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Knockout of 'metal-responsive transcription factor' MTF-1 in *Drosophila* by homologous recombination reveals its central role in heavy metal homeostasis

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'Metal-responsive transcription factor-1' (MTF-1), a zinc finger protein, is conserved from mammals to insects. In the mouse, it activates metallothionein genes and other target genes in response to several cell stress conditions, notably heavy metal load. The knockout of *MTF-1* in the mouse has an embryonic lethal phenotype accompanied by liver degeneration. Here we describe the targeted disruption of the *MTF-1* gene in *Drosophila* by homologous recombination. Unlike the situation in the mouse, knockout of *MTF-1* in *Drosophila* is not lethal. Flies survive well under laboratory conditions but are sensitive to elevated concentrations of copper, cadmium and zinc. Basal and metal-induced expression of *Drosophila* metallothionein genes *MtnA* (*Mtn*) and *MtnB* (*Mto*), and of two new metallothionein genes described here, *MtnC* and *MtnD*, is abolished in *MTF-1* mutants. Unexpectedly, *MTF-1* mutant larvae are sensitive not only to copper load but also to copper depletion. In *MTF-1* mutants, copper depletion prevents metamorphosis and dramatically extends larval development/lifespan from normally 4–5 days to as many as 32 days, possibly reflecting the effects of impaired oxygen metabolism. These findings expand the roles of MTF-1 in the control of heavy metal homeostasis.

Keywords: gene targeting/heavy metal/metallothionein/MRE/MTF-1

Introduction

Every organism must cope with environmental fluctuation of heavy metal concentrations. Non-essential, toxic heavy metals have to be exported or sequestered intracellularly, while the uptake, storage and distribution of essential heavy metals, such as zinc and copper, have to be ensured, with the additional problem that even these metals are toxic if present in excess.

Important components of the heavy metal homeostasis and detoxification system are the membrane-based heavy metal transporters (reviewed in Nelson, 1999; Puig and Thiele, 2002), intracellular metal chaperones for efficient distribution of scarce essential metals (reviewed in Harrison *et al*., 2000), and the metallothioneins, a group of small, cysteine-rich proteins that have the ability to bind and thereby sequester heavy metals (reviewed in Kägi, 1991; Palmiter, 1998; Andrews, 2000; Simpkins, 2000). There are >10 functional metallothionein genes in humans and four in the mouse (West *et al*., 1990; Quaife *et al*., 1994); in *Drosophila*, two genes were characterized before, designated *Mtn/MtnA* and *Mto/MtnB* (Lastowskiperry *et al*., 1985; Mokdad *et al*., 1987) (see also below). Transcription of metallothionein genes is strongly induced by heavy metal load. This induction is mediated via conserved DNA sequence motifs, so-called metal response elements (MREs) of consensus TGCRCNC (R = A or G, and N = any nucleotide) that are present in the promoters of all metallothionein genes from insects to mammals (Stuart *et al*., 1985).

Previously, our laboratory isolated and characterized a zinc finger transcription factor that binds to MRE sequences. This protein was referred to as metal response element-binding transcription factor-1 (MTF-1, also designated metal-responsive transcription factor-1 or metal transcription factor-1) (Westin and Schaffner, 1988; Radtke *et al*., 1993; Brugnera *et al*., 1994; Auf der Maur *et al*., 1999; reviewed in Andrews, 2001; Giedroc *et al*., 2001; Lichtlen and Schaffner, 2001). In the mouse, MTF-1 plays an essential role in liver development; targeted deletion of the *MTF-1* gene results in embryonic death due to liver degeneration (Günes *et al*., 1998). A search for MTF-1 target genes in the mouse has revealed metallothionein genes and a number of other genes, several of which contain MREs in the promoter and/or are involved in coping with cell stress (Andrews *et al*., 2001; Lichtlen *et al*., 2001). MTF-1 plays a role not only in heavy metal stress but also in other cell stress conditions such as oxidative stress, hypoxia and amino acid starvation (Murphy *et al*., 1999; Dalton *et al*., 2000; Adilakshmi and Laine, 2002; reviewed in Lichtlen and Schaffner, 2001).

