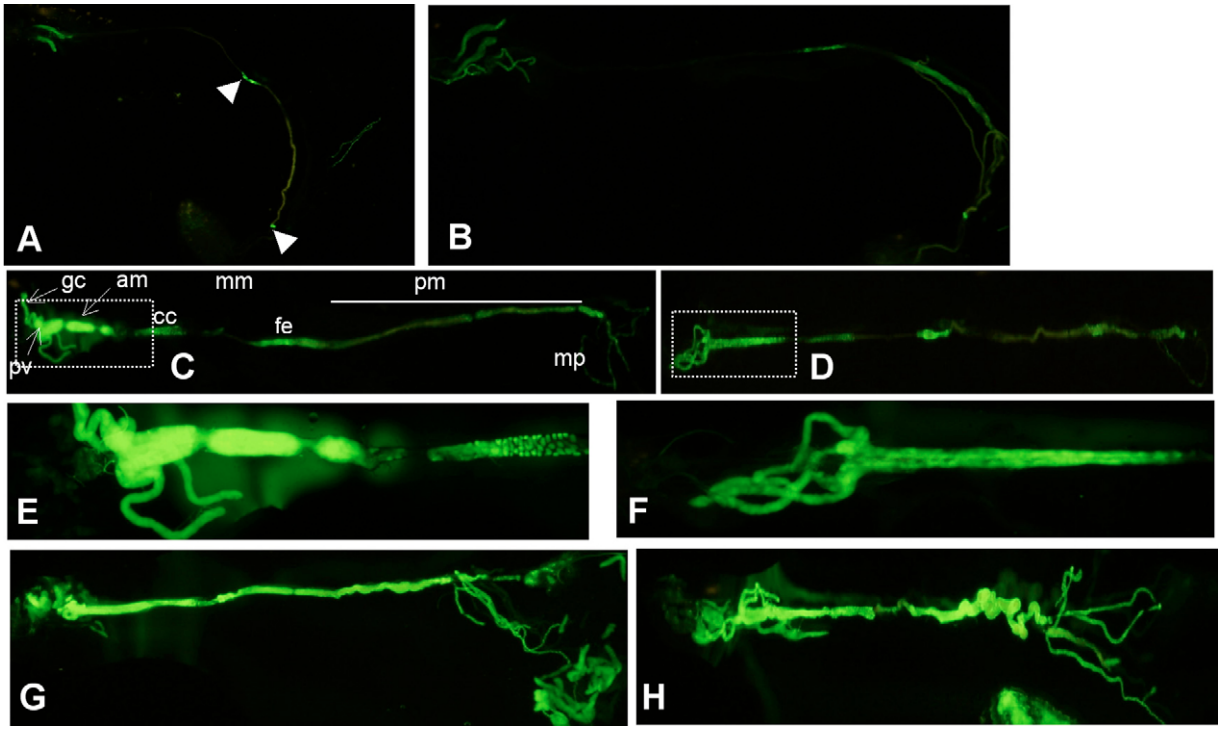

Fig. 6. Larval Cu distribution. Third instar larvae were raised under Cu-limited conditions with 100 μmol l^{-1} BCS (A,B), basal food (C,D and enlarged in E,F, respectively) or exposed to 1 mmol l^{-1} Cu for 4 h (G,H) and then dissected. MtnA-EYFP fluorescence was used as a proxy marker of Cu distribution in w^{1118} control larvae (A,C,E,G) and $Mvl^{97f/+}$ larvae (B,D,F,H). EYFP levels were not different between male and female larvae (data not shown). Regions of the gut are identified in C: pv, proventriculus; gc, gastric caecum; am, anterior midgut; cc, copper cell region; mm, middle midgut; fe, iron cell region; pm, posterior midgut; mp, Malpighian tubule. Under basal conditions, control larvae (C) showed a complex distribution of EYFP throughout the anterior, middle and posterior midgut as well as the proventriculus and Malpighian tubules. By contrast, $Mvl^{97f/+}$ larvae (D) did not show the same high levels of EYFP in the proventriculus and anterior midgut (clearly seen in the enlargements E and F). Under Cu-limited conditions, EYFP levels were reduced across all tissues of both control (A) and $Mvl^{97f/+}$ (B) larvae, with the exception of the iron cell region of the middle midgut and in the imaginal ring at the midgut–hindgut border (indicated by arrows in A). Under Cu-excess conditions EYFP levels were saturated in all tissues of both control (G) and $Mvl^{97f/+}$ (H) larvae.

compared to w^{1118}/MtnA-EYFP controls (Fig. 6). When larvae were raised on BCS to limit available Cu, EYFP could only be detected in the iron-cell region of the middle midgut and in the imaginal ring at the midgut–hindgut border of both control (Fig. 6A) and $Mvl^{97f/+}$ (Fig. 6B) larvae. The lack of EYFP expression in the remainder of the midgut and other tissues does not suggest that these tissues are not taking up Cu, but that all available Cu is being utilised. The EYFP detected in the iron-cell region and the imaginal ring suggests these were the only tissues where an excess of Cu was available to stimulate *MtnA* expression. Under basal conditions control larvae displayed significant EYFP in the proventriculus, gastric caecum, anterior midgut and Malpighian tubules (Fig. 6C,E). Importantly, $Mvl^{97f/+}$ larvae had dramatically reduced EYFP expression in the proventriculus and anterior midgut (Fig. 6D,F). We next exposed larvae to 1 mmol l^{-1} Cu for 4 h prior to dissection to see if excess Cu altered this distribution. There was a dramatic increase in EYFP levels in all tissues including the posterior midgut, fatbody, hindgut and larval epidermis. However, there was no detectable difference between $Mvl^{97f/+}$ (Fig. 6H) and control (Fig. 6G) larvae. This saturation of EYFP in all tissues supports the hypothesis that *Drosophila* essentially take up all available Cu rather than risk a deficiency (Balamurugan et al., 2007).

