

## Copper homeostasis in *Drosophila melanogaster* S2 cells

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Copper homeostasis was investigated in the *Drosophila melanogaster* S2 cell line to develop an insect model for the study of copper regulation. Real-time PCR studies have demonstrated expression in S2 cells of putative orthologues of human Cu regulatory genes involved in the uptake, transport, sequestration and efflux of Cu. *Drosophila* orthologues of the mammalian Cu chaperones, *ATOX1* (a human orthologue of yeast ATX1), *CCS* (copper chaperone for superoxide dismutase), *COX17* (a human orthologue of yeast COX17), and *SCO1* and *SCO2*, did not significantly respond transcriptionally to increased Cu levels, whereas *MtnA*, *MtnB* and *MtnD* (*Drosophila* orthologues of human metallothioneins) were up-regulated by Cu in a time- and dose-dependent manner. To examine the effect on Cu homeostasis, expression of several key copper homeostasis genes was suppressed using double-stranded RNA interference. Suppression of the *MTF-1* (metal-regulatory transcription factor 1), reduced both basal and Cu-induced gene expressions of *MtnA*, *MtnB* and

*MtnD*, significantly reducing the tolerance of these cells to increased Cu. Suppression of either *Ctr1A* (a *Drosophila* orthologue of yeast CTR1) or *Ctr1B* significantly reduced Cu uptake from media, demonstrating that both these proteins function to transport Cu into S2 cells. Significantly, Cu induced *Ctr1B* gene expression, and this could be prevented by suppressing *MTF-1*, suggesting that *Ctr1B* might be involved in Cu detoxification. Suppression of *DmATP7*, the putative homologue of human Cu transporter genes *ATP7A* and *ATP7B*, significantly increased Cu accumulation, demonstrating that *DmATP7* is essential for efflux of excess Cu. This work is consistent with previous studies in mammalian cells, validating S2 cells as a model system for studying Cu transport and identifying novel Cu regulatory mechanisms.

Key words: copper, copper homeostasis, *Drosophila*, gene expression, S2 cell, viability.

### INTRODUCTION

The maintenance of Cu homeostasis at the cellular level is crucial for aerobic organisms. The redox properties of Cu ensure that it is an essential cofactor for several key enzymes including cytochrome *c* oxidase (mitochondrial electron transport chain), CuZn superoxide dismutase (antioxidant defences), tyrosinase (pigmentation) and dopamine  $\beta$ -hydroxylase (catecholamine synthesis) [1]. However, an excess of Cu is toxic since these redox properties also allow Cu ions to generate reactive oxygen species [2].

Studies in yeast and mammalian systems have identified well-conserved mechanisms for maintaining Cu homeostasis, including regulation of uptake, distribution, sequestration and efflux [3]. In humans, several proteins have been identified as having an important role in Cu homeostasis: Cu enters the cell via the high-affinity uptake protein, hCTR1 (a human orthologue of yeast CTR1) [4]; the specific chaperones CCS (copper chaperone for superoxide dismutase), COX17 and ATOX1 (human orthologues of yeast COX17 and ATX1 respectively) are involved in the distribution of Cu to superoxide dismutase, cytochrome *c* oxidase and the secretory pathway respectively [5–7]; sequestration of excess Cu by metallothioneins prevents cellular damage; metallothioneins are encoded by several genes that are transcriptionally regulated by MTF-1 (metal-regulatory transcription factor 1) [8]; and the heavy metal P-type ATPase ATP7A (human copper-transporting ATPase), expressed in extrahepatic tissues, and ATP7B (human copper-transporting ATPase), expressed predominantly in the liver, allow efflux of excess Cu from the cell [9].

Orthologues of several key Cu homeostasis genes have been described in *Drosophila melanogaster*. Cu uptake genes *Ctr1A*, *Ctr1B* and *Ctr1C* (*Drosophila* orthologues of yeast CTR1) are classified as members of the Ctr1 family on the basis of conservation of sequence motifs present in mammalian and yeast Ctr1 genes [10,11]. Studies with these *Drosophila* Ctr genes transfected into S2 cells have shown that Ctr1A and Ctr1B could stimulate Cu uptake, whereas Ctr1C could not [10]. The human genome contains ten functional metallothionein genes, as well as seven pseudogenes [12]. In contrast, only four metallothioneins have been identified in *Drosophila*, designated *MtnA*, *MtnB*, *MtnC* and *MtnD*, all containing the metal response element TGRCNC (R = A or G and N = any nucleotide) [13–15]. *MtnB*, *MtnC* and *MtnD* share a high level of sequence similarity [15]. *MTF-1* has been identified as the *Drosophila* transcription factor capable of inducing expression of these *Mtn* genes in response to Cu [15]. In addition, the present study has identified a *Drosophila* orthologue of *ATP7A* and *ATP7B*, designated *DmATP7*. The *Drosophila* cell line S2 has been used to elucidate the function of the *Drosophila* Cu uptake proteins [10]; however, little else is known about Cu homeostasis in S2 cells.

The specific aims of the present study were to identify orthologues of mammalian Cu homeostasis genes, and use dsRNAi (double-stranded RNA interference) to suppress their gene expression in S2 cells. We suppressed *Ctr1A*, *Ctr1B*, *MTF-1* and *DmATP7* and examined Cu accumulation and retention, cell viability and gene expression. The results demonstrated the similarity between *Drosophila* S2 cells and mammalian systems

Abbreviations used: ATOX1, a human orthologue of yeast ATX1; ATP7A, human copper-transporting ATPase; CCS, copper chaperone for superoxide dismutase; CHO cells, Chinese-hamster ovary cells; dsRNAi, double-stranded RNA interference; MBS, metal-binding site; MTF-1, metal-regulatory transcription factor 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; SFM, serum-free media.

