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Copper sensing function of *Drosophila* metal-responsive transcription factor-1 is mediated by a tetranuclear Cu(I) cluster

Xiaohua Chen[1], Haiqing Hua[2], Kuppusamy Balamurugan[2], Xiangming Kong[1], Limei Zhang[3], Graham N. George[3], Oleg Georgiev[2], Walter Schaffner[2,*] and David P. Giedroc[1,4]

[1]Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843-2128, USA, [2]Institute of Molecular Biology, University of Zurich, Winterhurerstrasse 190, 8057 Zurich, Switzerland, [3]Department of Geological Sciences, University of Saskatchewan, Saskatoon, S7N 5E2, Canada and [4]Department of Chemistry, Indiana University, Bloomington, IN 47405, USA

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ABSTRACT

Drosophila melanogaster **MTF-1 (dMTF-1) is a copper-responsive transcriptional activator that mediates resistance to Cu, as well as Zn and Cd. Here, we characterize a novel cysteine-rich domain which is crucial for sensing excess intracellular copper by dMTF-1. Transgenic flies expressing mutant dMTF-1 containing alanine substitutions of two, four or six cysteine residues within the sequence ^{547}CNCTNCKCDQTKSCHGGDC^{565} are significantly or completely impaired in their ability to protect flies from copper toxicity and fail to up-regulate MtnA (metallothionein) expression in response to excess Cu. In contrast, these flies exhibit wild-type survival in response to copper deprivation thus revealing that the cysteine cluster domain is required only for sensing Cu load by dMTF-1. Parallel studies show that the isolated cysteine cluster domain is required to protect a copper-sensitive *S. cerevisiae ace1Δ* strain from copper toxicity. Cu(I) ligation by a Cys-rich domain peptide fragment drives the cooperative assembly of a polydentate [Cu_4-S_6] cage structure, characterized by a core of trigonally S_3 coordinated Cu(I) ions bound by bridging thiolate ligands. While reminiscent of Cu_4-L_6 (L = ligand) tetranuclear clusters in copper regulatory transcription factors of yeast, the absence of significant sequence homology is consistent with convergent evolution of a sensing strategy particularly well suited for Cu(I).**

INTRODUCTION

Metal ions play myriad essential roles in all of biology. As a result, all cell types have evolved the ability to extract specific metal ions from their environment and ultimately maintain the intracellular concentrations of each in a range compatible with cellular needs (1). This is critical for the survival of the organism since even essential transition metal ions, e.g. Fe, Cu and Zn are toxic in excess (2). The same is true for Ni (3) and Mn (4), although acquisition of these ions ensures that specialized microorganisms are capable of surviving in a strongly acidic or potently oxidizing environment, respectively. Cu and Fe are particularly toxic since their reduced forms, Cu(I) and Fe(II), when weakly chelated in an aerobic environment, will catalyze the production of damaging hydroxyl radicals via redox cycling; as a result, the 'free' or bioavailable concentrations of these ions, as well as Zn, may likely be vanishingly small (5,6). The control of metal homeostasis is mediated by the balancing of uptake, efflux and intracellular sequestration or compartmentalization of essential metal ions, and is largely regulated transcriptionally by gene regulatory proteins, collectively coined metal sensor proteins (2). Metal sensor proteins directly bind a particular metal ion, or groups of metal ions that form similar coordination complexes, to the exclusion of

*To whom correspondence should be addressed. Tel: +41 1 635 3140; Fax: +41 1 635 6811; Email: walter.schaffner@molbio.uzh.ch
Correspondence may also be addressed to David P. Giedroc. Tel: +1 812 856 5449; Fax: +1 812 855 8300; Email: giedroc@indiana.edu

The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors

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all others (7); this, in turn, allows an organism to turn on or turn off the expression of specific genes in order to mount a metabolic response to either deprivation or excess of a particular metal ion in the cell.

Metal-responsive transcription factor-1 (MTF-1) is a heavy metal sensing transcriptional activator that up-regulates the expression of genes that allow an organism to mitigate zinc, cadmium and copper toxicity (8) (for reviews, see (9–13)). MTF-1 has been identified and at least partially characterized from human, mouse (14), pufferfish *Fugu rubripes* (15), zebrafish *Danio rerio* (16,17) and *Drosophila* (18). Human and mouse MTF-1 as well as the *Drosophila* homolog, termed dMTF-1, contain multiple functional domains, including a highly conserved zinc-finger domain that recognizes the cognate DNA sequence termed metal response element (MRE) (8). MTF-1 also harbors multiple domains for transcriptional activation (19), and short sequences that mediate intra-cellular trafficking into and out of the nucleus (20).

How a particular metal ion mediates MTF-1-dependent metalloregulation of gene expression is the subject of current debate (13,21); however, multiple levels of regulation clearly exist (20,22,23). Zn(II) binding to the zinc-finger domains clearly stabilizes an MRE-MTF-1 complex (8), particularly in chromatin (24). Biochemical studies of the finger domain fragment (25–27) have revealed that at least part of the zinc-sensing mechanism is mediated by the zinc-finger domain itself (21). However, MTF-1 also senses other cell stress conditions including Cd(II) (28), oxidative stress (29), hypoxia (30), and the synergistic influence of heavy metal load and heat shock (31). It seems unlikely that such inducers would act directly on the finger domain. For example, it is known that Cd(II) does not bind to the finger domain in a way that preserves the canonical $\beta\beta\alpha$-structure for DNA binding (26). However, substantial data support an indirect sensing model, in which MTF-1 senses Zn(II) that is mobilized by other inducers from intracellular stores of cytoplasmic Zn(II) (29).

Previous functional studies of mammalian MTF-1 reveal that a 13-amino acid domain containing four conserved cysteines just C-terminal to a transcriptional activation domain is required for Zn(II)/Cd(II)-induced transcriptional activation in transiently transfected mouse MTF-$1^{-/-}$ cells (23). The mechanistic role of this domain in metalloregulation is not yet clear. However, it functions downstream of nuclear translocation and MRE-binding, perhaps activating transcription via a metal-dependent protein-protein interaction at the promoter. Indeed, when *Drosophila* S2 cells are stimulated with exogenous copper salts, dMTF-1 recruits TFIID to the *MtnA* (metallothionein A) promoter (32).

Drosophila MTF-1 differs from mammalian MTF-1 in two crucial respects. First, MRE- and MTF-1-dependent expression of metallothionein genes (*mtnA-D*) is strongly induced by Cu and Cd, relative to Zn, whereas Zn and Cd are the most potent inducers of mammalian metallothioneins (18). Second, disruption of the *MTF-1* gene by targeted insertional mutagenesis (MTF-1 KO flies) results in a strong sensitivity to not only Cu, Cd and Zn toxicity but also to Cu depletion (33,34). The requirement for dMTF-1 to mitigate the effects of Cu deprivation is unique to dMTF-1, and originates with the ability of dMTF-1 to activate expression of a high affinity Cu importer *Ctr1B* under normal or low-Cu growth conditions. As a result, dMTF-1 plays a central role in copper homeostasis in the fly by regulating both import and sequestration of this essential yet toxic metal (34,35).

