

Copper homeostasis gene discovery in *Drosophila melanogaster*

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Abstract Recent studies have shown a high level of conservation between *Drosophila melanogaster* and mammalian copper homeostasis mechanisms. These studies have also demonstrated the efficiency with which this species can be used to characterize novel genes, at both the cellular and whole organism level. As a versatile and inexpensive model organism, *Drosophila* is also particularly useful for gene discovery applications and thus has the potential to be extremely useful in identifying novel copper homeostasis genes and putative disease genes. In order to assess the

suitability of *Drosophila* for this purpose, three screening approaches have been investigated. These include an analysis of the global transcriptional response to copper in both adult flies and an embryonic cell line using DNA microarray analysis. Two mutagenesis-based screens were also utilized. Several candidate copper homeostasis genes have been identified through this work. In addition, the results of each screen were carefully analyzed to identify any factors influencing efficiency and sensitivity. These are discussed here with the aim of maximizing the efficiency of future screens and the most suitable approaches are outlined. Building on this information, there is great potential for the further use of *Drosophila* for copper homeostasis gene discovery.

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Introduction

There remain unanswered questions about the transport and maintenance of copper levels in multicellular organisms. For example, Ceruloplasmin is the major plasma form of copper but copper deficiency does not occur in its absence indicating these are not the only possible plasma sources of copper for tissues (Vargas et al. 1994;

Harris et al. 1998). Yet copper status is markedly different between tissues (Linder 1991), and organs have the ability to conserve copper during times of inadequate dietary intake (Levenson 1998). The known cellular copper transporters and chaperones are essential for the uptake, transport, and efflux of copper, but this does not explain how the appropriate amount of copper is taken into each organ and sub-cellular compartment and how the distinct levels are maintained. So what are the sensors of copper status and what are the regulators? In addition, few protein–protein interactions have been defined for the known copper homeostasis pathways. What other genes may be involved, and may be potential disease genes? This is particularly important in the case of copper transport proteins, which undergo copper regulated intracellular trafficking (Petrus et al. 1996). The genomic tools currently available have the potential to be highly productive in addressing these areas through the generation and characterization of a large number of mutations and through screening the transcriptional response to a wide variety of conditions. Mammalian models are not ideal for this type of forward approach to identifying novel copper homeostasis genes due to expense and ethical considerations. A suitable approach would be to use mice for the downstream analysis of novel mechanisms in comparison to humans and for the investigation of potential treatments after as much preliminary research as possible has been achieved in a simpler system.

The multicellular nature of the fly and the existence of effective mutagenesis, gene expression analysis, and transgenic systems make *Drosophila melanogaster* potentially a very useful tool for the isolation and characterization of novel genes as well as the further study of previously identified copper homeostasis mechanisms. A variety of recent studies demonstrate high conservation between insect and mammalian copper homeostasis mechanisms (e.g., Egli et al. 2003; Zhou et al. 2003; Southon et al. 2004; Norgate et al. 2006). These studies also highlight the suitability of *Drosophila* for efficiently characterizing copper homeostasis genes through mutagenesis and developmental studies. They have provided novel information on factors such

as gene expression patterns during development and previously unobserved maternal effects.

We investigated three different screening approaches to identify novel copper homeostasis genes using *Drosophila*. These included a study of the transcriptional response to copper in adult flies and cultured embryonic cells and two mutagenesis-based screens. Here we present the outcomes of this work with the aim of guiding future screens for copper homeostasis genes using *Drosophila*.

Materials and methods

Transcription

Four-day-old adult female Celera were fed 0, 0.8 or 2 mM CuSO₄ (Merck Pty Ltd, Kilsyth, VIC, Australia) in standard laboratory medium for 7 days. 0.8 mM CuSO₄ is the highest concentration on which *Drosophila* can live and breed normally (Turner and Gardner 1960) and 2 mM Cu is the LC50 after 2 weeks, but is not associated with an increase in mortality after 1 week (data not shown). *Drosophila* embryonic S2 cells were cultured in 0 and 0.8 mM added CuCl₂ (BDH Chemicals Ltd, London UK) in serum free media (SFM; Invitrogen, Paisley, UK) for 24 h as described in Southon et al. (2004). 0.8 mM CuCl₂ does not decrease cell viability compared to control and LC50 is 2.8 mM CuCl₂ (Southon et al. 2004). Paraquat and ageing experiments were conducted using fly heads as described in McCarroll et al. (2004). All samples were compared to an untreated control using *Drosophila* GeneChips (Affymetrix, Santa Clara, CA, USA) and according to the manufacturer's instructions. Expression values were normalized using the MASS5 algorithm and values were excluded from future analysis if labeled as 'Absent' in both samples. Differentially expressed genes were identified as those genes with expression levels, which exceeded twofold relative to the untreated control on both of two replicates for a given treatment.

Quantitative Real Time PCR was performed as described in Southon et al. (2004). For Real Time PCR experiments in S2 cells, cells were exposed

to 2, 39, 79, 157, 393, and 785 μM Cu for 12, 24, and 48 h and gene expression was normalized to *Actin42A*. General linear model univariate analysis was used to identify dependence of gene expression on copper concentration and exposure time. $P < 0.05$ was considered significant. Primers for Real Time PCR were designed using Primer3 software (Rozen and Skaletsky 2000) available at http://www.broad.mit.edu/genome_software/other/primer3.html. Primer sequences for *Actin42A*, *MtnA*, *MtnB*, *MtnC*, *CtrlA*, and *CtrlB* have been previously published (Southon et al. 2004). Forward and reverse primer sequences for novel genes were: *CG7194*: CCTTCGCCAGG-TATTCG, ACGGTGAGATCAGCCTCTAAA; *CG14757*: GCGCCAACTACGGTTGA, CACGGAGGAAGATCCAA; *CG11825*: CCTTTATGCTGGTGGGTATTG, GTCTGGCCTGGTATTGCTTT; *CG14036*: TTGCCCTTACGACAAATCG, CGATGCTGCGGAGATAGTAGT.