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and establishes S2 cells as a model system for exploring Cu homeostatic mechanisms.

## EXPERIMENTAL

### Cell culture

*Drosophila* S2 cells were propagated in *Drosophila* SFM (serum-free media Invitrogen); supplemented with 367  $\mu\text{M}$  L-glutamine and penicillin/streptomycin (Sigma). Cells were maintained at 27 °C and passaged every 7 days. S2 cells were loosely attached to the surface of tissue culture flasks and could be dislodged by pipetting. For all experiments, Cu was added to SFM as  $\text{CuCl}_2$  at the concentration specified in the Figure legends.

### Real-time PCR

Total RNA was extracted using the RNeasy kit, including DNase treatment, according to the manufacturer's instructions (Qiagen, Chartsworth, CA, U.S.A.). cDNA was transcribed from 1  $\mu\text{g}$  of total RNA using AMV Reverse Transcriptase in a 20  $\mu\text{l}$  reaction according to the manufacturer's instructions (Promega, Charbonnières, France). Primers for real-time PCR were designed using Primer3 software [16] available at [http://www.broad.mit.edu/genome\\_software/other/primer3.html](http://www.broad.mit.edu/genome_software/other/primer3.html).

Forward and reverse primer sequences were: Ctr1A: GCTGGA-ATATCGACCTGTGA, ATCGAAGGTGGTTGCTTGT; Ctr1B: AGCAGCGTAGGAAGAACA, CAGGGACTGGACGATGTG; Ctr1C: GGCGATTCAGAGACCATT, GAGCACAGCAACCATGAAA; CG32444: GGCGATAAGGTCGAGAAAGT, ACGTAGGTGGTGCTCTTGC; CCS: TGGACAAGACACCAATCCAG, ACAACCACCTCCAGGCTTCTT; CG9065: GCAGCTC-CAAGTGTTCG, ATCGGGTGGTTGTGGAT; CG8885: GGC-AGTTGGGAATTAGTTGAT, CTCTAGCTCATCGGGACAAA; DmATP7: CCCACTGACCTTCTTCGATAC, GGTCTTTCCCTTGGCTATGT; MtnA: CCTGCAACTGCGGATCT, CGCAGGCGGATTTCTT; MtnB: ATGGTTTGCAAGGGTTGTG, TTGCAGGCGCAGTTGT; MtnC: CAAAGGCTGCGGAACAA, GCACTTGCAGTCCTGATTACAG; MtnD: AGTGCTCCGCCACCAA, TGTCCTTGGGTCCGTTCT; MTF-1: ACAAGTGGCGAGCAA-CAG, GGCTGTAGGAAGTGAGGAATG; and Actin42A: GCTTCGTTGCTACTTCCA, CAGCCGACTACTGCTTAGA.

Real-time PCR was performed using the Rotor Gene 3000 (Corbett Research, Mortlake, NSW, Australia). Reverse-transcribed total RNA (20 ng) was amplified in a 25  $\mu\text{l}$  reaction containing 1  $\mu\text{M}$  of each primer and 12.5  $\mu\text{l}$  of 2  $\times$  QuantiTect SYBR Green PCR Master Mix (Qiagen).

The amount of gene product in each sample was determined using two different methods, the established  $\Delta\text{CT}$  method [17] and the comparative quantification method using the Rotor Gene 5.0 software (Corbett Research). These two methods provided very similar results, and all data shown in this paper were obtained using the comparative quantification method.

*Actin42A*, a gene that does not transcriptionally respond to copper, was used as a housekeeping gene. The amount of gene product for the gene of interest was expressed relative to that of *Actin42A* to normalize for differences in total cDNA between samples.

### dsRNA production and RNA interference

Production of dsRNA was conducted as reported previously [18]. The gene of interest was PCR-amplified using forward and reverse primers, each containing the T7 RNA polymerase promoter sequence at the 5'-end and pSK(bluescript)-specific sequence at the 3'-end. Forward and reverse primer gene-specific sequences were

TGTAATACGACTCACTATAGGGCAATTAACCCTCACTAAAGGGAA and TGTAATACGACTCACTATAGGGC respectively. PCR products were purified using Ultra-Clean PCR Clean Up (Mo Bio Laboratories, Carlsbad, CA, U.S.A.). RNA was transcribed *in vitro* from 1  $\mu\text{g}$  of DNA template using MEGAscript T7 (Ambion, Austin, TX, U.S.A.). RNA was precipitated, dissolved in water, incubated at 65 °C for 30 min and slowly cooled to 25 °C to allow annealing of the complementary RNA strands.

The method described by Worby et al. [18] to introduce dsRNA into S2 cells for suppressing native RNA was adapted for use with SFM. S2 cells were seeded at a concentration of  $1 \times 10^6$  cells/ml in SFM. dsRNA (15  $\mu\text{g}$ ) was added and the media were swirled vigorously for 10 s. Cells were incubated at 27 °C for 48 h before subsequent experiments.