We reasoned that some aspect of copper-dependent metalloregulation of dMTF-1 requires the direct binding of Cu(I), analogous to the direct binding of Zn(II) to the zinc fingers of hMTF-1. Such a Cu(I)-sensing mechanism is however unlikely to function through the zinc finger domain itself, which is predicted to have a low affinity for Cu(I); thus, some other Cu(I)-binding domain would have to be present in dMTF-1. Inspection of the amino acid sequence reveals two candidate cysteine-rich Cu(I)-binding domains, both located in the C-terminal one-third of the protein (9). Here, we present evidence that the six cysteine residues from residues 547-565 are necessary for dMTF-1 to sense copper load. When challenged with copper stress, flies harboring Cys-to-Ala substitutions are unable to up-regulate the transcription of metallothionein *MtnA*, the major effector of copper-resistance (36,37). We also show that a peptide harboring this Cys-rich domain protects a Cu-sensitive *S. cerevisiae* strain (38) from Cu-toxicity, presumably by mediating intracellular storage/chelation of the metal. Binding studies show that the Cu-sensing domain of dMTF-1 binds four Cu(I) ions tightly and highly cooperatively to form a Cu_4-Cys_6 polynuclear cluster. This cluster is reminiscent of known Cu-sensing domains of *S. cerevisiae* Mac1 and Ace1 and paralogs in other organisms (39–41). The mechanistic implications of these findings are discussed.

MATERIALS AND METHODS

Plasmids and fly transformation

Cys-to-Ala mutations were generated using pUAST-dMTF-1 as a template by a quick change mutagenesis technique. pUAST-dMTF-$1^{4C\text{-}4A}$, pUAST-dMTF-$1^{2C\text{-}2A}$ and pUAST-dMTF-$1^{6C\text{-}6A}$ constructs were used to generate transgenic flies with P-element mediated transformation as described earlier (37).

Fly food, fly stocks and genetics

One liter of standard fly food was composed of 55 g corn, 10 g wheat, 100 g yeast, 75 g glucose, 8 g agar and 15 ml anti-fungal agent nipagin (15% in ethanol). For toxicity experiments, food was supplemented with $CuSO_4$ or CdCl2 or bathocuproinedisulfonate (BCS) disodium salt hydrate (Sigma-Aldrich No. 14,662-5) to the indicated concentrations. BCS is a specific copper chelator used to deplete copper in the food. Flies were raised at 25°C and 65% humidity. *UAS-dMTF-1*, *UAS-dMTF-1^{4CA}*, *UAS-dMTF-1^{2CA}*, and *UAS-dMTF-1^{6CA}* transgenes were crossed into *dMTF-$1^{140\text{-}1R}$* (*dMTF-1* null allele) background respectively. The expression of the transgenes was induced by a ubiquitous Gal4 transactivator (*actin-Gal4*).

Drosophila toxicity experiments

The flies that were homozygous for *dMTF-1*$^{140\text{-}1R}$ and *UAS-dMTF-1* (or its derivatives) were crossed with *y w;; dMTF-1*$^{140\text{-}1R}$*, actin-Gal4/TM6B,y+* flies on standard food or food containing copper or BCS. From the cross, two types of progeny could be obtained: Progeny (A) flies that were expressing the transgene and were *dMTF-1* null mutant. Progeny (B) flies that were not expressing the transgene and contained endogenous *dMTF-1*. The survival index (*I*s) was calculated as follows: $Is = 2A/(A + B)$.

Quantitation of MtnA and dMTF-1 transcripts in transgenic flies

To determine the level of MtnA transcripts, larvae were raised on either standard food or food containing 100 µM $CuSO_4$. Only third instar stage larvae were collected for analysis. Total RNA was extracted using the TRIzol reagent (Life Technologies) and nuclease S1 mapping of transcripts (100 µg of total RNA) was performed as described previously (42). The gels were developed using FLA-7000 system and bands were quantified using ImageGauge software (Fuji Film). The transcripts of the endogenous *actin5c* gene were measured and used for normalization of MtnA transcript levels. To monitor *dMTF-1* expression levels, the gut tissue was dissected from the third instar stage larvae raised on standard food. Total RNA was extracted using TRizol and first-strand cDNA synthesis was performed with 5 µg total RNA using reverse transcriptase (RT). mRNA levels were measured by quantitative (q) PCR using a SybrGreen Q-PCR reagent kit (Sigma) in combination with the MX3000P light cycler (Stratagene, Amsterdam, The Netherlands). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To verify RNA integrity and equal input levels, *actin* mRNA was used as a reference.

Cu(I) binding experiments by absorption and luminescence spectroscopies

All Cu(I) titration samples of C-dMTF_81 were prepared anaerobically in a glovebox ($[O_2] < 2$ ppm) with deoxygenated buffers and solvents in 10 mM MES, 0.1 M NaCl, pH 6.3, 25°C. The samples were kept in sealed containers, including during transfer from the glovebox for characterization. Then, 500 µM Cu(I) was titrated into 800 µL of 20 µM apo-protein in anaerobic environment and the absorption was monitored over the wavelength range 200–500 nm on a Hewlett-Packard model 8452A spectrophotometer. In magfura-2 competition experiments, Zn(II) was titrated into the mixture of 15.8 µM C-dMTF_131 and 16.3 µM magfura-2 (43). For the competition experiments with BCS, 282 µM C-dMTF_81 was titrated into a mixture of 100 µM BCS and 30 µM Cu(I) and the absorption spectra recorded from 250 to 600 nm. Luminescence spectra were recorded on an ISS PC1 Photon Counting spectrofluorometer. Also, 1.0 mM Cu(I) was titrated into 1700 µL of 20 µM apo-C-dMTF_81 and the full emission spectra were collected from 400 to 800 nm with excitation at 300 nm essentially as described (44).

RESULTS

Domain structure of Drosophila MTF-1

The domain structure of *D. melanogaster* MTF-1 (dMTF-1) is shown in Figure 1 (18). The functional domains of dMTF-1 have not yet been extensively mapped and the amino acid sequence has diverged considerably from mammalian MTF-1 outside of the DNA-binding zinc finger domain (18). However, dMTF-1 contains a cluster of six cysteines within 19 consecutive amino acids (residues 547-565) that bears some