Recently, we have characterized the MTF-1 homolog from *Drosophila* and demonstrated that it can activate metallothionein gene promoters in cell transfection experiments (Zhang *et al*., 2001). Here we show that inactivating the *Drosophila MTF-1* gene using the technique of targeted gene disruption by homologous recombination (Rong *et al*., 2002) yields viable flies that are sensitive not only to high concentrations of copper, cadmium and zinc but, unexpectedly, also to copper depletion. We also describe two novel metallothionein genes *MtnC* and *MtnD* and show them to be, like the known metallothionein genes *MtnA* and *MtnB*, targets of MTF-1.

Results

Generation of MTF-1 mutant alleles

We decided to mutate the gene for MTF-1 in *Drosophila* by homologous recombination, since there are no

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P-elements close by in the corresponding 67B region of chromosome 3. For gene targeting, a 17 kb genomic fragment was modified by replacing the initiator ATG with TTA and inserting an I-*Sce*I recognition site into the second intron of *MTF-1*. Targeting of the *MTF-1* locus was achieved by a procedure essentially corresponding to that described by Rong and Golic (2000, 2001) and Rong *et al.* (2002) (Figure 1). By screening a total of 40 000 flies, we recorded 23 independent targeting events (frequency of one event in 1700 flies). Because of the design of an 'ends-in' recombination, the gene targeting led to a tandem duplication of the locus, resulting in a mutant and a wild-type gene copy. In a final step, one of the two gene copies could be removed by cleavage with subsequent recombination (Figure 1). Unexpectedly, in five independent lines, homologous integration of the targeting DNA into the *MTF-1* locus resulted in additional sequence alterations, namely large deletions of 1–4 kb in the supposedly wild-type copy of *MTF-1* (Figure 2). Similar deletions as side products of homologous gene targeting apparently were also observed by others (Rong and Golic, 2000; Seum *et al.*, 2002). The origin of such deletions is enigmatic at present. In any case, they were a welcome addition to the intended ATG site mutation and allowed for an independent test of the mutant phenotype.

Isogenic DNA does not increase targeting efficiency at MTF-1

In the mouse, the best targeting efficiency is observed if targeting and recipient DNA are isogenic, i.e. do not display DNA sequence polymorphisms (te Riele *et al.*, 1992). In our case, the targeting construct used was not isogenic to recipient DNA, but differed by no less than 215 single nucleotide substitutions and 25 insertions/deletions of up to 12 bp within a segment of 17 kb. To test whether an isogenic background would increase the targeting efficiency, we performed the same experiment in a fly strain that does not contain any polymorphisms relative to the targeting construct (a gift of R.Jiao and M.Noll). Surprisingly, in our hands, isogenicity did not improve the frequency of targeting by homologous recombination: among 15 000 flies, merely two events were recorded. It might be that as the length of homology increases, there is less dependence on isogenic DNA.

MTF-1 mutant flies are sensitive to heavy metals

Disruption of the *MTF-1* gene in the mouse results in embryonic lethality (Günes *et al.*, 1998). Unexpectedly, this was not the case in *Drosophila*. We observed viable and fertile flies with single or tandemly arranged mutant genes, irrespective of whether they were kept over the