### Cell viability

Cell viability following Cu exposure was determined colorimetrically using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma] by a method adapted from La Fontaine et al. [19]. Cells were seeded in a 96-well plate at a concentration of  $2 \times 10^5$  cells (0.2 ml per well) and exposed to basal media or media supplemented with Cu for 48 h. The media were replaced with 0.3 ml SFM containing 2.4 mM MTT for 4 h. Media were removed and cells were solubilized in 0.3 ml DMSO (Sigma). The absorbance was read at 600 nm using an EIA plate reader (model no. 2550, Bio-Rad) and the percentage viability was determined by expressing the average absorbance from a given Cu treatment relative to the average absorbance from a reference sample treated with basal media.

### $^{64}\text{Cu}$ accumulation and retention

$^{64}\text{Cu}$  accumulation and retention experiments were conducted using a method adapted from Camakaris et al. [20]. Cells ( $1 \times 10^6 \text{ ml}^{-1} \cdot \text{well}^{-1}$ ) were seeded in a 24-well plate and incubated with approx. 0.4 MBq of  $^{64}\text{Cu}$  (Australian Radioisotopes) and various concentrations of unlabelled Cu for 1 or 24 h. Cu accumulation was stopped by washing cells four times with 1 ml of cold Hanks balanced salt solution containing 1 mM histidine (Sigma). Cells were then lysed in 100  $\mu\text{l}$  of 0.1 % SDS, containing 2 mM EDTA. For Cu-retention experiments, cells were incubated with various concentrations of Cu for 24 h, washed as described above, and incubated for an additional 24 h in 1 ml of basal media. Cells were then washed and lysed as described above. Radioactivity was measured with a  $\gamma$ -counter (1282 CompuGamma, LKB Wallac) and  $^{64}\text{Cu}$  content was determined as described previously [20]. Cu levels were standardized to total cellular protein, which was determined using a Bio-Rad protein reagent according to the manufacturer's instructions (Bio-Rad).

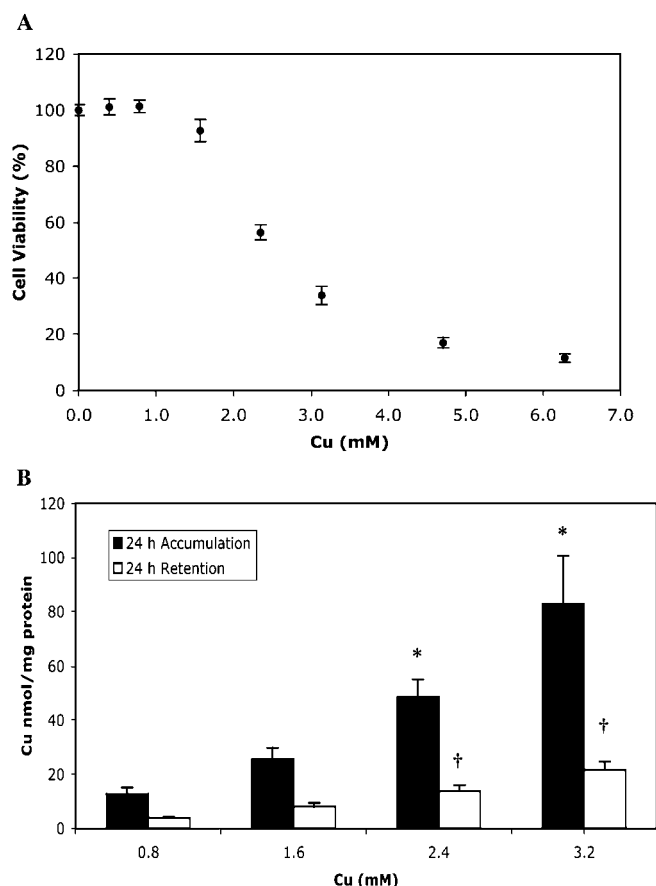
### Statistics

Statistical analysis was conducted using SPSS v11 (SPSS). A one-sample Kolmogorov-Smirnov test was used to assess whether data were normally distributed. Statistical analyses are described in the Figure legends.  $P < 0.05$  was deemed statistically significant.

## RESULTS AND DISCUSSION

### S2 cells are highly Cu tolerant

S2 cells were exposed to Cu concentrations ranging from basal to 6.3 mM and viability was measured 48 h later (Figure 1A). The



**Figure 1** Cell viability and Cu accumulation and retention in S2 cells

(A) Cell viability was measured with an MTT assay after 48 h exposure to 0.2, 0.4, 0.8, 1.6, 2.4, 3.2, 4.8 and 6.4 mM Cu, and is expressed as a percentage of viable cells compared with cells exposed to basal media. Values are means  $\pm$  S.E.M. of six replicates from two independent experiments. (B) Intracellular Cu accumulation and retention in cells exposed to 0.8, 1.6, 2.4 and 3.2 mM Cu labelled with  $^{64}\text{Cu}$ . Accumulation was measured after 24 h Cu exposure. Retention was measured after 24 h Cu exposure and an additional 24 h exposure to basal media. Intracellular Cu was normalized to total cellular protein as described in the Experimental section. Values are means  $\pm$  S.E.M. of six replicates from two independent experiments. A one-way ANOVA with Games–Howell post-hoc test was used to determine statistically significant differences between groups. \* $P < 0.05$  compared with 0.8 mM Cu accumulation; † $P < 0.05$  compared with 0.8 mM Cu retention. A Pearson correlation showed Cu exposure was positively correlated with Cu accumulation ( $r = 0.98$ ,  $P < 0.05$ ) and Cu retention ( $r = 0.99$ ,  $P < 0.05$ ).