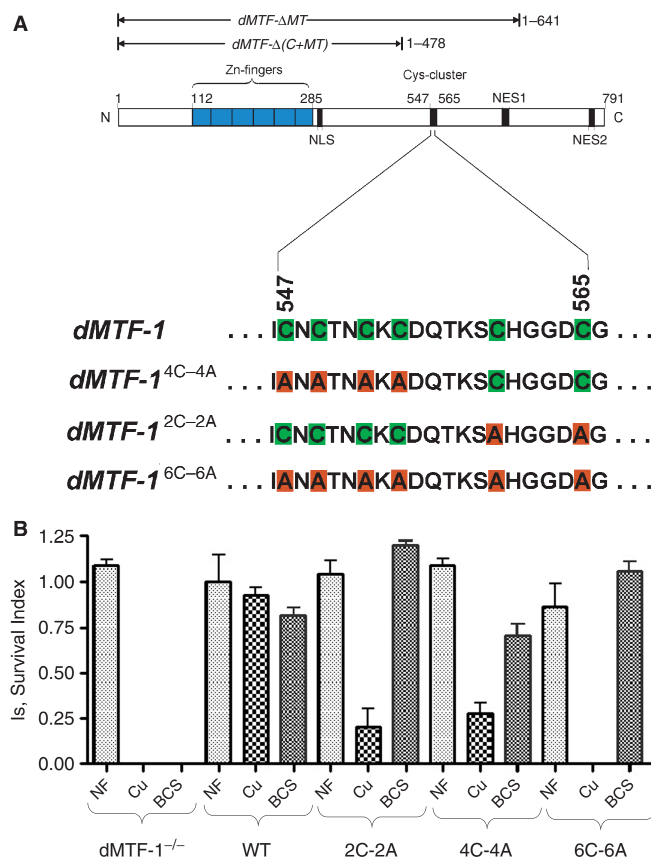

Figure 1. The cysteine-rich region plays a critical role in protecting *Drosophila* from copper toxicity. (**A**) Domain structure of *Drosophila melanogaster* (dMTF-1) highlighting the short Cys-cluster region (residues 547-565) and *dMTF-1* Cys-to-Ala substitution alleles characterized in transgenic flies. In addition to the zinc-finger DNA-binding domain, a putative nuclear localization signal (NLS) and two nuclear export signals (NES1 and NES2) are also indicated (V. Günther and W.S., unpublished). 131- (C-dMTF_131), 81- (C-dMTF_81) and 51- (C-dMTF_51) residue constructs of dMTF-1 characterized here correspond to amino acid residues 499-629, 499-579 and 529-579, respectively. Two C-terminal domain deletion mutants of dMTF-1, *ΔMT* and *Δ(C + MT)* characterized in *S. cerevisiae* (see Figure 3) are also shown. *MT*, metallothionein-like segment (residues 642-791); *C*, Cys-rich domain (residues 479-641). (**B**) Survival of *dMTF-1* null flies and flies expressing *dMTF-1*$^{2C\text{-}2A}$, *dMTF-1*$^{4C\text{-}4A}$ or *dMTF-1*$^{6C\text{-}6A}$ on a standard food source (NF), or on food supplemented with 400 µM $CuSO_4$ (Cu) or 160 µM bathocuprione disulfonate (BCS).

resemblance to the Cys_4 cluster that has been functionally characterized in hMTF-1 (23). This cluster is followed by a Thr/Ser-rich domain (13 Thr/Ser in 19 residues), which is connected via a seryl-glycyl linker to a C-terminal metallothionein (MT)-like domain, which also contains several cysteines (residues 641-791). This C-terminal MT-domain bears strong resemblance to domain IV of *S. pombe* Pccs, a copper chaperone for copper-zinc superoxide dismutase (SOD1) which have been shown to protect *S. pombe* and a *S. cerevisiae ace1Δ* mutant strain from copper toxicity (45). To probe the copper-binding ability of the cysteine cluster (residues 547-565), we performed a functional analysis of this region of dMTF-1 both in *Drosophila* and in *S. cerevisiae*.

Transgenic flies expressing wild-type and mutant dMTF-1 genes

To investigate whether the Cys-cluster in *Drosophila* MTF-1 plays any role in copper homeostasis, we generated transgenic flies with constructs in which subsets of cysteines, or all six of them, are substituted by alanines (Figure 1A). As mentioned, *dMTF-1* mutant flies are sensitive not only to excess copper but also to copper depletion (34). This is due to the fact that dMTF-1 activates two sets of genes that are working in opposing conditions, namely, metallothioneins at high copper, and the copper importer *Ctr1B* at times of copper deprivation (35). The sensitivity of *dMTF-1* mutants can be rescued by co-expression of wild-type *dMTF-1* transgene. To examine the role of the cysteine-rich domain, we introduced the mutant constructs $dMTF\text{-}1^{2C\text{-}2A}$, $dMTF\text{-}1^{4C\text{-}4A}$ or $dMTF\text{-}1^{6C\text{-}6A}$ encoding double (C560A/C565A), quadruple (C547A/C549A/C552A/C554A) or complete (C547A/C549A/C552A/C554A/C560A/C565A) alanine substitutions (Figure 1A), into *dMTF-1* mutant flies lacking endogenous dMTF-1 and tested whether these constructs could rescue the sensitivity to either copper supplementation or copper depletion. All of the three *dMTF-1* derivatives are able to rescue the sensitivity to copper starvation as well as the wild-type *dMTF-1* transgene (Figure 1B). This result demonstrates that the wild-type and mutant forms of dMTF-1 are expressed to similar levels since a functional dMTF-1 is required for this.

In contrast, $dMTF\text{-}1^{2C\text{-}2A}$ and $dMTF\text{-}1^{4C\text{-}4A}$ could only partially rescue the sensitivity to copper while *dMTF-1* mutant flies expressing $dMTF\text{-}1^{6C\text{-}6A}$ failed to survive to adulthood in copper supplemented food (Figure 1B). To further understand the molecular mechanism of the copper sensitivity phenotype, we examined the expression of metallothionein A (MtnA) in flies expressing either the wild-type or mutant alleles of *dMTF-1* (Figure 2A). *MtnA* transcript abundance was measured by quantitative S1 nuclease mapping experiments. These data show that under copper stress (100 μM), the wild-type *dMTF-1* transgene strongly activates the transcription of *MtnA* while $dMTF\text{-}1^{6C\text{-}6A}$ transgene is completely unable to induce *MtnA* transcription. Interestingly, $dMTF\text{-}1^{4C\text{-}4A}$ and $dMTF\text{-}1^{2C\text{-}2A}$ transgenes mediate some Cu(I)-induced *MtnA* expression, but to a lesser extent than