The methods used for characterization of candidate genes in S2 cells are described in Southon et al. (2004). Double-stranded RNA interference (dsRNAi) was used to knock-down gene expression, following which changes in copper accumulation and cell viability were measured in cells cultured in different concentrations of copper. Cells were exposed to 2 μM copper labeled with ^{64}Cu (ARI, Lucas Heights, NSW, Australia) and intracellular accumulated radio-copper was measured after 1 h and normalized against total cellular protein. Data shown include six replicates from two independent experiments. An independent *t*-test was used to determine the significance of changes in copper levels relative to control GFP dsRNA treated cells. $P < 0.05$ was considered significant.

Deficiency Kit

The 196 heterozygous deletion mutants that make up the Deficiency Kit were obtained from the Bloomington Stock Center. The applications of this kit are described in Results and Discussion and the complete stock list and mortality data are available on request. For each strain, five replicates of 50 first instar larvae were scored for survival to adulthood on 0, 1, 2, 3, and 4 mM CuSO_4 . Mortality of each mutant on 0, 1, and

2 mM copper was compared to that of wild-type Armenia (Arm^{60} ; obtained from the European *Drosophila* Stock Center, Umeå, Sweden) by a one-way ANOVA using a Dunnett comparison. $P < 0.05$ was considered significant. Resistance/sensitivity was assigned to strains that showed no significant difference in mortality on control medium, but significantly lower/higher than Armenia on copper.

EMS mutagenesis

Virgin Armenia were treated with 100 μL of ethyl methanesulfonate (EMS) whilst under a vacuum, as described by Segal and Lee (1970) with modifications described by Smyth et al. (1992). Embryos were collected every 12–14 h for 7–10 days from mass mated EMS-treated flies and transferred to 4 mM CuSO_4 in standard laboratory media. Approximately 650,000 embryos were screened from 13 independent EMS treatments. Survivors were individually crossed to wild-type Armenia and re-screened on 4 mM copper before being maintained on 3 mM copper for higher numbers. To confirm resistance, these lines were screened as for the Deficiency Kit on 0, 3 and 4 mM copper and mortality compared to Armenia using a one-sided Mann–Whitney test. $P < 0.05$ was considered significant. Chromosomal mapping was performed using a reciprocal cross (for the X Chromosome) and a back cross to a *bw st* marker stock (for Chromosomes 2 and 3). A contingency Chi-square test was used to identify deviation from expected ratios. $P < 0.05$ was considered significant.

Statistical analysis

Real Time PCR analysis was performed as described in Southon et al. (2004). SPSS v11 (SPSS) was used for all statistical analysis of S2 cell data. PriProbit v1.63 (Masayuki Sakuma; available for download from <http://bru.usgmr1.isu.edu.au/throne/>) was used to plot dosage-mortality curves and calculate LC50 and LC95 to compare mortality between different *Drosophila* strains. Other statistical analysis involving live flies was performed using Minitab Release 14.1 (Minitab Inc., State college, PA, USA). Statistical analyses are described in the

above methods and the Results and Discussion where appropriate.

Results and discussion

The global transcriptional response to copper in *Drosophila*

To investigate the *Drosophila* transcriptional response to copper, this study employed Affymetrix *Drosophila* GeneChips, which interrogate more than 13,000 genes on a single array. These data were compared to the effects of oxidative stress in adults. Here we assess the sensitivity of examining the global transcriptional response by using whole flies, illustrate the potential for confirmation of candidates using *Drosophila* S2 cells, and compare the transcriptional responses to copper and oxidative stress in adult flies.

Table 1 shows the *Drosophila* orthologues of known copper homeostasis genes that have a change in transcription in response to at least one of the treatments. As expected, all four known *Drosophila* metallothioneins respond to copper, showing the trend of a greater increase in expression for 2 mM than 0.8 mM copper. *MtnA* has previously been suggested to be more important for the response to toxic levels of copper than *MtnB*, as it has a lower transcriptional response to excess copper (Silar et al. 1990; Durliat et al. 1995). Examining the fold-changes in expression shown in Table 1 suggests the opposite in this case. The raw data for these genes is shown in

Table 2. The ‘signal’ values (expression before normalization against the control sample) indicate *MtnA* expression is in fact far higher than *MtnB* on both control and copper treatments and does follow the expected trend in expression levels. Because the expression of *MtnB* increases from undetectable on control medium to much higher on copper, the fold-change is greater for this gene. Microarray analysis is superior in measuring fold-changes compared to previously used methods such as in situ hybridization and northern analysis. This approach allowed us to observe the dramatic change in *MtnB* expression. The exact fold-change is not accurate as this is calculated by dividing the copper result by the control result, which requires dividing by an undetectable value. However, the result indicates the *MtnB* response, while not reaching the absolute levels of *MtnA*, may be more important than previously thought.