Cu concentration needed to reduce cell viability to 50 % of control ( $\text{EC}_{50}$ ) was 2.8 mM, which is consistent with a previous study that found that viability of S2 cells decreased to 70 % of control after 48 h exposure to 2 mM  $\text{CuSO}_4$  [21]. Cu tolerance of S2 cells was much higher than that seen in CHO cells (Chinese-hamster ovary cells), which have a 24 h  $\text{EC}_{50}$  of approx. 0.33 mM  $\text{CuCl}_2$  [19]. However, comparisons between different cell types are difficult due to different measures of viability, different cell-culture media and the bioavailability of Cu. Both  $\text{CuCl}_2$  and  $\text{CuSO}_4$  are used for such studies and Cu is sometimes added as a complex. Comparable levels of Cu tolerance have been found with other cell lines: HL-60, a human leukaemia cell line, has an approximate  $\text{EC}_{50}$  of 1 mM when exposed to copper sulphate/nitrilotriacetate for 14 h [22], and the rat hepatoma cell line HAC has an  $\text{EC}_{50}$  of 0.4 mM when exposed to Cu(II) acetate for 72 h [23].

Variations in Cu tolerance may be due to regulation of Cu uptake, sequestration of free Cu or regulating efflux of excess Cu. Both CHO and HAC cell lines can be adapted to tolerate increased Cu levels. The increased tolerance of CHO-Cur3 cells occurs as a result of *ATP7A* gene amplification, resulting in increased Cu efflux [20]. In contrast, HAC<sub>800</sub> cells have increased Cu tolerance as a result of metallothionein gene amplification. Increased metallothionein mRNA levels in these cells were positively correlated with intracellular Cu concentrations, demonstrating that tolerance is due to efficient Cu sequestration [24].

### S2 cells accumulate and retain excess Cu

Cu accumulation was examined in S2 cells exposed to 0.8, 1.6, 2.4 or 3.2 mM Cu, labelled with  $^{64}\text{Cu}$  (Figure 1B). A 24 h exposure resulted in a dose-dependent increase in Cu accumulation. Cells exposed to 3.2 mM Cu accumulated five times as much Cu as those exposed to 0.8 mM. These results demonstrate that S2 cells do not appear to regulate intracellular Cu via changes in uptake, since exposure to high levels is sufficient to cause cells to accumulate Cu in excess of cellular requirements. Similar results were obtained with CHO cells for 6, 20 and 72 h [25,26].

Cu retention was measured in S2 cells exposed to Cu for 24 h, and then returned to basal media for another 24 h (Figure 1B). Cu 'efflux', the amount of Cu exported from the cell over that period, could be calculated from the difference between accumulation and retention. Cu retention was positively correlated with the initial Cu exposure. Cells were able to export some Cu at all exposure concentrations; however, cells initially exposed to 3.2 mM Cu retained 5-fold more Cu than those exposed to 0.8 mM. These results demonstrate that S2 cells have a mechanism for exporting excess Cu; however, this mechanism is not sufficient to maintain optimal intracellular Cu levels when exposed to a very high Cu concentration. The high levels of Cu accumulation and retention of S2 cells exposed to increased Cu concentrations suggests that the relatively high Cu tolerance of these cells is probably a result of efficient intracellular Cu sequestration than decreased uptake or increased efflux.

### S2 cells express orthologues of Cu homeostasis genes

The sequencing of the *D. melanogaster* genome has facilitated the identification of orthologues of mammalian Cu homeostasis genes involved in uptake, distribution, sequestration and efflux of Cu [27]. These *Drosophila* orthologues show significant protein sequence identity and similarity (Table 1). Three copper uptake genes, *Ctr1A*, *Ctr1B* and *Ctr1C*, have been identified [10,11]. Four metallothioneins, designated *MtnA*, *MtnB*, *MtnC* and *MtnD* [13–15], and *MTF-1*, a transcription factor capable of inducing the expression of these *Mtn* genes, have also been identified [15].

A default BLASTn search of the *Drosophila* genome identified putative orthologues of the human Cu chaperones *ATOX1*, *CCS*, *COX17* and *SCO1* and *SCO2*. One putative Cu-transporting P-type ATPase was also identified, designated *DmATP7*, which shows strong sequence similarity to both *ATP7A* and *ATP7B* of humans (Table 1). The sequence similarity between human *ATP7A* and *ATP7B* was recently reviewed, and across the entire protein sequence, *ATP7A* and *ATP7B* are 54 % identical; however, sequence similarity is much higher for several key motifs [9,28]. These include six N-terminal MBSs (metal-binding sites) characterized by a CXXC motif, eight transmembrane domains and the TGES/A, DKTG, TGDN and AMXGDGVND motifs in the major cytosolic loop. An alignment of the human *ATP7A* and *ATP7B* and the putative *DmATP7* protein sequences using CLUSTAL W [29] demonstrated that all these key motifs were

**Table 1** *D. melanogaster* Cu homeostasis genes are expressed in S2 cells

Putative *D. melanogaster* orthologues of human copper homeostasis genes were identified with a default BLASTn search of the *D. melanogaster* genome (<http://flybase.bio.indiana.edu>). Percentage protein identity and similarity were determined using a default Gap alignment of human and *Drosophila* sequences (<http://www.accelrys.com>).