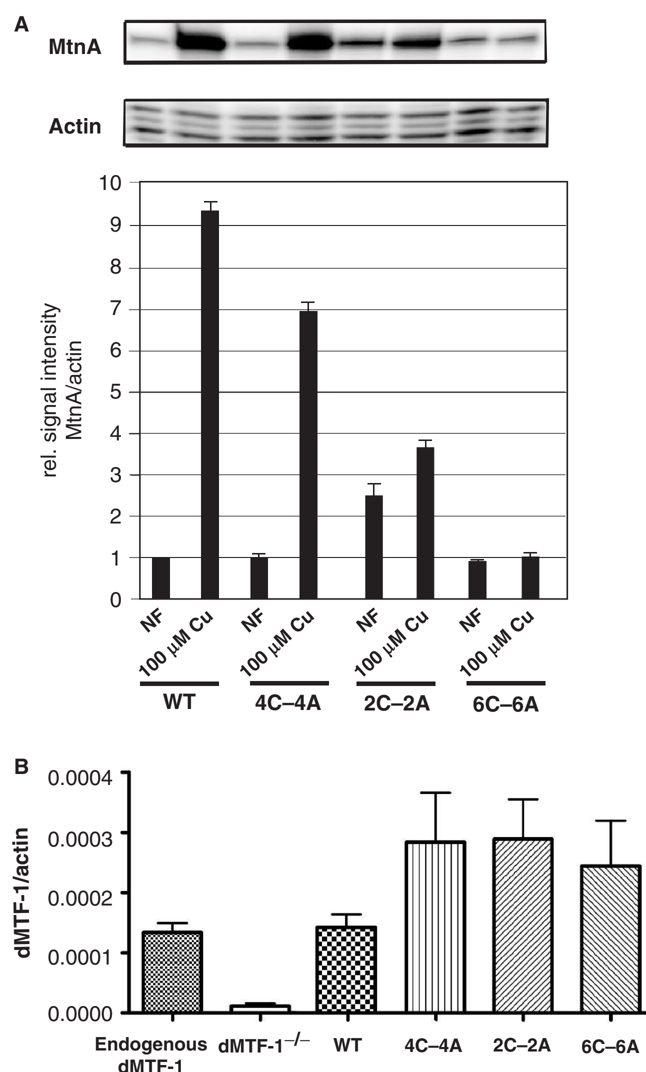

Figure 2. The Cys-rich domain of dMTF-1 is required to activate *MtnA* expression in transgenic flies. (**A**) Total RNA was isolated from transgenic *Drosophila* at the third instar larval stage expressing either a wild-type *dMTF-1*, $dMTF\text{-}1^{4A\text{-}4A}$, $dMTF\text{-}1^{2C\text{-}2A}$ or $dMTF\text{-}1^{6C\text{-}6A}$ allele raised on normal food (NF) or on 100 μM $CuSO_4$ (Cu). MtnA and *actin5c*-specific transcripts were measured by S1 nuclease mapping and are shown as a ratio of transcript abundance. (**B**) *Drosophila* with indicated genotypes was allowed to develop on standard food until third instar larval stage. Total RNA was isolated from larval gut and analyzed by quantitative RT-PCR to quantify transcripts of *dMTF-1*. *Actin-5c* transcripts served as a normalization reference.

wild-type *dMTF-1*. Control experiments reveal that the wild-type and mutant *dMTF-1* transgenes are expressed to similar levels in the larval gut, with the expression of the mutant *dMTF-1* alleles perhaps even slightly (≈2-fold) higher; this and the fact that all transgenes equally confer resistance to copper starvation (see Figure 1B) render unlikely the possibility that the observed phenotypes could be due to insufficient expression of mutant alleles (Figure 2B). Taken together, these data show that the cysteine-rich domain of dMTF-1 is critical for copper-induced transcriptional activation but is clearly dispensable for sensing copper scarcity.

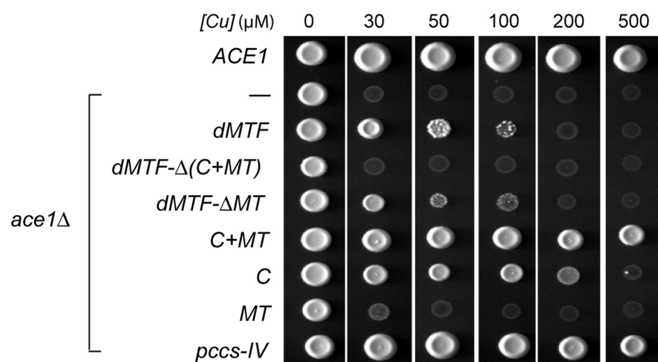

Figure 3. The Cys-rich domain of dMTF-1 protects a Cu-sensitive strain of baker's yeast from the effects of Cu toxicity. *S. cerevisiae* strain DTY59 (*ace1*Δ) was transformed with a plasmid expressing either intact *dMTF-1* (dMTF) or the indicated domain fragments of *dMTF-1* and spotted onto agar plates in a defined medium containing the indicated concentration of $CuSO_4$. A fragment encoding domain IV of the copper chaperone for SOD1 in *S. pombe* Pccs (labeled *pccs-IV*) is a positive control for this experiment. –, empty vector control; *ACE1*, isogenic wild-type strain DTY7 transformed with empty vector.

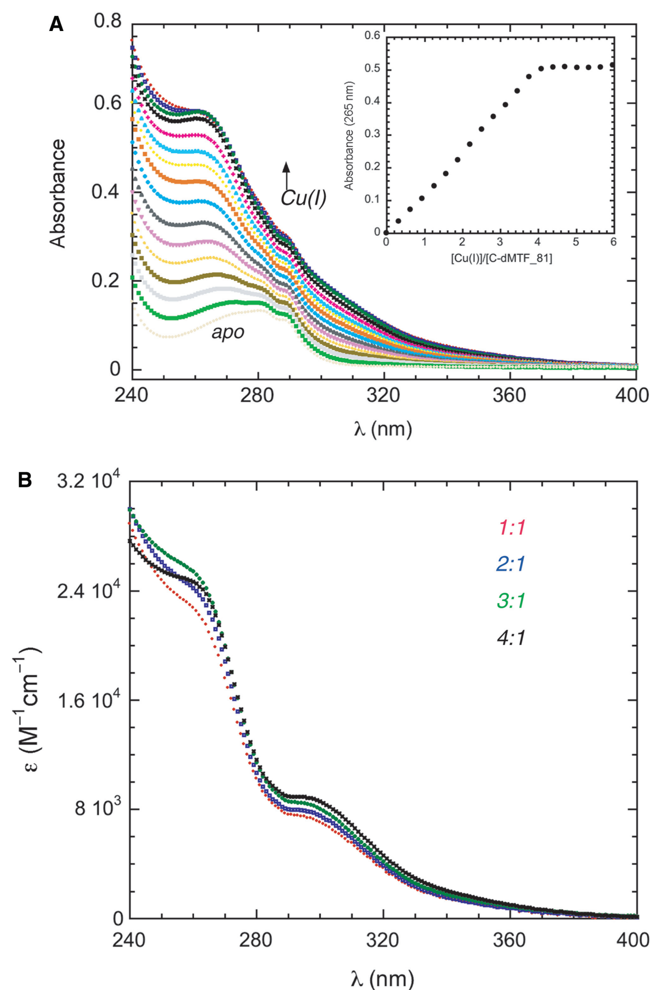

Figure 4. Representative anaerobic titration of C-dMTF_81 with Cu(I). (**A**) Full absorption spectra are shown corrected for dilution, with the apoprotein contribution (*gray curve*) not subtracted. *Inset*, apoprotein-subtracted absorbance at 265 nm from the main body of the figure plotted as a function of Cu(I)/C-d-MTF-1 ratio. (**B**) Apoprotein-subtracted corrected molar absorptivity spectra of Cu(I):C-dMTF_81 mixtures at 1 : 1 (*red*) 2 : 1 (*blue*), 3 : 1 (*green*) and 4 : 1 (*black*) molar ratios. Conditions: 20 μM apo C-dMTF_81, with Cu(I) concentrations ranging from 0.3 to 6.0 molar equivalents, pH 6.0, 22°C.