No other transcriptional changes are detected from known copper homeostasis genes in the copper-treated samples. Downregulation of *CtrlB* and *C* and *MtnB* and *D* is detected in the paraquat-treated sample, and aged flies also have lower expression of *CtrlB* than young flies. The downregulation of copper uptake genes may reflect a benefit of reducing copper, known to mediate paraquat toxicity, in the presence of excess paraquat (Zer et al. 1991). In addition, the Glutathione S transferases GstD4, GstD5, GstD9, and an uncharacterized Gst (CG6776) all responded to copper. This is in agreement with the suggestion that glutathione is important

Table 1 Changes in gene expression for known copper homeostasis genes

Gene	0.8 mM Cu	2 mM Cu	S2 cells	Paraquat
Total upregulated	17	38	142	782
Total downregulated	5	56	167	727
CtrlB	–	–	–	0.399
CtrlC	–	–	–	0.171
MtnA	–	2.558	–	–
MtnB	31.785	260.418	6.202	0.168
MtnC	5.173	13.635	–	–
MtnD ^a	3.214	5.473	3.047	0.386

The total number of genes upregulated and downregulated is shown, along with the fold-change values for the known copper homeostasis genes that respond to at least one treatment

^a All *MtnD* values refer to the antisense transcript CG15921

Table 2 A comparison of the MtnA and MtnB expression data

MtnA	0 mM Cu	0.8 mM Cu	2 mM Cu
Signal	3994.7	6492.3	12920.2
Detection (<i>P</i> -value)	<i>P</i> (0.001)	<i>P</i> (0.001)	<i>P</i> (0.001)
Expression change (<i>P</i> -value)	–	I (0.000)	I (0.000)
MtnB	0 mM Cu	0.8 mM Cu	2 mM Cu
Signal	10	784.5	4233.6
Detection (<i>P</i> -value)	A (0.223)	<i>P</i> (0.001)	<i>P</i> (0.001)
Expression change (<i>P</i> -value)	–	I (0.000)	I (0.000)

Signal is the amount of fluorescence detected from the microarray. The detection *P*-value is used to calculate whether the level of Signal is above the detection level and a ‘present’ (P) or ‘absent’ (A) flag is given accordingly. The expression change is assigned based on a *P*-value, where ‘I’ is an increase compared to control

in responding to the toxic effects of copper (Freedman et al. 1989).

The inability to detect a response from *MtnA* on 0.8 mM copper, due to its falling below the twofold significance threshold, suggests the sensitivity of the screen may be insufficient to detect important responses. This may explain the larger number of genes identified by screening S2 cells rather than whole flies (Table 1). The sensitivity could be improved most simply by increasing the number of replicates, which would allow the significance threshold to be lowered. Alternatively, dissecting and screening different tissues separately would reduce the ‘dilution’ effect of combining multiple tissues not all of which have a response. This would most easily be done in third instar larvae.

The next steps for genes of interest

While microarrays are powerful for screening the entire genome, the major disadvantage is the expense, which can limit the number of conditions and replicates investigated. We used quantitative Real Time PCR to further examine ten genes identified through microarray analysis of copper treatment. The treatment conditions used for microarray were replicated, but the data set was also expanded to include more time-points and copper concentrations. The candidates identified through the S2 cell microarray proved more reproducible than those identified in adults as the fold-change was greater. In adults, only *MtnB* and *MtnC* dramatically exceed a twofold increase in expression, and when the experiment was replicated by Real Time PCR the response,

although following the same trend, was below the twofold significance threshold (data not shown). In S2 cells, several interesting genes showed a reproducible response, which was examined on a greater number of concentrations and exposure times. This was then followed up by examining the effects of dsRNAi knock-down on copper transport in S2 cells.

S2 cell microarray analysis revealed *CG14036* was upregulated 8.0-fold and this was reproduced with Real Time PCR (Fig. 1). The gene expression profile of *CG14036* was significantly related to copper concentration and time of exposure ($P < 0.05$). dsRNAi knock-down of the metal-responsive transcription factor MTF-1 (Egli et al. 2003) did not inhibit copper induced expression of *CG14036*, indicating its expression is not regulated by this key copper homeostasis transcription

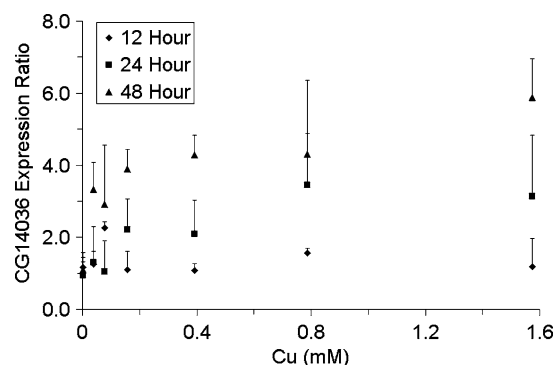


Fig. 1 Transcriptional response of *CG14036* in S2 cells. Gene expression was normalized against *Actin42A* and expressed relative to control medium, so the Y-axis represents the fold change (1.0 = no change). The values shown are the mean $\pm$ SEM of two replicates

factor (not shown). Interestingly gene expression was increased not only by copper but also following copper chelation using 10 μ M Diamsar. This expression change was not significant at 4 days (1.33 ± 0.07) but was at 6 days (2.15 ± 0.07 , $P < 0.05$). This may suggest a role under conditions of both excess and limiting copper. MTF-1 and Ctr1B mutants show sensitivity to both excess and limiting copper (Egli et al. 2003; Zhou et al. 2003), indicating it is possible for a gene to have a significant role under both conditions.

To further elucidate the role of *CG14036* in copper homeostasis we examined copper accumulation in S2 cells following dsRNAi knock-down. Copper accumulation was significantly reduced relative to control GFP dsRNA treated cells when exposed to 2 μ M Cu for 1 h (0.87 ± 0.04 , $P < 0.05$). Cell viability following 48 h exposure to copper concentrations up to 6.4 mM was not affected by dsRNAi of *CG14036*.