Human gene (GenBank® accession ID)	Protein function	Drosophila orthologue	Protein sequence identity/similarity (%)	Fold induction by Cu (means ± S.E.M.)
<i>hCTR1</i> (AI831843)	Cu uptake	<i>CG3977 Ctr1A</i>	47.6/57.8	1.2 ± 0.1
		<i>CG15551</i>	PA 38.1/48.4	Not detected
		<i>Ctr1C*</i>	PB 37.5/47.2	
		<i>CG7459 Ctr1B</i>	38.4/46.5	4.3 ± 1.6
<i>ATOX1</i> (AY165037)	Cu chaperone	<i>CG32444</i>	22.4/35.8	1.2 ± 0.1
<i>CCS</i> (AF002210)	Cu chaperone	<i>CG17753 CCS</i>	51.0/64.1	1.1 ± 0.1
<i>COX17</i> (L77701)	Cu chaperone	<i>CG9065</i>	57.1/60.3	0.9 ± 0.1
<i>SCO1</i> (AF026852)	Cu chaperone	<i>CG8885</i>	51.0/62.4	1.1 ± 0.1
<i>SCO2</i> (AL021683)	Cu chaperone	<i>CG8885</i>	44.7/51.6	
<i>ATP7A</i> (L06133)	Cu efflux	<i>CG1886</i>	49.9/58.9	1.7 ± 0.6
<i>ATP7B</i> (U11700)	Cu efflux	<i>CG1886</i>	49.9/59.1	
<i>MT</i> †	Cu sequestration	<i>CG9470 MtnA</i>	N/A	46 ± 12‡
	Cu sequestration	<i>CG4312 MtnB</i>	N/A	1443 ± 517*‡
	Cu sequestration	<i>CG5097 MtnC</i>	N/A	Not detected
	Cu sequestration	<i>CG33192 MtnD</i>	N/A	474.4 ± 149.3*‡
<i>MTF-1</i> (X78710)	MT transcription factor	<i>CG3743</i>	PA 32.0/38.5	1.4 ± 0.2
		<i>MTF-1*</i>	PB 27.0/32.4	

\* FlyBase identified two protein sequences, designated PA and PB, for both *Ctr1C* and *MTF-1*. Gene expression was measured with real-time PCR and normalized to *Actin42A* expression as described in the Experimental section. Gene expression was measured in S2 cells exposed to basal media and 0.8 mM Cu for 24 h. Values are gene expression in cells exposed to Cu relative to cells exposed to basal media and are the means ± S.E.M. of four replicates from two independent experiments. A one-sample *t* test was used to determine if gene expression was significantly different with Cu exposure.

† *Drosophila Mtn* sequences were similar to multiple members of the human *MT* family and were not assigned (N/A) to a single putative orthologue.

‡ *P* < 0.05.

conserved in DmATP7 (results not shown). The conservation of these key motifs in *Drosophila* suggests that this protein is probably a functional Cu-transporting ATPase. Interestingly, the protein sequence alignment showed that DmATP7 has only four N-terminal MBS, corresponding to MBS 3–6 in human ATP7A and ATP7B.

S2 cells are of embryonic origin, and it was important to establish whether these putative *Drosophila* Cu genes were expressed in these cells. Real-time PCR was used to examine gene expression in cells under basal conditions and after exposure to 0.8 mM Cu. All the genes in Table 1, with the exception of *Ctr1C* and *MtnC*, were expressed in S2 cells. PCR failure was unlikely to be responsible for the inability to detect expression of these genes since expression was detected using adult *D. melanogaster* cDNA under the same PCR conditions (results not shown). A previous study using an RNA blot also failed to detect either *Ctr1B* or *Ctr1C* expression in S2 cells [10]. However, using real-time PCR, *Ctr1B* was detectable and expression was also induced by Cu exposure (Table 1).

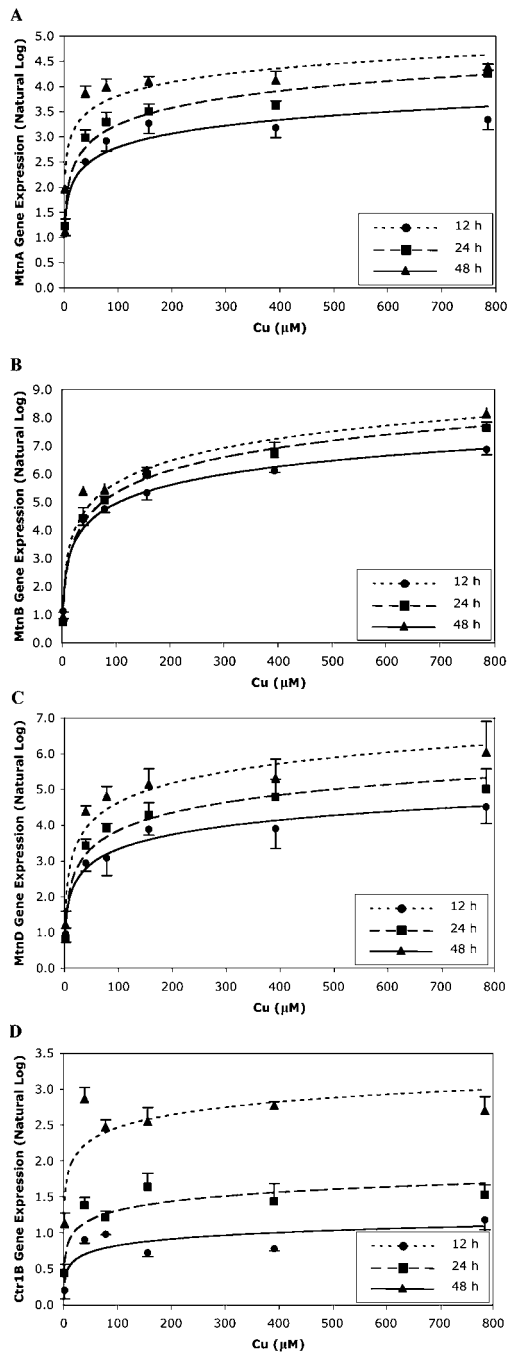
Orthologues of the mammalian Cu chaperones *ATOX1*, *CCS*, *COX17* and *SCO1* and *SCO2* did not significantly respond transcriptionally to increased Cu levels. As expected, the expression of *MtnA*, *MtnB* and *MtnD* genes in S2 cells was significantly increased after Cu exposure (Table 1). *Drosophila Mtn* expression has been shown to be temporally and tissue-specifically induced by Cu [13–15]. To explore the *Mtn* gene expression profile further, we exposed S2 cells to various Cu concentrations for 12, 24 and 48 h and measured gene expression with real-time PCR (Figures 2A–2C). Gene expression increased in a time- and dose-dependent manner for all three *Mtn* genes, although the expression of *MtnB* and *MtnD* increased more than that of *MtnA* at all time points. Interestingly, under these Cu conditions, *Ctr1B* gene expression also increased in a time- and dose-dependent manner