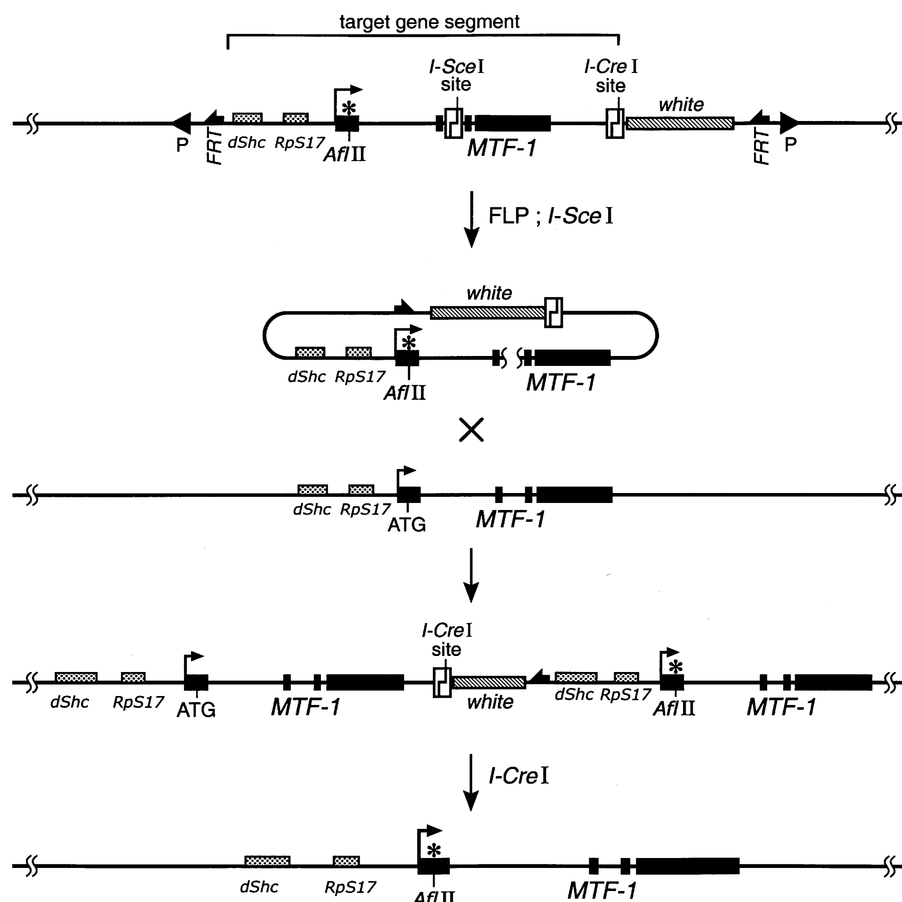

Fig. 1. Schematic view of the *MTF-1* locus and targeting strategy. A transgene containing a mutant *MTF-1* and the marker gene '*white*' (w^+) is circularized by FLP recombinase and linearized by the rare-cutter restriction enzyme I-*Sce*I in germ cells. Alignment of the targeting DNA and resident *MTF-1* locus by 'ends-in' recombination results in a duplication of *MTF-1*, with concomitant integration of the w^+ gene into the *MTF-1* locus. After identification of successful targeting events, the duplication is reduced to a single copy by means of homologous recombination, after inducing a double strand break in the genomic DNA with the other rare-cutter (I-*Cre*I). The w^+ marker gene is thereby lost. *, mutation of start colon.

large deficiency *Df(3L)AC1* that also removes the *MTF-1* locus, or were bred to homozygosity. In the mouse, MTF-1-deficient cells can be taken into culture before the deadly crisis of the embryo; such cells are sensitive to heavy metal load (Günes *et al.*, 1998). We thus wanted to test whether the mutant flies are sensitive to elevated concentrations of cadmium, copper or zinc. Cadmium is a non-essential, highly toxic metal; in contrast, copper and zinc are essential trace metals whose homeostasis is regulated carefully (Palmiter and Findley, 1995; reviewed in Eide, 1998; Nelson, 1999). Indeed, as seen in Figure 3, *MTF-1* mutant flies are more sensitive to cadmium toxicity than wild-type flies. Even though generally higher concentrations of copper and zinc were required to achieve toxic effects, the mutant flies were again considerably more sensitive to these trace metals. At the concentrations shown here, wild-type flies are not affected (Figure 3B). Severe toxicity for *MTF-1* mutant flies (tested with allele *MTF-1^140-1R^*) is seen with 50 μM $CdCl_2$, 250 μM $CuSO_4$ and 4 mM $ZnCl_2$, whereas the wild-type is able to grow on food supplemented with 300 μM $CdCl_2$, 3 mM $CuSO_4$ and 8 mM $ZnCl_2$, respectively. The metal sensitivity phenotype of the mutant could be completely rescued by a P-element containing a genomic *MTF-1* transgene (not shown), confirming that it was indeed lack of MTF-1 that caused the phenotype.