Table 1. Metal accumulation in *Drosophila*

	Cu limitation*		Basal		6 mmol l^{-1} Cu†	
	w^{1118}	Mvl^{97f}	w^{1118}	Mvl^{97f}	w^{1118}	Mvl^{97f}
Cu	2.24±0.09	2.04±0.05	3.41±0.08	3.19±0.09	31.2±1.16	63.1±2.66§
Fe	32.2±1.43	23.5±1.84§	35.5±1.76	25.3±1.39§	33.9±1.54	37.6±2.71
Mn	4.91±0.12	7.62±1.61	3.96±0.10	4.11±0.22	3.40±0.43	5.48±0.23‡
Zn	13.4±0.43	14.1±0.56	14.6±0.29	14.4±0.53	8.89±0.57	13.1±1.41§

*Measurements were made on pupae raised on medium containing 100 μmol l^{-1} bathocuproinedisulfonic acid (BCS) to limit Cu availability and compared to pupae raised on basal medium.

†Response to excess copper was measured in 7-day-old adults after a 24 h exposure to medium containing excess Cu (6 mmol l^{-1}). Cu, Fe, Mn and Zn levels were determined by ICP-AES and expressed as ng/fly.

Values are mean ± s.e.m. of five replicates of 50 *Drosophila*. An independent samples *t*-test was used to determine significant differences. ‡P<0.05, §P<0.01 compared to w^{1118} control.

We next raised Mvl^{97f} and w^{1118} control larvae under various Cu conditions and measured metal levels at pupation and adulthood by ICP-AES (Table 1). When raised on basal food, Fe levels were 40% lower in Mvl^{97f} pupae than controls. Cu levels were 7% lower, however, this was not statistically significant (P=0.09). Mn and Zn levels were unchanged. Raising larvae under Cu-limited conditions significantly reduced Cu levels in both control and Mvl^{97f} pupae. Cu levels were 10% lower in Mvl^{97f} pupae than control, however, again this was not statistically significant (P=0.10). Cu levels could not be assessed in Mvl^{97f} pupae following Cu exposure as viability was too severely reduced (Fig. 5). We therefore raised larvae under basal condition and exposed the adults to 6 mmol l^{-1} excess Cu for 24 h. Cu levels were dramatically higher in Mvl^{97f} and w^{1118} flies than that seen in pupae. Interestingly Mvl^{97f} flies also contained significantly more Mn and Zn than controls, and Fe levels were no longer lower in Mvl^{97f}, demonstrating a general elevation of metal levels. The elevated Cu seen in Mvl^{97f} flies exposed to short-term excess Cu is consistent with the hypothesis that reduced Cu transport into the fat body or Malpighian tubules leads to systemic accumulation and reduced viability. Unfortunately we could not detect any difference in MtnA-EYFP fluorescence between $Mvl^{97f/+}$ and control larvae at high Cu levels as the EYFP signal was saturated in all tissues because of a systemic upregulation of the Mtn Cu sequestration system.

The relatively small reduction in Cu levels seen in Mvl^{97f} pupae relative to controls, shows impaired Mvl-mediated Cu uptake does not result in a systemic Cu deficiency, presumably because of a compensatory Ctr1-mediated Cu uptake. The reduced MtnA-EYFP expression in the anterior midgut, a tissue known to express *Mvl* (Folwell et al., 2006), and the proventriculus of Mvl^{97f} larvae suggests that impaired Mvl-mediated Cu uptake in one or both of these regions is likely to be responsible for the reduced viability of female *Drosophila* under Cu limiting conditions. *Ctr1A* and *Ctr1B* are also expressed in the midgut (Chintapalli et al., 2007) and the loss of function of either of these Cu transporters confers sensitivity to Cu deficiency (Turski and Thiele, 2007; Zhou et al., 2003), suggesting these transporters are unable to fully compensate for the absence of another in this tissue.

Conclusion

These results demonstrate Malvolio is a physiologically important Cu transporter both *in vitro* and *in vivo*. The similarity between *Mvl* and *Ctr1B* mutant *Drosophila* with respect to their sensitivity to both Cu limitation and excess Cu, despite similar expression profiles, suggests each of these pathways are essential for optimal Cu uptake and distribution with only partial redundancy, and this is supported by *in vitro* experiments with dsRNAi knockdown in S2 cells. These results, together with the recent study redefining the role of human CTR1 (Zimnicka et al., 2007), suggest the role of DMT1 in mammalian dietary Cu uptake and distribution should be investigated further.

LIST OF ABBREVIATIONS

BCS	bathocuproinedisulfonic acid
DMT1	Divalent metal ion transporter 1
dsRNAi	dsRNA interference
ICP-AES	inductively coupled plasma atomic emission spectrophotometry
Mtn	metallothionein
Mvl	Malvolio
SFM	serum free medium

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