(Figure 2D). In contrast, *Ctr1A* gene expression did not change after Cu exposure (results not shown). To the best of our knowledge, this is the first time a *Ctr1* gene has been shown to be up-regulated by Cu. Studies using *Drosophila* larvae found that *Ctr1B* gene expression increased after Cu depletion and decreased with excess Cu [10] and similar results are seen in yeast [30].

To investigate the role of Cu homeostasis genes in S2 cells, we utilized dsRNAi to suppress gene expression of *Ctr1A*, *Ctr1B*, *MTF-1* and *DmATP7*. S2 cells were treated with dsRNA for 48 h before analysis. Real-time PCR was used to measure the gene expression, which was expressed as a percentage of that in control cells treated with non-specific dsRNA. Mean *Ctr1A* gene expression was reduced to 9.9% of control (S.E.M. = 2.8%), *Ctr1B* was reduced to 15.0% (S.E.M. = 4.6%), *MTF-1* was reduced to 24.1% (S.E.M. = 2.6%) and *DmATP7* was reduced to 6.9% (S.E.M. = 2.0%). Suppression was maintained at these levels for at least 4 days and often up to 7 days or more (results not shown). Similar dsRNAi experiments in S2 cells have also shown gene suppression that lasted over 7 days [21].

### *Ctr1A* and *Ctr1B* transport Cu into S2 cells

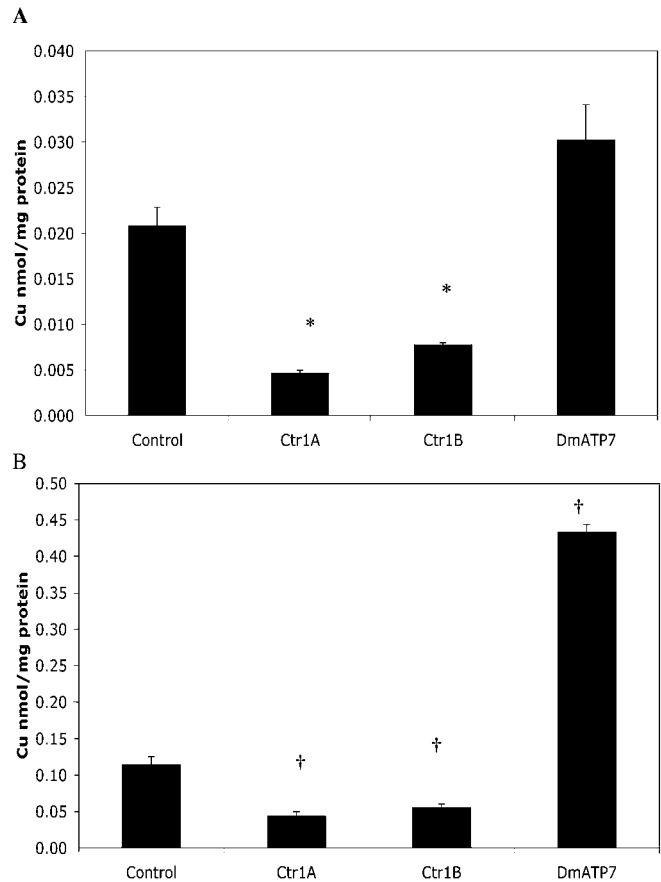
The expression of *Ctr1A* and *Ctr1B* was suppressed using dsRNAi, and Cu accumulation in S2 cells was then determined after exposure to 2 μM Cu. As expected, both *Ctr1A*- and *Ctr1B*-dsRNAi-treated cells took up significantly less Cu than control cells over 1 h (Figure 3A), and when exposed to 2 μM Cu for 24 h, only accumulated half as much Cu as control cells (Figure 3B). Suppression of each *Ctr1* gene did not affect the gene expression of the other (results not shown), suggesting that the unaffected *Ctr1* could function to transport Cu into the cell. However, it appears that both *Ctr1A* and *Ctr1B* are necessary for maximal Cu accumulation, under physiological Cu conditions. Cu tolerance