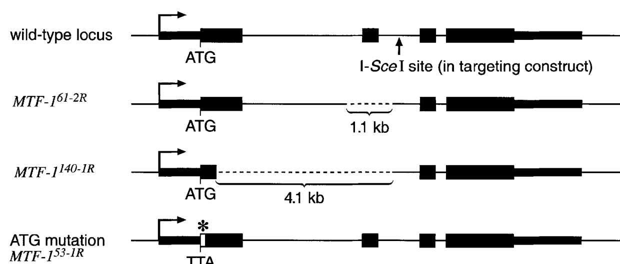

Fig. 2. Overview of wild-type and mutant *MTF-1* alleles. The gene structure of the wild-type *MTF-1* allele is shown on top; below, two examples of spontaneous deletions that had occurred upon integration of the targeting DNA by homologous recombination are depicted. These deletions are missing 1.1 kb including exon 2 (allele *MTF-1^61-2^* and *MTF-1^61-2R^*), or 4.1 kb including a substantial fraction of exon 1, together with the entire first intron and exon 2 (allele *MTF-1^140-1^* and *MTF-1^140-1R^*). The targeting mutation in the initiator codon ATG→TTA in the original targeting construct is shown at the bottom (alleles *MTF-1^140-1^*, *MTF-1^61-2^* and *MTF-1^53-1R^*). Alleles *MTF-1^61-2^* and *MTF-1^140-1^* are duplications of the locus (as indicated in Figure 1) containing a ATG→TTA mutation in one copy and a deletion in the other copy of *MTF-1*. *MTF-1^140-1R^*, *MTF-1^61-2R^* and *MTF-1^53-1R^* are alleles reduced to a single copy.

Metallothionein gene transcription depends on MTF-1

The question arose as to what target genes are most obviously affected by the lack of MTF-1. In mammals, the best characterized targets are the metallothionein genes and, also in cultured *Drosophila* cells, transcription of metallothionein genes *MtnA* and *MtnB* (formerly *Mtn* and *Mto*, respectively) is activated by MTF-1 via the typical MREs in their promoters (Zhang *et al.*, 2001). When we searched the database for metallothioneins using the known sequences of *MtnA* and *MtnB*, two new metallothionein genes were identified that were accordingly named *MtnC* and *MtnD* (Figure 4A). Initially, the significance of *MtnD* was in doubt because of a premature stop codon (TAG) replacing AAG (lysine) at amino acid position 32. Otherwise, *MtnD* has all the attributes of a functional metallothionein gene, including MRE sequences in the promoter, which prompted us to determine the gene's sequence in other fly strains as well. Interestingly, we found that this stop codon, most probably resulting in a non-functional pseudogene, is confined to the OregonR *Drosophila* strain, while the CantonS strain, as well as a wild catch in the village of Küsnacht, Switzerland, contain a lysine codon instead. This identifies MtnD as a novel metallothionein closely related to MtnB and MtnC (Figure 4B). We tested the effect of heavy metal load on the expression of these metallothionein genes and found that all four of them displayed greatly elevated transcript levels upon exposure to cadmium or copper