CG14036 encodes a 93 amino acid protein, that is, orthologous to the human hypothetical protein LOC121355 (37.3% identity, 45.2% similarity). These proteins contain a conserved domain, UPF0224, the function of which has not been characterized (CDART, <http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>). Other microarray studies have found *CG14036* transcription is related to cell division and development (Stathopoulos et al. 2002; Dimova et al. 2003). Interestingly, another gene with a copper concentration and exposure time dependent downregulation in S2 cells (*CG14545*; not shown) was also identified by Dimova et al. (2003) as being related to cell division and development. dsRNAi of *CG14545* had no effect on either copper accumulation or cell viability in response to copper (data not shown). Further studies are underway to determine whether *CG14036* is involved in copper homeostasis directly, or whether these effects are secondary to a more general role in cell division.

CG11825 was downregulated 5.3-fold by copper in S2 cells and 2.4-fold in adults exposed to 2 mM copper. Real Time PCR demonstrated that gene expression was significantly decreased at all copper concentrations and time points ($P < 0.05$; Fig. 2). *CG11825* gene expression was not affected by copper chelation (not shown). dsRNAi

knock-down of *CG11825* significantly reduced copper accumulation when cells were exposed to 2 μ M Cu for 1 h (0.83 ± 0.03 , $P < 0.05$). Cell viability following 48 h exposure to copper was not affected by dsRNAi of *CG11825* (data not shown).

CG11825 encodes a 93 amino acid protein, that is, orthologous to the human Hypoxia Induced Gene (HIG1) domain family, member 1 A (58.9% identity, 50.0% similarity). This gene is induced by hypoxia in cultured mouse cerebral cortical cells (Jin et al. 2002). Hypoxia is induced by several metals including nickel and cobalt (Goldberg et al. 1988). Induction of HIG1 and other hypoxia-induced genes is due to the transcription factor Hypoxia Inducible Factor (HIF)-1. Recent studies have shown copper is important for HIF-1 stability in the nucleus, and that copper is important for HIF-1 induction of Ceruloplasmin (Martin et al. 2005). It is unclear why *CG11825* was downregulated by copper or how dsRNAi knock-down of this gene can reduce copper accumulation. Further studies are aiming to explore this intriguing aspect of copper homeostasis.

CG14757 and *CG7194* both show a transcriptional response to copper on the S2 cell microarray (3.1-fold and 3.8-fold upregulated, respectively). This result was reproduced by Real Time PCR (Figs. 3, 4) and was not affected by MTF-1 knock-down (not shown). *CG7194* encodes a 346 amino acid protein that contains no known copper binding motifs, but it does contain a Mab-21 domain. Mab-21 has been shown to be required for the choice of cell fate alternatives in *Caenorhabditis elegans* (Chow et al. 1995) and a tri-peptide repeat in the human homolog of this *C. elegans* protein, CAGR1, is associated with developmental disorders (Margolis et al. 1996). *CG14757* encodes a 163 amino acid, 16 kDa protein with a single MxxM motif, a TPR-div 1 domain and a COG2938 domain. The TPR, or tetra trico peptide repeat domain is a structural motif that mediates protein–protein interactions and the assembly of multiprotein complexes (D’Andrea and Regan 2003; Kanz et al. 2005). The mammalian conserved oligomeric Golgi complex (COG) is a multiprotein complex important for Golgi structure and function (Ungar et al.

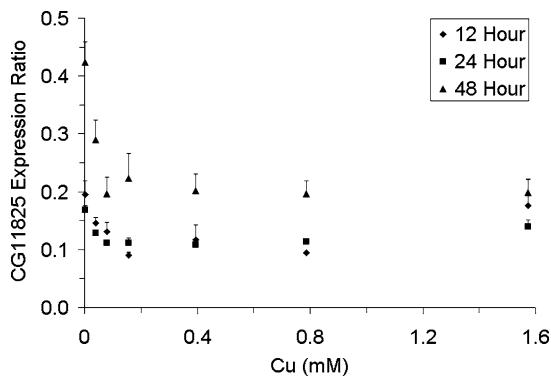


Fig. 2 Transcriptional response of CG11825 in S2 cells. These data are expressed and analyzed as described in the Fig. 1 legend

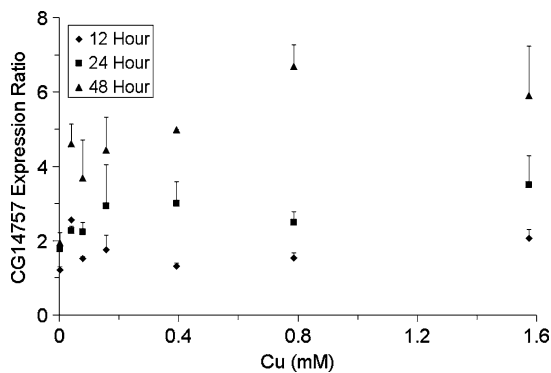


Fig. 3 Transcriptional response of CG14757 in S2 cells. These data are expressed and analyzed as described in the Fig. 1 legend

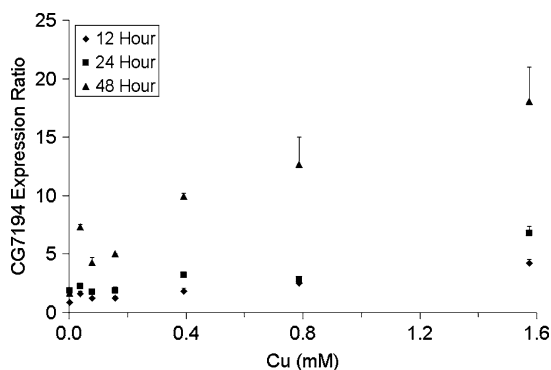


Fig. 4 Transcriptional response of CG7194 in S2 cells. These data are expressed and analyzed as described in the Fig. 1 legend