**Figure 2** Metallothionein gene expression in S2 cells after Cu exposure

S2 cells were exposed to basal media and 2, 39, 79, 157, 393 and 785  $\mu\text{M}$  Cu for 12, 24 and 48 h, and then *MtnA* (A), *MtnB* (B), *MtnD* (C) and *Ctr1B* (D) gene expressions were measured with real-time PCR. Expression was normalized to *Actin42A* and expressed relative to cells exposed to basal media. Data were  $\log_e$ -transformed and values are the means  $\pm$  S.E.M. of three replicates. The gene expression profiles of *MtnA*, *MtnB*, *MtnD* and *Ctr1B* were significantly related to Cu exposure concentration and time of Cu exposure, as determined from a general linear model univariate analysis,  $P < 0.05$ .

was not significantly affected by suppression of either *Ctr1A* or *Ctr1B* (Figure 4). Unfortunately, attempts to use dsRNAi to suppress both *Ctr1A* and *Ctr1B* simultaneously were unsuccessful; hence it was not possible to determine if there are other mechanisms of Cu uptake active in these cells.

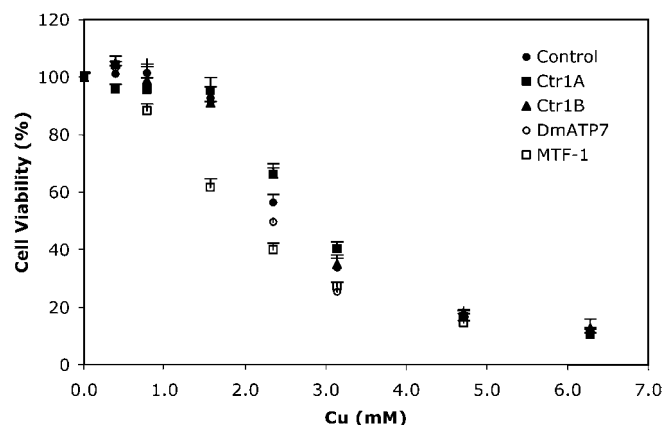


**Figure 3** Cu accumulation in S2 cells after dsRNAi treatment

S2 cells were pretreated with *Ctr1A*, *Ctr1B*, *DmATP7* or control dsRNA. (A) Cells were exposed to 2  $\mu\text{M}$  Cu-labelled with  $^{64}\text{Cu}$ , and intracellular Cu was measured after 1 h. Cu was normalized to total cellular protein. Values are means  $\pm$  S.E.M. of nine replicates from three independent experiments. Data were normally distributed and a one-way ANOVA with Games–Howell post-hoc test was used to determine significant differences. \* $P < 0.05$  compared with control. (B) Cells were exposed to 2  $\mu\text{M}$  Cu labelled with  $^{64}\text{Cu}$ , and intracellular Cu was measured after 24 h. Cu was normalized to total cellular protein. Values are means  $\pm$  S.E.M. of nine replicates from three independent experiments. Data were not normally distributed and a Mann–Whitney test was used to determine significant differences. † $P < 0.05$  compared with control.

### *MTF-1* suppression reduces Cu tolerance

Cu tolerance was examined after suppression of *MTF-1*. The viability of treated cells was significantly lower than that of control when exposed to Cu (Figure 4), probably due to an inhibition of Cu-induced *Mtn* expression. To test this hypothesis, we exposed *MTF-1*-dsRNAi-treated cells to basal media and 0.8 mM Cu and measured the expression of *MTF-1* and the *Mtn* genes. Relative to basal conditions, Cu significantly increased expression of *MtnA*, *MtnB* and *MtnD* in control cells; however, suppression of *MTF-1* significantly reduced this response (Table 2). After *MTF-1* suppression, *MtnA*, *MtnB* and *MtnD* expressions were also reduced under basal conditions, demonstrating an additional role for this transcription factor in the absence of increased Cu. Expression of the *Mtn* genes tended to increase slightly in *MTF-1*-dsRNAi-treated cells after Cu exposure, which is probably due to the ability of residual *MTF-1* protein to induce some *Mtn* expression. Alternatively, there may be another, as yet unidentified mechanism capable of inducing a limited amount of *Mtn* expression in response to Cu.



**Figure 4** S2 cell viability after dsRNAi treatment

S2 cells were pretreated with *MTF-1*, *DmATP7*, *CtrlA*, *CtrlB* or control dsRNA. Cell viability was measured with an MTT assay after 48 h exposure to 0.2, 0.4, 0.8, 1.6, 2.4, 3.2, 4.8 and 6.4 mM Cu and expressed as a percentage of viable cells compared with those exposed to basal media. Values are means  $\pm$  S.E.M. of six replicates from two independent experiments. Viability was significantly dependent on Cu exposure concentration and different between groups as determined from a general linear model univariate analysis;  $P < 0.05$ . A Dunnett post-hoc test found that viability of *MTF-1*-treated cells was significantly different from control cells;  $P < 0.05$ .