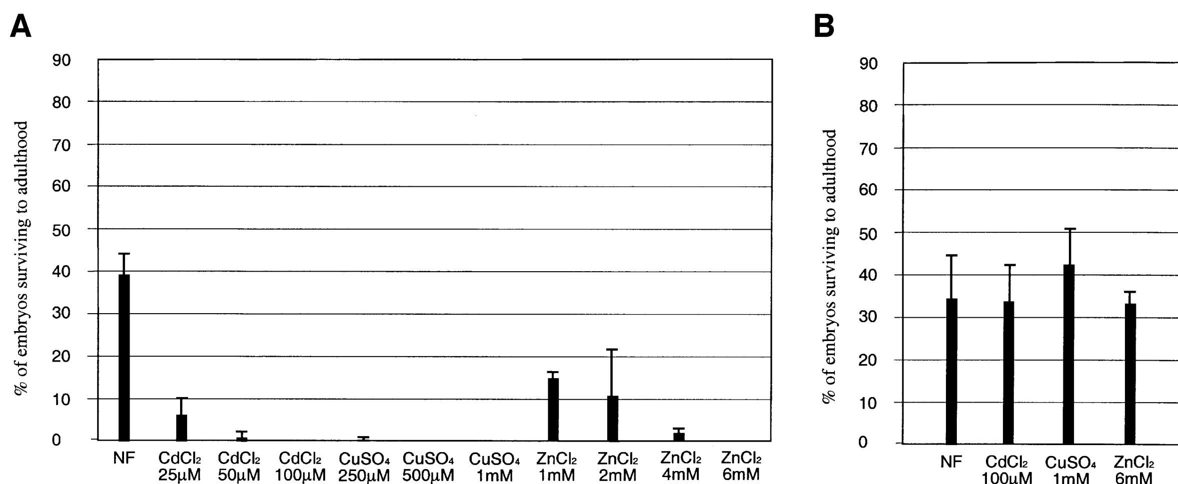

Fig. 3. Relative viability of *MTF-1* mutant versus wild-type at different metal concentrations. The bar diagrams depict the percentage survival of mutant and wild-type embryos to adulthood. *MTF-1^–/–^* mutant (allele *MTF-1^140–1R^*) (**A**) or y w control flies (**B**) were allowed to deposit 150–300 eggs on food containing the indicated concentrations of metals, and eclosing adults were counted. NF = normal food. Error bars represent standard deviations calculated from the number of flies in at least three different tubes.

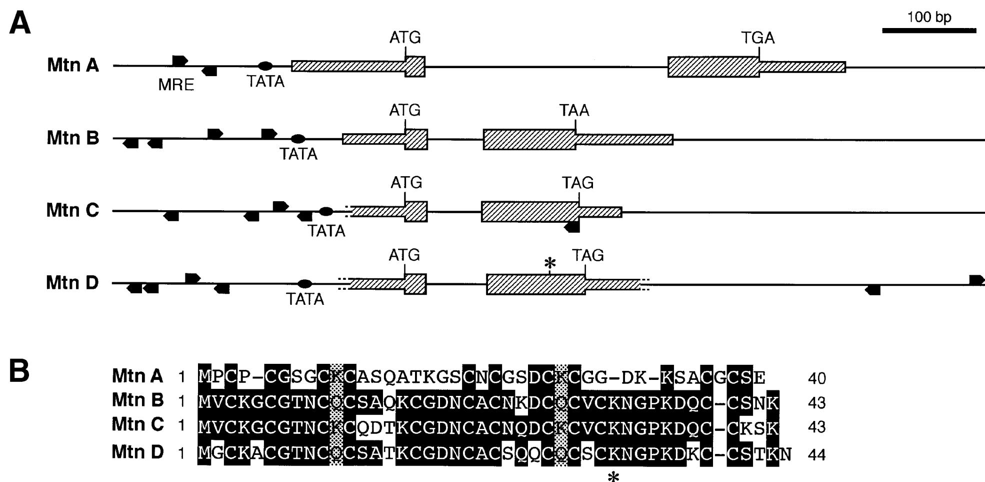

Fig. 4. *Drosophila* metallothionein genes and proteins. (**A**) Overview of the four metallothionein genes; genomic location on 3R (*MtnA* at 85E9, *MtnB* at 92E12, *MtnC* at 92E4, *MtnD* at 92F1). In addition to the previously known *MtnA* and *MtnB*, two new metallothionein genes are present in the region of *MtnB*, designated *MtnC* and *MtnD*. (**B**) Comparison of metallothionein protein sequences. All four metallothioneins have a similar pattern of cysteines that are involved in metal complexation. The amino acid sequences of MtnB, C and D are closely related, indicating that these genes arose by duplication. However, several genes between them are not duplicated. OregonR contains a premature TAG stop codon at position 32 of Mtn D, indicated by an asterisk in the gene structure and in the amino acid sequence. In the CantonS strain and a Swiss wild catch, a conserved lysine is present instead of this premature stop codon.

(Figure 5A). Zinc is a relatively poor inducer of *Drosophila* metallothioneins (Zhang *et al.*, 2001) and was therefore not tested in this experiment. Mutation of *MTF-1* had a striking effect on the expression of all four metallothionein genes. As seen in Figure 5B, the heavy metal response was abrogated and even the basal expression level was strongly reduced.