2002). The predicted *CG14757* protein also contains the sequence MxxM, which may represent a copper binding motif as found in the Ctr proteins

and CutC (Li et al. 2005). This description of a low-molecular weight protein with motifs for copper binding and protein–protein interaction is typical of chaperone proteins such as Atox1 and CCS. Neither dsRNAi knock-down nor transient overexpression of these genes affected copper uptake, accumulation or viability on copper in S2 cells (not shown), suggesting these genes do not have a critical role in copper homeostasis.

The characterization approaches using S2 cells have successfully demonstrated conservation of function between mammals and insects for genes involved directly in cellular copper uptake, efflux, and sequestration (Ctr1A, Ctr1B, and DmATP7; Southon et al. 2004). However, this may be less likely to show a phenotype for genes involved in intracellular transport pathways unrelated to copper uptake/efflux or with chaperone function. When the *Drosophila* Atox1 and CCS chaperones were examined in the same way in our laboratory, no phenotype was observed (A. Southon, unpublished). In addition, yeast Superoxide dismutase 1 (SOD1) can function independently of its chaperone, CCS, under conditions of excess copper (Rae et al. 1999). We are extending the characterization experiments to include limiting copper as this may be more successful in identifying a phenotype for genes such as *CG14757* and *CG7194*.

Comparing copper and oxidative stress

As high concentrations of copper can cause oxidative stress, it is important to assess the extent of the transcriptional response that may be due to oxidative stress rather than copper directly. To achieve this, the microarray data were analyzed in comparison to a previous study involving the oxidative agent Paraquat (McCarroll et al. 2004; Table 3). The effect of ageing is also shown as this illustrates the level of overlap expected between two conditions affecting similar processes. The results show a large number of genes (~30% of the genes surveyed) respond in a similar way to ageing and oxidative stress. However, there is little overlap between the responses to copper and either ageing or oxidative stress in adult flies; a similar number of genes respond in the opposite way as in the same way. This

Table 3 A comparison of the transcriptional response to copper and oxidative stress

	Adult 0.8 mM Cu	Adult 2 mM Cu	Cells 0.3 mM Cu	Adult 15 mM paraquat	Adult aged
Adult 0.8 mM Cu	17/5				
Adult 2 mM Cu	3/0/5/0	38/56			
Cells 0.3 mM Cu	1/0/0/1	5/3/2/2	142/167		
Adult paraquat	0/3/1/0	10/6/9/3	16/14/11/13	782/727	
Adult aging	1/0/0/2	2/3/0/5	7/11/1/9	149/179/26/6	485/433

The number of genes upregulated or downregulated compared to control samples is given in relation to the samples named for the intersecting rows and columns. For each separate treatment the numbers represent: upregulated/downregulated. Where different treatments intersect, the numbers for numbers of genes responding are presented in the following format: A/B/C/D; (A) the number of genes upregulated in both samples; (B) the number of genes downregulated in both samples, (C) the number of genes upregulated in the column sample and downregulated in the row sample, (D) the number of genes downregulated in the column sample and upregulated in the row sample

indicates the level of copper exposure is not sufficient to cause significant oxidative stress. A relatively small transcriptional response to copper was observed in adult flies, which may indicate the sensitivity is limited when all tissues are combined in the screen (although similar was done for the oxidative stress and ageing treatments). *Drosophila* embryonic S2 cells do not have this problem and a greater number of genes did respond in these cells; however, there remains minimal overlap with the oxidative stress and ageing. Of those genes that do overlap, there is no apparent trend in function with 30% having an unknown function.

This result supports that seen in mammalian cell lines when copper accumulation is caused by a mutation in the Menkes copper transporter rather than the addition of high concentrations of copper in the medium (Armendariz et al. 2004). Amenderiz et al conclude that previous studies showing oxidative stress to be one of the major mechanisms of copper toxicity may be a reflection of artificially high-copper treatment. The copper concentrations to which the flies were exposed for this study were artificially high, but nonetheless provide additional evidence against oxidative stress as the major cause of toxicity.

Mutagenesis approaches to identifying novel genes

We have investigated mortality on excess copper as a method to screen for genes involved in copper resistance and sensitivity in *Drosophila*.

This phenotype was selected based on increased tolerance seen in *Drosophila* with a natural *MtnA* duplication (Otto et al. 1986; Maroni et al. 1987, expression levels).

Deletion mutants

Overlapping chromosomal deletions have been used successfully in the past to narrow regions of interest when mapping insecticide resistance in *Drosophila* (Ffrench-Constant and Roush 1991). An efficient approach is to initially screen large deletions and quickly identify regions of interest, then to screen smaller deletions to limit the number of candidate genes within each region. The Deficiency Kit is a set of 196 strains of *Drosophila*, each homozygous for a large chromosomal deletion removing many genes. Because these large mutations are lethal, each mutation is maintained over a balancer chromosome, which typically contains an inversion to inhibit recombination. This means only dominant or dosage sensitive phenotypes will be detected by screening these lines, but has the advantage of allowing a quick screen of ~80% of the genome.