Interestingly, *MTF-1* suppression also reduced the basal and Cu-induced expressions of *CtrlB* (Table 2). *CtrlB* contains several copies of the metal-response elements that are bound by *MTF-1*, suggesting that this transcription factor may regulate *CtrlB* as well as the *Mtns* [10]. Studies in *Drosophila* have shown that *CtrlB* [10] and *MTF-1* [15] knockout flies are sensitive to both increased and decreased Cu levels. Zhou et al. [10] proposed that sensitivity of *MTF-1* null flies to low Cu levels may be due to loss of *CtrlB* regulation and that sensitivity of *CtrlB* null flies to high Cu levels may be due to a potential role of this protein in Cu detoxification. This is supported by studies with yeast *Ctr1* and human *Ctrl1* that show that excess Cu stimulates the endocytosis of these proteins [31,32]. If *CtrlB* translocates in response to Cu it would be very interesting to explore whether this occurs to prevent excess Cu uptake or to sequester and detoxify intracellular Cu actively. The *MTF-1*-mediated up-regulation of *CtrlB* expression found in S2 cells exposed to Cu supports an active detoxification role for *CtrlB*, where this protein may mediate a sequestration of excess Cu.

**Table 2** Gene expression in S2 cells after *MTF-1* dsRNAi treatment

S2 cells were pretreated with *MTF-1* or control dsRNA and then exposed to either basal media or 0.8 mM Cu for 24 h. *MTF-1*, *MtnA*, *MtnB* and *MtnD* gene expressions were measured with real-time PCR and normalized to *Actin42A* expression. Values were expressed relative to control cells exposed to basal media and are the means  $\pm$  S.E.M. of four replicates from two independent experiments. *MTF-1*, *MtnA* and *CtrlB* gene expression were normally distributed and a one-way ANOVA with Games-Howell post-hoc test was used to determine significant differences. \*  $P < 0.05$  compared with control cells exposed to basal media. †  $P < 0.05$  compared with *MTF-1* cells exposed to basal media. ‡  $P < 0.05$  compared with control cells exposed to basal media. §  $P < 0.05$  compared with *MTF-1* cells exposed to basal media.

dsRNA	Cu (mM)	<i>MTF-1</i>	<i>MtnA</i>	<i>MtnB</i>	<i>MtnD</i>	<i>CtrlB</i>
Control	Basal	1.00 $\pm$ 0.05	1.00 $\pm$ 0.04	1.00 $\pm$ 0.04	1.00 $\pm$ 0.10	1.00 $\pm$ 0.03
Control	0.8	1.87 $\pm$ 0.05*	5.79 $\pm$ 0.23*	402 $\pm$ 44.6†	1326 $\pm$ 156†	2.71 $\pm$ 0.11*
<i>MTF-1</i>	Basal	0.24 $\pm$ 0.01*	0.20 $\pm$ 0.01*	0.18 $\pm$ 0.02†	0.34 $\pm$ 0.05†	0.32 $\pm$ 0.05*
<i>MTF-1</i>	0.8	0.26 $\pm$ 0.04*	1.51 $\pm$ 0.14*‡	3.43 $\pm$ 0.27†§	6.54 $\pm$ 1.29†§	0.60 $\pm$ 0.08*

### *DmATP7* is required for Cu efflux from S2 cells

Cu accumulation and retention studies with wild-type S2 cells showed that these cells were capable of effluxing Cu (Figure 1A). Given that bioinformatic analysis suggests that *Drosophila* has only one putative Cu translocating P-type ATPase (Table 1), it seemed likely that this protein is responsible for Cu efflux from S2 cells, similar to the role of ATP7A and ATP7B in mammalian cells [20]. Cu accumulation was examined in *DmATP7*-dsRNAi-treated cells. After exposure to 2  $\mu$ M Cu for 1 h, cells did not show a significant increase in Cu accumulation (Figure 3A); however, 24 h exposure to Cu resulted in a 4-fold increase in Cu accumulation (Figure 3B). These results demonstrate that *DmATP7* is required for efflux of Cu from S2 cells.

Suppressing *DmATP7* gene expression did not significantly affect S2 cell viability in response to Cu (Figure 4), suggesting that it may not be the major copper detoxification mechanism. It is possible that a complete knockout of *DmATP7* would cause reduced tolerance to increases in copper; however, the level of *DmATP7* gene suppression in the present study was sufficient to increase intracellular Cu levels significantly without any significant effect on cell viability. Since wild-type S2 cells continue to accumulate Cu when exposed to increased concentrations, it is probable that the excess intracellular Cu must be efficiently sequestered to prevent cellular damage from reactive oxygen species. Unfortunately, we were not able to suppress the expression of both *DmATP7* and *MTF-1* with dsRNAi simultaneously. We predict that S2 cells lacking significant levels of *DmATP7* and *MTF-1* would be extremely sensitive to Cu exposure, as there does not appear to be another P-type ATPase for Cu efflux, and *Mtn* gene expression could not be significantly induced by Cu in S2 cells with reduced *MTF-1* expression (Table 2).

We have examined mechanisms of Cu homeostasis in *Drosophila* S2 cells and found that they not only accumulate and export Cu in a comparable way to mammalian cells, but also there is an expression of orthologues of mammalian genes involved in Cu uptake, transport, sequestration and efflux. Although both *CtrlA* and *CtrlB* are involved in Cu uptake, Cu-induced expression of *CtrlB* is mediated by *MTF-1*, suggesting a dual role in Cu uptake and detoxification. Sequestration appears to be the primary method of Cu detoxification in S2 cells, as preventing induction of *Mtn* expression by suppressing *MTF-1* reduces copper tolerance. However, *DmATP7* is also important, as it is essential for efflux of excess Cu. This work is consistent with previous studies in mammalian cells and validates S2 cells as an efficient and malleable system for further exploring mechanisms of Cu homeostasis.

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