MTF-1 mutants are also sensitive to copper depletion

To see whether *MTF-1* mutant flies were perhaps more resistant to a scarcity of an essential trace metal, flies were raised on food containing various concentrations of metal chelators. Three chelators were tested: bathocuproinedisulfonic acid (BCS), an extracellular copper chelator (Labbe *et al.*, 1997); ammonium tetrathiomolybdate (TTM), a strong copper chelator also used in the treatment of Wilson's disease patients who suffer from copper accumulation in the liver (Brewer *et al.*, 1991; Jeannin *et al.*, 1992); and bathophenanthrolinedisulfonic acid (BPS), an iron (II) chelator that also, however, has affinity for copper (Alcain *et al.*, 1994; Georgatsou and Alexandraki, 1999). Unexpectedly, *MTF-1* mutant larvae were exquisitely sensitive to the copper chelators TTM and BCS (Figure 6); they were also more sensitive to the iron (copper) chelator BPS, although >5-fold higher BPS concentrations were needed to achieve lethality as compared with the chemically very similar compound BCS (not shown).

Larvae died at first and second instar larval stages with 5 or 10 μM TTM, and at second or third instar and even the pupal stage in the case of 50 or 100 μM BCS, with a decreasing number of larvae escaping to the next stage. The effect of copper depletion was aggravated by breeding mutant flies for more than one generation: flies that had been raised under a sublethal BCS chelator condition for one generation failed to produce viable larvae if these were subjected to the same low copper conditions, whereas wild-type flies could be grown continuously in the same food. This suggests a maternal copper effect: if raised in normal food, both wild-type and mutant mothers might deposit copper in the egg. If raised in low copper food, however, the mutant mothers would be unable to provide the necessary copper for their offspring, resulting in early larval death. To see whether *MTF-1* mutants were more sensitive to adverse nutrient conditions in general, we also raised them in a 10-fold dilution of the food in a constant amount of supporting agar. Even under such hardship which required extra activity of the larvae to collect enough nutrients, the mutant was not at a disadvantage: a few larvae from both wild-type or *MTF-1* mutants survived and reached pupation and fertile adulthood, indicating that *MTF-1* mutants do not suffer from a general weakness (not shown). The effect of the copper chelators is specific, because addition of copper but not zinc to food containing either BCS or TTM rescued the phenotype (Figure 6).

Another unexpected finding was a dramatically extended larval period of *MTF-1* mutants upon copper depletion. Instead of the usual 4 days for passing through instar stages 1–3 to pupation at 25°C, some mutant larvae were roaming through the food cake for up to 32 days, apparently unable to continue on to pupation. While their size was approaching that of third instar larvae, other features, especially the tracheal system and mouth apparatus, corresponded to second instar larvae (Figure 7). These findings show that loss of MTF-1 makes animals hypersensitive to too high and too low concentrations of heavy metals, thus suggesting that MTF-1 plays a dual role in metal homeostasis.

Discussion

To study the function of MTF-1 in *Drosophila*, we mutated the *MTF-1* locus via homologous recombination. *Drosophila* had long been refractory to this kind of