The cysteine-rich region protects *S. cerevisiae* against copper toxicity

The above results indicate that the cysteine-rich domain plays an essential role in protecting *Drosophila* from copper toxicity by mediating up-regulation of metallothionein genes. In order to assess the importance of this domain relative to other domains in dMTF-1, we have carried out a parallel experiment in a Cu-sensitive *S. cerevisiae* strain, *Δace1*, which lacks the gene for the Cu-dependent activator of *CUP1*, the Cu-binding yeast metallothionein (Figure 3) (38). This strain exhibits severely attenuated survival on Cu-supplemented growth media (first two rows, Figure 3). Expression of *dMTF-1* reverses some of this sensitivity in a manner that absolutely requires the Cys-rich domain (rows 3–5, Figure 3). Interestingly, expression of the entire C-terminal domain of dMTF-1 (C + MT) induces resistance to Cu-toxicity equivalent to that of the MT-like domain of the Cu-chaperone *pccS* of *S. pombe* (45), with most of the protection mediated by the Cys-rich domain itself (rows 6–8, Figure 3).

The cysteine-rich region of dMTF-1 binds four mol•equiv of Cu(I)

We hypothesized that the direct binding of Cu(I) by the sequence encompassing residues 547-565 in dMTF-1 is the basis for copper sensing in cells. To test this, we purified three recombinant dMTF-1 fragments of 131, 81 and 51 amino acids each of which contains the Cys-rich motif, encompassing residues 499-629 (denoted C-dMTF_131), 499-579 (C-dMTF_81) and 529-579 (C-dMTF_51). C-dMTF_81 was chosen for detailed study. Cu(I) titration of C-dMTF_81 (carried out at pH 6.0, 22°C) exhibits intense metal-to-ligand charge transfer absorption (Figure 4A), indicative of coordination to Cys thiolates (44). Similar spectra were obtained for C-dMTF_131 and C-dMTF_51 as well (data not shown). The absorption spectra for C-dMTF_81 saturate at 4 mol•equiv of Cu(I) and the binding is stoichiometric (tight) under these conditions (Figure 4A). Further examination of the absorption spectra at subsaturating amounts of Cu(I) added are consistent with the formation of a single molecular species throughout the course of the titration since molar (per bound Cu(I)) absorptivity spectra of the species formed at 1:1, 2:1, 3:1 and 4:1 Cu(I):C-dMTF_81 molar ratios are identical (Figure 4B) (*vide infra*). These spectra are virtually identical to previously published spectra of Cu_4-Ace1 (46), and are consistent with highly cooperative assembly of $Cu(I)_4$ polynuclear cluster in C-dMTF_81.

C-dMTF_81 also binds Zn(II) ($K_{Zn} \geqslant 10^{10}\,M^{-1}$) and Cd(II) ($K_{Cd} \approx 3 \times 10^6\,M^{-1}$) to form saturating 1:1 complexes under the same solution conditions (Supplementary Figure S1). However, preincubation of C-dMTF_81 with 4 mol•equiv of Zn(II) has virtually no influence on the

Cu(I) binding titration; *i.e.*, Cu(I) still binds stoichiometrically (Supplementary Figure S2A). This suggests that the Cu_4 complex is far more thermodynamically stable than other metallated complexes of C-dMTF_81. Consistent with this, 30 µM C-dMTF_81 is capable of stripping ⩾80% of the Cu(I) from 30 µM Cu(I)-$(BCS)_2$, the latter of which forms with an affinity constant $K_{Cu} \approx 10^{19}\ M^{-1}$(Supplementary Figure S2B). This suggests that the affinity constants for BCS and C-dMTF_81 may be comparable.

Anaerobic titrations like those shown in Figure 4 were also acquired using luminescence spectroscopy ($\lambda_{ex} = 300$ nm). The results of a representative titration are shown in Figure 5, with full luminescence emission spectra (Figure 5A) and a plot of the $\lambda_{em,\ 600}$ vs. Cu(I):C-dMTF_81 molar ratio (Figure 5B) shown. These spectra reveal an intensely luminescent species that shows maximum intensity at a molar ratio of 4:1, after which point the intensity sharply decreases. These data reveal that the Cu(I) ions in the Cu_4 polynuclear cluster are significantly shielded from solvent, as has been previously observed for other polynuclear metalloregulatory clusters in *S. cerevisiae* Mac1 and Ace1 (47). Further titration beyond four mol•equiv of Cu(I) results in significant bleaching of the luminescence intensity, which is not observed in an anaerobic optical titration (Figure 2). This suggests that Cu(I) ions that are added beyond saturation induce significant reorganization in the structure, which leads to a less solvent-shielded average environment for the Cu(I) ions. Addition of greater than 4 mol•equiv of Cu(I) to apo-C-dMTF_81 also leads to significant degradation of the ^{1}H-^{15}N HSQC spectrum (data not shown) consistent with conformational exchange broadening at greater than saturating Cu(I). These complexes may well by oligomeric in nature.

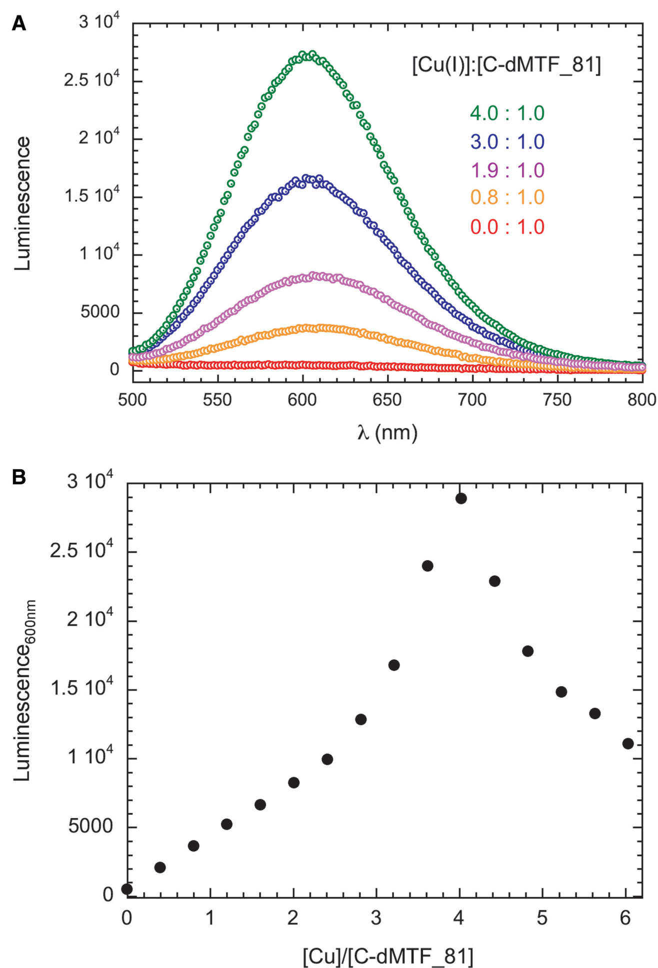

Figure 5. Representative anaerobic titration of apo C-dMTF_81 with Cu(I) as monitored by luminescence spectroscopy. (**A**) Full luminescence spectra ($\lambda_{ex} = 300$ nm) acquired as a function of Cu(I):C-dMTF_81 ratio, as indicated. (**B**) Luminescence emission intensity at 600 nm (from panel *A*) plotted a s function of Cu(I):C-dMTF_81 ratio. Conditions: pH 6.0, 25°C.