The Deficiency Kit yielded 31 resistant stains and 33 sensitive strains (Table 4, Fig. 5). In addition, 49 strains were ruled out as having a similar significant change in mortality as was seen on control medium; one strain had lower mortality than the wild-type Armenia on both control and copper medium, and 48 had higher mortality on both. Based on these data, the Deficiency Kit appears to be more reliable in screening for

Table 4 Summary of Deficiency Kit results

Chromosome	Resistant	Sensitive	Increased fitness	Decreased fitness
Total	33	31	1	48
X	4	9	0	15
2	12	11	1	16
3	17	11	0	17

‘Resistant’ strains are defined as having lower mortality than Armenia on 2 mM copper but not on 0 mM copper, and ‘Sensitive’ strains are those that have higher mortality than Armenia on 1 mM copper but not on 0 mM copper. ‘Increased fitness’ is assigned to strains that have lower mortality than Armenia on 0 and 2 mM copper, and ‘Decreased fitness’ is assigned to strains with higher mortality than Armenia on 0 and 1 mM copper

resistance than for sensitivity. However, the greatest changes in LC50 were 2.5-fold lower for the most sensitive strain (Df(2L)esc10; Bloomington stock BL3129) and 1.5-fold higher for the most resistant strain (Df(3R)3–4; BL4787). High-level resistance is not observed, despite ~80% of the genome being screened through these deletions.

A strain with a deletion including *MtmB*, *C* and *D* (Df(3R)H-B79; BL4962) was more sensitive than the control, in agreement with the phenotype

of a mutant lacking the *MTF-1* transcription factor for these genes (Egli et al. 2003). Resistance was associated with a deletion including *Ctr1B* (Df(3R)p712; BL1968), whereas Zhou et al. (2003) have shown the homozygous mutant to be sensitive to excess copper. It is possible that the heterozygous mutants have sufficient activity to overcome the cause of the homozygote sensitivity (e.g., accumulation of copper in particular tissues), but are less efficient at uptake than wild-type when the concentration in the medium is very high, and are consequently more resistant. However, as the resistance level of heterozygous *Ctr1B* mutants has not been published, it is not clear whether the phenotype observed in the Deficiency Kit mutant is due to *Ctr1B* or another gene within the deletion without screening this specific mutant. Deletions including the *Drosophila* orthologues of either *Cox17*, *GC9065*, (Df(1)RK4; BL1039) or *Sco1* and *Sco2*, *CG8885*, (Df(2L)sc19-8; BL693) were more sensitive to copper than wild-type. Specific mutants must be screened to prove these genes are the cause of the phenotype. If they are, it may be due to either loss, sequestration by the chaperones or to a combination of

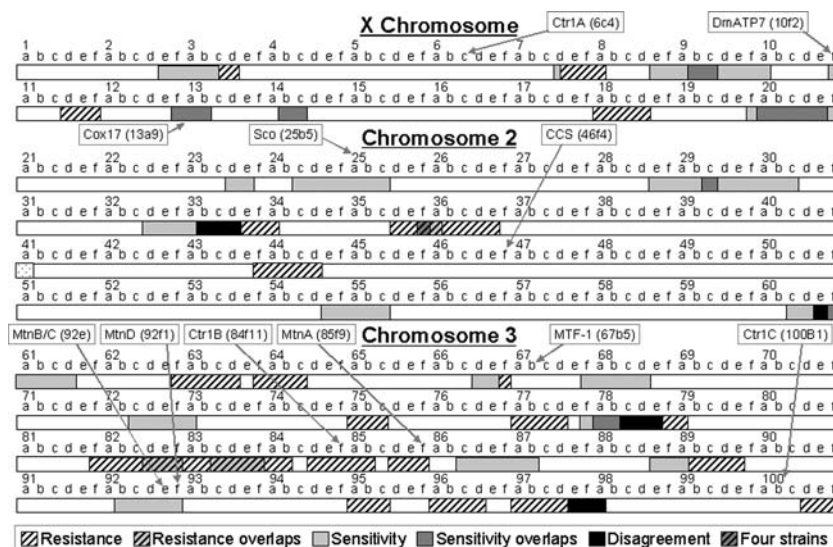


Fig. 5 Regions of the genome in which deficiency is associated with a change in copper susceptibility. These results are based on a single screen of the Deficiency Kit, and overlapping deficiencies are shown. Refer to the shading key to identify regions for which a heterozygous deficiency is associated with resistance or sensitivity and

where overlapping deficiencies yielded a similar result. The 2L35F region is associated with resistance in four strains. 2L32D-34A was also examined in overlapping deficiencies, but the resistant and sensitive regions shown here are from single strains, as the overlaps did not narrow the region

stress caused by the loss of Cytochrome C oxidase activity and excess copper.

To assess the reproducibility of the Deficiency Kit and mapping by comparing overlapping deletions, two regions associated with resistance and deficiency were examined further using additional deletion mutants. Nine mutants with deletions overlapping Df(2L)r10 (BL1491, Chromosome 2L35F) were screened. All four strains with deletions including the small region containing *Syntaxin 5* (*Syx5*) had increased copper resistance, effectively narrowing the resistance association to a single locus. We are currently investigating the copper phenotype of a specific *Syx5* mutant.

Syx5 encodes a 467 amino acid protein that is highly conserved in eukaryotes and belongs to the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family of proteins involved in vesicle trafficking (Jahn et al. 2003). SNAREs are located on both vesicles and target membranes and are critical for vesicle docking. The human orthologue is involved in vesicle trafficking between the ER and Golgi as well as between the TGN and endosomes (Dasscher et al. 1994; Tai et al. 2004). *Drosophila Syx5* is also involved in vesicle trafficking and is essential for cytokinesis and spermatid differentiation (Xu et al. 2002). Recently, RNA interference of mammalian *Syx5* was shown to induce Golgi fragmentation without inhibiting vesicle trafficking, suggesting this protein may be primarily involved in maintenance of the COG (Suga et al. 2005). We are investigating whether the copper sensitivity seen in *Drosophila* following the deletion of *Syx5* is due to impaired vesicle transport of a copper regulatory protein such as DmATP7 or whether this sensitivity is a consequence of a more general impairment of Golgi function.