X-ray absorption spectroscopy reveals a Cu_4-S_6 polynuclear cluster

X-ray absorption spectroscopy was carried out to structurally characterize the copper binding to C-dMTF_81. Figure 6A shows that the Cu K-edge near-edge spectra from Cu(I)-C-dMTF_81 complex prepared with 1.0 and 3.5 mol•equiv of Cu(I) are essentially identical. The peak centered at around 8983 eV, is a 1s→4p transition that is commonly used as a fingerprint for determining the coordination environment of Cu(I) compounds (48). The spectra of the Cu(I)-peptide complexes are very similar to trigonally-coordinated $[Cu_4(SPh)_6]^{2-}$ and distinct from digonally-coordinated $[Cu(SC_{10}H_{12})_2]^{2-}$ (48,49) (Figure 6A), suggesting the former coordination environment in the peptide.

More structural detail is available from analysis of the Cu K-edge extended X-ray absorption fine structure (EXAFS) spectra. Figure 6B and C show the EXAFS, and corresponding Fourier transforms of the Cu(I)-C-dMTF_81 complexes with both 1 : 1 and 3.5 : 1 Cu:peptides, together with best fits. EXAFS curve-fitting parameters are listed in Table SI (Supplementary Material). As with the near-edge spectra, the EXAFS of the two stoichiometries are essentially identical, and gave curve fitting analysis (discussed below) that were also very similar. Two major Fourier transform peaks are observed at ≈2.3 and ≈2.7 Å, and are attributable to Cu—S and Cu ··· Cu interactions, respectively. In agreement with the near-edge spectra (Figure 6A), EXAFS curve fitting indicates three Cu—S at 2.26 Å. Inclusion of lighter scatterers such as N or O resulted in unreasonably small Debye-Waller factors for Cu—S, indicating a sulfur-only $Cu(SR)_3$ coordination. The 2.7 Å Fourier transform peak is best fitted by including two different types of Cu ··· Cu interactions, with two short and one long Cu ··· Cu interactions at 2.70 Å and 2.82 Å, respectively, for 1:1 Cu(I):C-dMTF_81; similar fitted parameters characterize 3.5 : 1 Cu(I):C-dMTF_81 sample as well. The overall similarity of the XAS for both Cu(I):C-dMTF_81 stoichiometries suggests the same Cu center structure and provides direct evidence that C-dMTF-1 binds to Cu(I) cooperatively. Based on the XAS results a Cu_4S_6 polynuclear cluster is proposed to form in Cu(I)-C-dMTF_81, as shown in inset of Figure 6C. MALDI-TOF mass spectroscopy

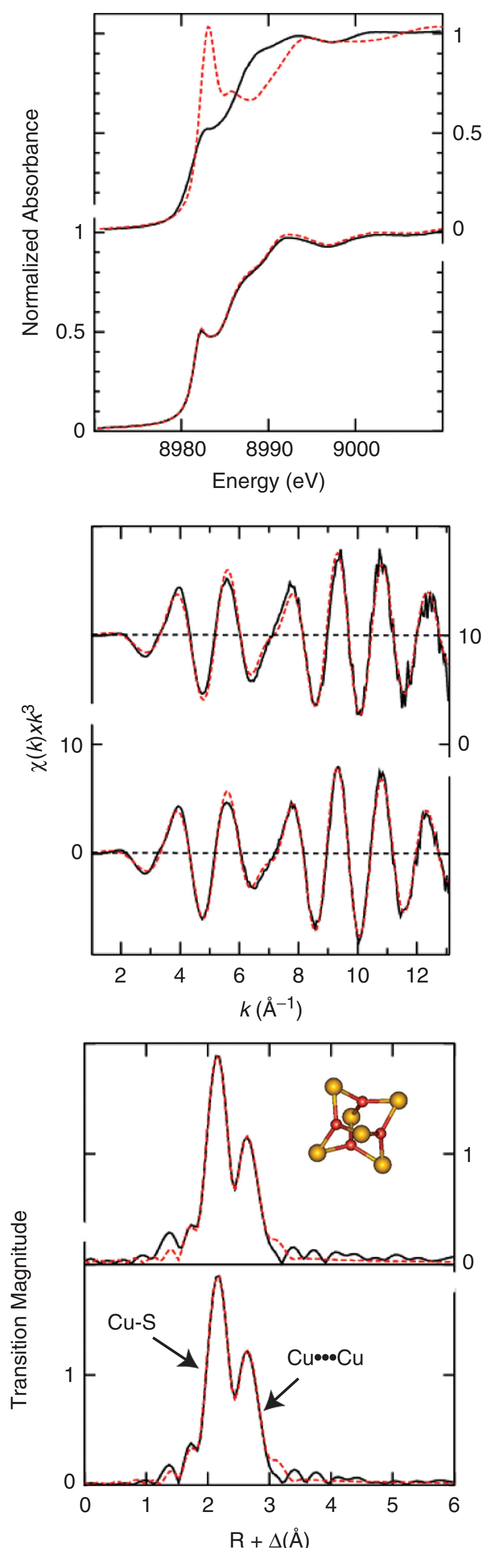

Figure 6. X-ray absorption spectroscopy (XAS) of Cu(I)- C-dMTF_81 complex. (**A**) Cu K-edge near-edge comparison of Cu(I)- C-dMTF_81 complex with two model Cu(I) thiolate compounds. In the upper panel are the trigonal Cu(I) thiolate model $[Cu_4(SPh)_6]^{2-}$ (or $[Cu_4(SR)_6]^{2-}$, black solid line) forming a four-Cu(I) cluster, and the diagonal Cu(I) thiolate model $[Cu(SC_{10}H_{12})_2]^{2-}$ (or $[Cu(SR)_2]^{2-}$, *red dash line*) containing a single Cu(I) ion. The lower panel shows Cu(I)- C-dMTF_81 complex with metal stoichiometries of 1.0 (black solid line) and 4.0 (red dash line), respectively. (**B**) Copper K-edge EXAFS spectra and C) Cu-S phase-corrected EXAFS Fourier Transforms of Cu(I)-C-dMTF_81 complex mixing with 1 mol·equiv. Cu (upper panel) and 4 mol·equiv. Cu (lower panel), respectively. Black solid curves represent the experimental data, while the red dash curves are for best fits with the parameters listed in Supplementary Table S1. The inset shows a structural model representing the proposed metal coordination of the Cu(I)- C-dMTF_81 complex based on the XAS data. The red balls represent copper atoms, while the yellow ones are for sulfur atoms.

of a 1:1 Cu(I):C-dMTF_81 mixture, i.e. identical to the 1:1 sample probed by XAS, as well as a 2:1 Cu(I): C-dMTF_131 mixture, is consistent with this picture, and further suggests that an intramolecular (monomolecular) polynuclear cluster is the dominant conformer in solution (see Supplementary Figure S3).