No consistent results were observed between strains with deletions overlapping Df(2L)esc10 (Chromosome 2L32D-34A). This suggests the results are due to other aspects of the genetic background in this strain and highlights the importance of follow-up experiments to confirm results of the Deficiency Kit. However, the more consistent results for the *Syx5* region indicate the Deficiency Kit does hold potential for quick screening and rough mapping experiments across

the entire genome. Several other candidates are suggested by compatible results between microarray analysis and the Deficiency Kit screen (Table 5): downregulation in response to copper and resistance associated with halving the copy number; or upregulation in response to copper and sensitivity associated with halving the copy number. (Table 6)

de novo mutagenesis

We also performed *de novo* mutagenesis using the chemical mutagen EMS. EMS induces a high proportion of point mutations whilst being of fairly low toxicity (Bentley et al. 2000). This approach was chosen to generate a range of copper resistant *Drosophila* mutants in a defined genetic background. As F1 EMS mutants were screened, this approach detected only dominant phenotypes.

The EMS treatment yielded ten copper lines from 650,000 embryos for which survival was slightly higher on copper (Fig. 6). Four of these were mapped to Chromosome 3. However, analysis of the mapping approaches identified four interfering factors that prevented further mapping (mapping data are available on request). First, none of the ten lines had greater than a twofold increase in LC50 compared to the wild-type parental strain (Armenia). This severely limited the number of surviving individuals that made up each mapping class. Second, a putative maternal effect was observed when investigating sex-linkage; offspring from a mutant female/wild-type male cross always survive better than those from a wild-type female/mutant male cross. This suggests the presence of a resistance-conferring maternal protein in the embryo, or an effect on the level of copper deposited in the embryo. Third, females always survive better than males. This has been commonly observed in tolerance studies in *Drosophila* and has been attributed to differences in body size and amount of reproductive tissue (P. Batterham, unpublished). Finally, sequencing *Ctr1B* on Chromosome 3 revealed four silent base pair changes between an EMS mutant line and the parental strain. As this is higher than the number of mutations expected from EMS treatment (Bentley et al. 2000), this is

Table 5 Candidate copper homeostasis genes identified through this study

Gene	Screen	Human orthologue	Evidence	Putative function
Syx5	Deficiency Kit	Syntaxin 5	Four deficiencies associated with resistance	Snare protein involved in vesicular trafficking
CG11825	S2 microarray	Hypoxia induced gene	Time/concentration-dependent transcriptional downregulation, dsRNAi reduces copper accumulation	Hypoxia induced gene
CG14036	S2 microarray	Hypothetical protein: LOC121355	Time/concentration-dependent upregulation, dsRNAi reduces copper accumulation	Cell division and development
CG7194	S2 microarray	Hypothetical protein: chromosome 6 open reading frame 150	Time/concentration-dependent transcriptional upregulation	Gonad development choice of cell fate
CG14757	S2 microarray	Hypothetical protein: FLJ20487	Time/concentration-dependent transcriptional upregulation MxxM motif, TPR domain, Low MW	Copper chaperone?
CG6908	Df Kit/array	Unknown	Upregulated/sensitive	Unknown*
CG13758	Df Kit/array	Calcitonin receptor	Upregulated/sensitive	G-protein coupled receptor protein signaling
CG30377	Df Kit/array	Unknown	Downregulated/resistant	Unknown*

'Df Kit/array' indicates genes found to be differentially expressed on the adult microarray ('array') in a way, that is, compatible with the copper susceptibility of at least one strain from the Deficiency Kit ('Df Kit'). Human orthologues were identified as described in Southon et al. (2004)

* The FlyBase consortium (2003)

Table 6 Genes responding to two different stresses

0.8 and 2 mM Cu	0.8 mM Cu and S2 cells	0.8 mM Cu and paraquat	2 mM Cu and S2 cells	2 mM Cu and paraquat	S2 cells and paraquat
Downregulated: yellow-G	Upregulated: MtnB	Downregulated: CG6639, CG3625, CG4757	Downregulated: CECC, CG11825, VM26AB	Downregulated: CecC, CG11892, CG4734, Jon99FII, Lsp1 β , OBP99A	Downregulated: CecC, CG10332, CG13476, CG2209, CG30485, CG3131, CG31522, CG4622, CG5404, CG7045, CG7441, CG8654, IM10/CG33470, Jon66CII
Upregulated: MtnB, MtnC, CG15921			Upregulated: CG17531, CG2064, GstD5, GstD9, MtnB		Upregulated: CG11975, CG12728, CG17531, CG2064, CG30295, CG31216, CG32636, CG9351, CG9801, CYP28A5, DGP-1, GstD5, IMD, JHI-26, Neur, W