C-dMTF_81 binds Cu(I) in an all-or-none manner

We first performed a preliminary NMR analysis of 131, 81 and 51 residue fragments of dMTF-1 encompassing residues 499-629 (C-dMTF_131), 499-579 (C-dMTF_81) and 529-579 (C-dMTF_51) by acquiring ^{1}H-^{15}N HSQC and ^{1}H-$\{^{15}N\}$heteronuclear NOE (ssNOE) spectra in the presence and absence of Cu(I). The latter experiment carried out with C-dMTF_81 revealed that only ≈27 crosspeaks were characterized by positive ^{1}H-$\{^{15}N\}$ ssNOE values and were significantly shifted following the addition of 4.0 mol•equiv of Cu(I). This finding is consistent with the idea that Cu(I) folds the region immediately around the Cys cluster with little additional long-range folding evident in these spectra (Supplementary Figures S4-S5); in the absence of Cu(I), all resolvable crosspeaks have strongly negative ssNOE values revealing little or no stable structure in the absence of Cu(I) (spectra not shown). Further evidence for limited and localized Cu-dependent folding is that amide resonances that shift upon addition of Cu(I) have virtually identical chemical shifts in the context of a fusion protein in which 27-residues of dMTF-1 (542-568) are C-terminally appended to protein G B1 domain (GB1) (spectra not shown) (50).

We next used NMR spectroscopy to investigate the cooperativity of Cu_4 cluster formation by acquiring ^{1}H-^{15}N HSQC spectra as a function of Cu(I):C-dMTF_81 molar ratio (Figure 7). These spectra reveal that at subsaturating Cu(I), the spectrum corresponds to a superposition of apo- and Cu_4 conformers with no evidence of a non-native structural intermediate. Quantitation of the crosspeak intensities of selected resonances (Supplementary Figure S6) as a function of Cu(I) loading is fully compatible with scenario, i.e. the intensity of apo-C-dMTF_81 crosspeaks decrease monotonically as Cu_4 crosspeak areas increase. The assembly of the Cu_4 cluster is therefore highly cooperative, a result consistent with the findings by XAS and mass spectrometry, which reveal significant Cu_4 polynuclear cluster upon addition of sub-stoichiometric Cu(I). Despite the highly cooperative Cu-binding by C_dMTF_81, the peptide is characterized by a high degree of internal dynamics, a characteristic not unprecedented from previous studies of Cu- and Zn/Cd-loaded metallothioneins (51).

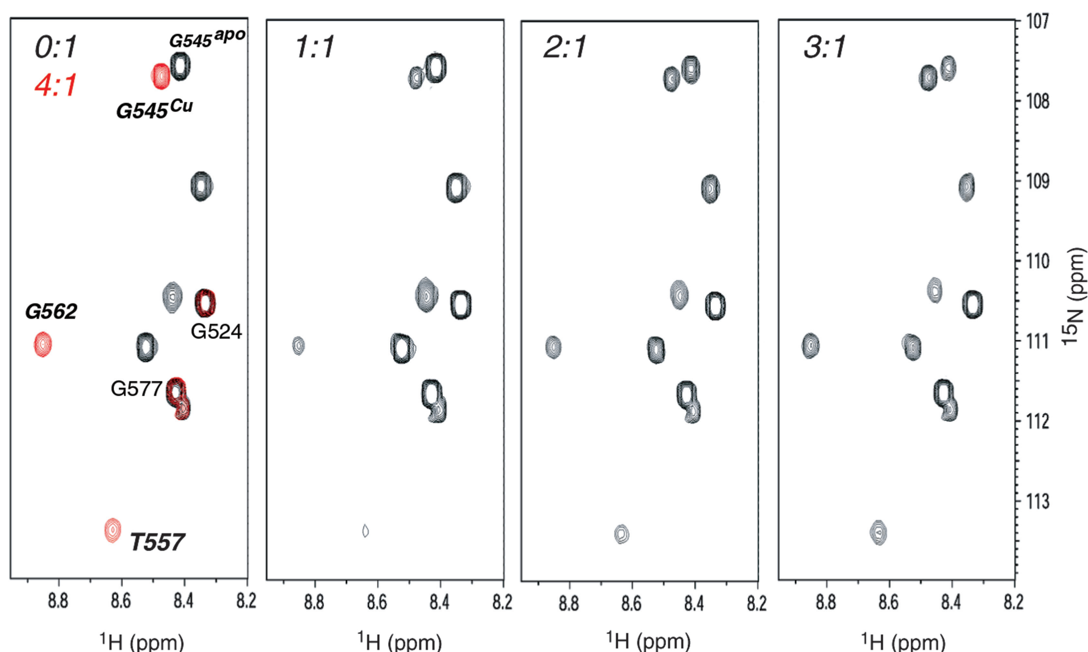

Figure 7. Substoichiometric addition of Cu(I) to apo-C-dMTF_81 results in cooperative assembly of a Cu_4 cluster from the apoprotein. A subregion of ^{1}H-^{15}N HSQC spectrum is shown as a function of Cu(I): C-dMTF_81 molar ratio indicated. Select resonances within the Cys cluster domain are *italicized*, while those outside this region (G524, G577) do not shift upon Cu(I) binding. The three remaining crosspeaks are unassigned apoprotein crosspeaks.

DISCUSSION

MTF-1 of *Drosophila* is capable of activating the transcription of distinct metallothionein genes (*MtnA-D*) in response to several metal ions, including Cu(I), Cd(II) and Zn(II). A recent characterization of transgenic flies carrying deletions of one of the four *Mtn* genes reveals that *MtnA* (the expression of which is studied here) is primarily responsible for protecting flies against exogenous copper load, while $MtnB^{-/-}$ flies are most sensitive to cadmium toxicity. The biological roles of MtnC and MtnD, which are closely related to MtnB, remain enigmatic since flies harboring a deletion of one or both of these genes exhibit near wild-type resistance against Cu and Cd toxicity (37). Binding studies revealed that MtnA is most strongly stabilized by Cu(I) binding, while MtnB binds Cd(II) preferentially over Zn(II) and Cu(I). These findings are generally consistent with the characteristics of flies harboring a metallothionein gene family knockout; these flies are viable and develop normally on standard food, but are highly sensitive to copper and cadmium toxicity. In particular, these experiments establish that MtnA and its regulator MTF-1 are responsible for the intense orange copper-mediated luminescence (when excited in the ultraviolet; see Figure 5) associated with specialized cells from the intestinal tract, termed midgut 'copper cells' (36). These cells likely function as storage depots for excess Cu(I), essentially protecting the organism against the effects of Cu(I)-mediated oxidative stress as well as a source of intracellular copper under conditions of copper deprivation (35). Interestingly, in contrast to mammalian MTF-1, *Drosophila* metallothioneins appear to play only a minor role against zinc toxicity (36). On the other hand, the expression of the zinc efflux transporter ZnT35C, thought to be analogous to the mammalian zinc exporter ZnT1, is strongly induced by Zn in an MTF-1-dependent manner (52).

How a single transcriptional activator, dMTF-1, is capable of up-regulating the expression of specific genes in response to distinct metal ions is unclear. One plausible scenario is that the metal selectivity of gene expression is dictated by the promoter-specific nature of the protein complex containing MTF-1 that mediates a specific transcriptional response. There is some evidence in support of this idea, since when MREs are excised from their context in the *Ctr1B* promoter (which is induced by Cu-scarcity) and placed in a non-native, mini-promoter context, they simply function as activating elements in response to copper overload, just like those derived from metallothionein genes (which are activated upon Cu-overload) (34). Along this vein of thought, MTF-1 might function as a promoter-specific adaptor molecule, in which the Zn(II)-bound zinc fingers mediate a direct interaction with the MRE, and another domain of the molecule mediates a Cu- or Cd- or Zn-specific complex with a putative co-activator or co-repressor. The foundational tenet of this hypothesis is that MTF-1 should be capable of forming complexes with Cu or Cd/Zn, with the distinct structures of each coordination complex (Cu vs. Zn/Cd) required to mediate metal-specific protein-protein interactions.

In the work presented here, we show that a C-terminal Cys-cluster of dMTF-1 encompassing six closely spaced cysteines forms a very stable, highly cooperative brightly luminescent Cu_4-S_6 polynuclear cluster. This cluster is essential for dMTF-1 to drive the expression of its target

gene *MtnA*, because a complete Cys substitution (6C-6A) in dMTF-1 abolishes its activity under copper stress and keeps the *MtnA* gene uninduced. Such a defect at molecular level results in a copper sensitive phenotype of mutant *Drosophila*. Partial alanine substitution of two (2C-2A) or four (4C-4A) of the Cu(I)-liganding cysteines also results in a severely attenuated survival index; this suggests that formation of the Cu_4-L_6 (L = ligand) complex optimally protects flies against copper toxicity by inducing *MtnA* expression. This short 19-amino acid domain is necessary and sufficient to bind four mol•equiv of Cu tightly and stoichiometrically *in vitro* and *in vivo*, the latter measured by examining the viability of Cu-sensitive *S. cerevisiae* strain on Cu-supplemented media.

Strikingly, the cysteine-rich domain of dMTF-1 is reminiscent of Cu-sensing domains of other Cu-regulators from lower eukaryotes, including Mac1 and Ace 1 from *S. cerevisiae*, Cuf1 from the fission yeast *S. pombe* (40), GRISEA from *Podospora anserina* (39), and Amt1 from *Candida glabrata* (41), in the complete absence of amino acid sequence homology. Spectroscopic studies of Amt1, Mac and Ace1 reveal that each forms intensely luminescent tetranuclear Cu_4•L_6 'cage-like' clusters containing trigonally coordinated solvent-shielded Cu(I) ions, with significant Cu•••Cu interactions, that either stimulate (Ace1, Amt1) or inhibit (Mac1) promoter DNA binding and/or transcriptional activation (41,47). A characteristic feature of the Cu complexes formed by Amt1, Ace and Mac1 is a short 2.7 Å Cu-Cu distance, also found here for dMTF-1 (41,47). Extensive molecular genetic studies have been carried out on *S. cerevisiae* Mac1, and these experiments are consistent with a model in which the Cu-binding domain forms a direct intramolecular protein–protein interaction with the N-terminal DNA-binding and nearby transactivation domain that allosterically blocks Mac1 function at multiple levels (53–55). Since Mac1 regulates the expression of the two high affinity Cu-importers CTR1 and CTR3, Cu-replete cells turn off the transcriptional activity of Mac1 in a Cu-dependent manner. In contrast, Cu-binding to both Ace1 and Amt1 strongly activates binding to the CuREs (copper response elements) positioned upstream of the genes encoding two metallothioneins, *CUP1* and *CRS5*, and superoxide dismutase *SOD1*. It seems plausible that Cu-binding to the C-terminal domain in dMTF-1 might unmask a critical transcription activation domain that allows the recruitment of TFIID to the promoter (32) or perhaps the chromatin remodeling enzymes, Swi5/Snf and Gcn5, as has been demonstrated for *C. glabrata* Amt1 (56).

The Cu-regulatory complexes formed by dMTF-1 and yeast transcriptional activators contrast sharply with those found in known copper metalloregulatory proteins in prokaryotes, which form either digonal (57), mononuclear trigonal planar (44), or binuclear Cu_2•S_4 coordination complexes (2,58). Unlike each of these systems which are highly specific for Cu(I) (and its structural surrogate Ag(I)) (46), the intrinsic metal specificity of the metal sensing domain of dMTF-1 may well be relaxed since dMTF-1 has to bind and metalloregulate gene expression from a variety of promoters in response to a number of different metal ions, including Cd(II) and Zn(II). A direct role of the Cys-cluster in sensing both Cd(II) and Zn(II) would require that the Cys-cluster of dMTF-1 bind these metal ions as well. In fact, the Cys cluster in dMTF-1 forms stoichiometric 1:1 complexes with both Cd(II) and Zn(II), rather than a polynuclear cluster; however, Cu(I) easily outcompetes Zn(II), with Zn(II) binding considerably more tightly than Cd(II) (Supplementary Figure S1). It seems likely then that Cu(I) is the 'cognate' metal and others may have to be recruited under specialized intracellular conditions at specific promoters. It will be interesting to determine the degree to which inactivation of the Cys-cluster by mutagenesis influences the metal-selectivity and inducibility at other promoters, in particular those that respond to other metal ions. In any case, under the chelator conditions tested, the cysteine mutants of dMTF-1 were no more sensitive to copper starvation than wild-type flies (Figure 1B). This likely indicates that the regulation of the *Ctr1B* copper importer gene by dMTF-1 (34) involves protein domain(s) other than the cysteine cluster characterized here.

Figure 8. Cysteine clusters of metalloregulatory transcription factors. (**A**) Conservation of the Cys-rich region in MTF-1 of *Drosophilidae* and a mosquito. Dm, *Drosophila melanogaster*; Dps, *Drosophila pseudoobscura*; Dmo, *Drosophila mojavensis*; Dgr, *Drosophila grimshawi*; An, *Anopheles gambiae* (23). (**B**) Cys-rich domains of yeasts and a filamentous fungus (39,40,47). Mac1 and Cuf1, copper-regulated transcription factors of baker's yeast (*S. cerevisiae*) and fission yeast (*S. pombe*), respectively. Grisea, copper-responsive transcription factor of the fungus *Podospora anserina*. (**C**) Tetracysteine cluster of human and mouse MTF-1, required for transcriptional response to zinc and cadmium load (9,23).

In conclusion, we have identified a novel Cu-binding domain in dMTF-1 derived from a cluster of six cysteines that is required to regulate metallothionein expression in transgenic flies in response to toxic intracellular levels of Cu(I). The structural features of this Cu-sensing domain while reminiscent of those previously identified in a number of fungal copper regulators, occurs in the absence of significant sequence homology and is therefore consistent with convergent evolution (Figure 8). These findings reveal a functional conservation of Cu homeostasis and detoxification from fungi to flies, with the added twist that just one transcription factor, dMTF-1, which must have evolved independently of the fungal regulators, handles both the uptake and detoxification arms of the Cu homeostasis system in *Drosophila* (34).

Ongoing studies in our laboratories are directed toward understanding the molecular mechanism of differential sensing and regulation performed by MTF-1 in response to a variety of inducers.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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