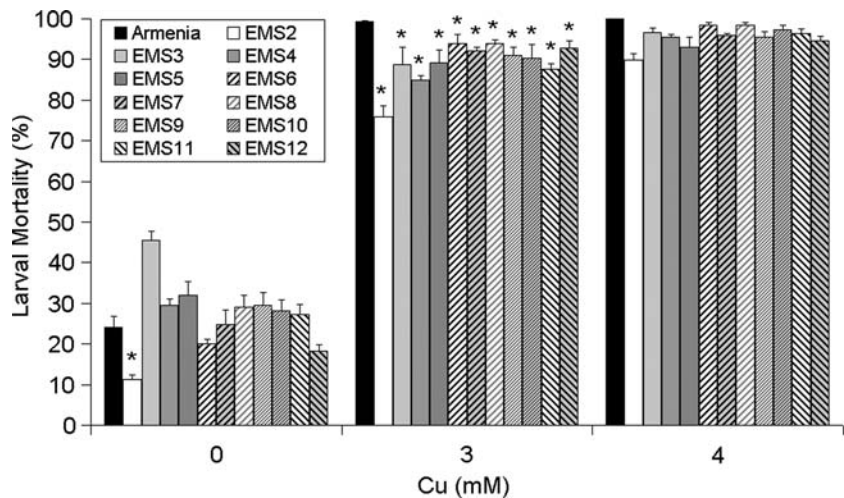
A Celera Gene (CG) number is allocated to genes predicted based on sequence alone. The microarray data values are available on request

likely to be due to variation from the parental strain becoming fixed when the EMS line was established from a single individual.

A hypopigmentation and cuticular defect was visible in one EMS strain when it was raised for several generations on control medium (Fig. 7).

The similarity to the phenotypes of *Ctr1B* mutant (Zhou et al. 2003) and *DmATP7* overexpressing (Norgate et al. 2006) flies indicates this EMS strain suffers a copper deficiency. This is supported by the complete rescue on copper medium (not shown) and suggests the mutation has a

Fig. 6 EMS induced copper resistant mutants. About 11 EMS lines survived several rounds of screening on 4 mM copper. EMS2 was excluded as it had lower mortality than Armenia on both control and copper media, indicating a non-specific effect. * Significantly lower mortality than Armenia ($P < 0.05$)



constitutive effect involved in removing copper from the fly or sequestering copper. Like *Ctr1B* and *Ctr1C*, this mutation maps to Chromosome 3. However, Real Time PCR revealed no change in expression for these genes and sequencing *Ctr1B* showed no causal mutations.

As mortality is not a copper-specific phenotype, other factors are able to influence the results. Had the level of resistance been higher, copper would have been the predominating factor affecting survival. However, with the no greater than twofold resistance observed for these mutants, mapping experiments were unreliable. The strains mapped to Chromosome 3 were made isochromosomal for this Chromosome, but this did not increase the level of resistance (not shown). In the case of the hypopigmentation phenotype, the individuals varied from wild-type and mild hypopigmentation, with only 1% displaying the severe phenotype shown in Fig. 7. Therefore, this phenotype did not provide sufficient easily scorable individuals to reliably map the mutation. However, it does suggest hypopigmentation may be an alternative phenotype to mortality for future mutagenesis screens.

Approaches for future gene discovery screens

Neither EMS nor the Deficiency Kit yielded greater than twofold resistance, which was surprising as previous studies have shown threefold resistance associated with an *MtnA* duplication

(Otto et al. 1986; Maroni et al. 1987) and the potential to gradually acclimatize flies to survive on 5 mM copper (Wallace 1982). The most likely cause of this discrepancy is that both the screens presented here are likely to involve loss-of-function mutations; the Deficiency Kit deletions halve copy number and EMS generated random point



Fig. 7 Disrupted cuticle phenotype in a copper resistant EMS mutant. a Wild-type Armenia and b homozygous EMS11 mutant raised on control medium. Approximately 1% of EMS11 adults show this cuticular developmental defect and hypopigmentation on control medium. This is completely rescued by growth on copper

mutations are more likely to disrupt protein function than improve it. In contrast, the *MtnA* duplication is a gain of function mutation and acclimatization of a strain over many generations also allows gain of function mutations to be selected. Another potential limitation of the screens presented here is that both examine heterozygous mutations. There is evidence that heterozygotes often have a more subtle phenotype than homozygotes (Camakaris et al. 1980, copper accumulation levels; Gunes et al. 1998, resistance levels in cells; Hamza et al. 2001; Kuo et al. 2001, mouse mutants; Egli et al. 2003, expression levels). Based on the results of this study, it may be advantageous to base future screens on gain-of-function, recessive phenotype or a phenotype such as hypopigmentation rather than mortality.

Conclusions

In screening for novel copper homeostasis genes, there were several limitations to the approaches presented here, the most significant being the sensitivity of microarray analysis of whole flies and mortality as a phenotype. In the context of previously published data and current technology, specific suggestions have been made that are likely to improve these screens. Despite the limitations encountered, the Deficiency Kit and microarray screens yielded a number of interesting novel genes. The evidence for each is summarized in Table 5. Using the known copper homeostasis genes previously identified in mammals, we have developed cell-based and whole-fly approaches for efficiently investigating the role of these candidate genes in copper transport and development, and the tissue-specific expression patterns (Southon et al. 2004; Norgate et al. 2006). The involvement in copper transport can be investigated by dsRNAi knock-down in S2 cells, then examining copper uptake and accumulation, and viability in response to copper (Southon et al. 2004). This has already been effective for confirming CG11825 and CG14036 as genes of interest. The adult fly provides a suitable system for examining tissue-specific gene expression and the effects of loss of function (dsRNAi, P-element

mutagenesis) or overexpression (GAL4-UAS) on copper tolerance, pigmentation, and development (e.g., Egli et al. 2003; Zhou et al. 2003; Norgate et al. 2006). This study has indicated several promising candidate genes. Applying the recommendations presented here, *Drosophila* has great potential for novel copper homeostasis gene discovery and characterization in the future.

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