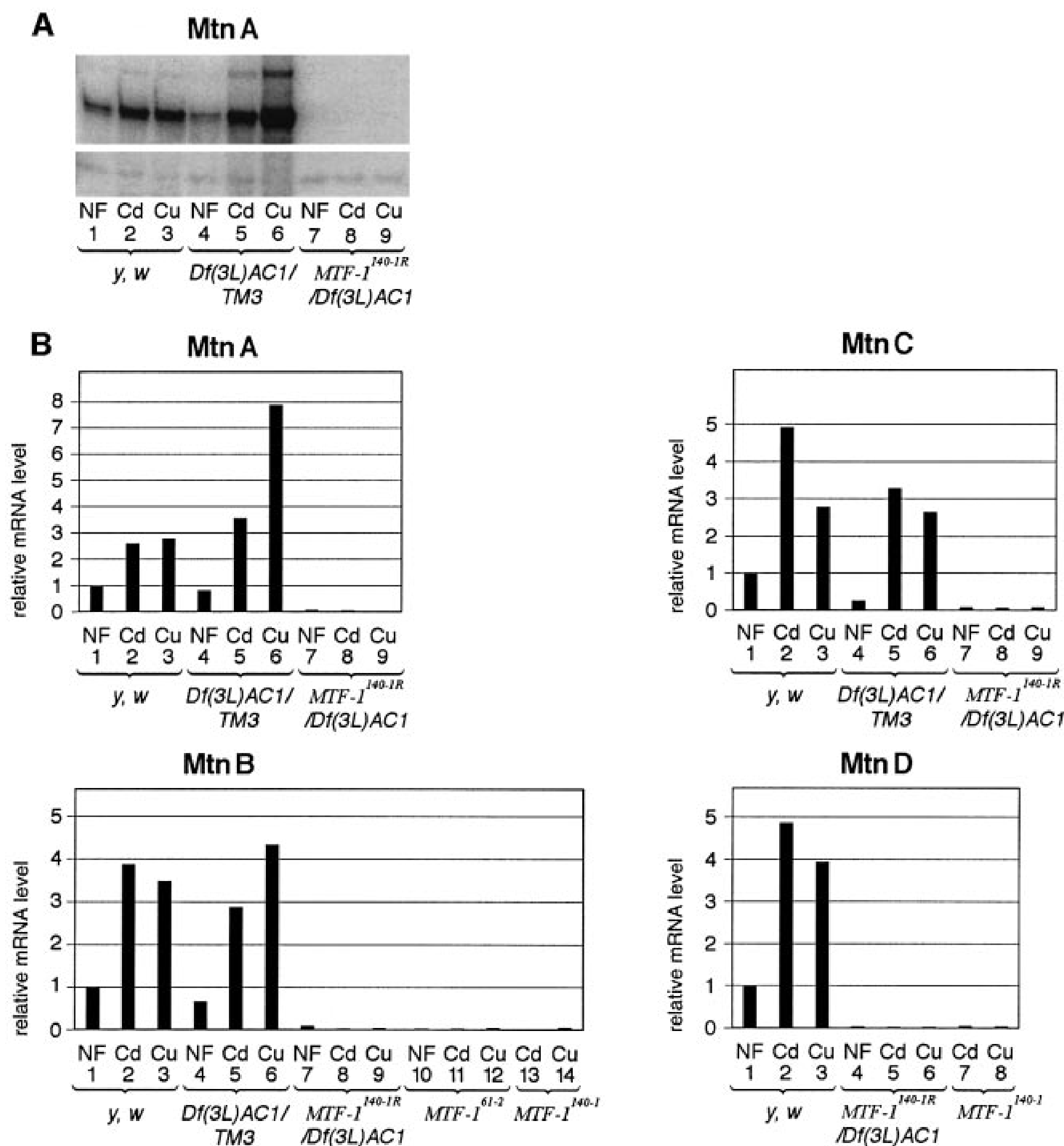

Fig. 5. All four *Drosophila* metallothionein genes respond to heavy metal load and are dependent on *MTF-1*. Transcripts of metallothionein genes in *Drosophila* third instar larvae were determined by S1 nuclease protection (Weaver and Weissmann, 1979). Several *MTF-1* mutant genotypes were used for this analysis, as indicated in the figure. NF = normal food; Cd = 50 μM $CdCl_2$; Cu = 500 μM $CuSO_4$. (**A**) Quantification of metallothionein (*MtnA*) transcripts. The lower part of the figure shows the signal from α-tubulin84B (loading control). (**B**) Compilation of the expression values of all four metallothionein genes (*MtnA–D*). Due to different specific activities of probes and hybridization optima, absolute transcript levels could not be compared easily between metallothionein genes. Therefore, the wild-type expression level in normal food was set arbitrarily to 1. Note that metallothionein expression in flies heterozygous for the *MTF-1* locus is about the same as in y w flies wild-type for *MTF-1* (compare lanes 1–3 of *MtnA*,*B*,*C* with lanes 4–6 of the same metallothioneins), while in *MTF-1* mutants basal and metal-induced transcription activities of metallothionein genes are strongly reduced (compare lanes 1–3 of *MtnA*,*B*,*C*,*D* with lanes 7–9 of *MtnA*, lanes 7–14 of *MtnB*, lanes 7–9 of *MtnC* and lanes 4–8 of *MtnD*, respectively). *MtnB* expression is equally low with different mutant alleles: *MTF-1*$^{61\text{-}2}$, *MTF-1*$^{140\text{-}1}$ and *MTF-1*$^{140\text{-}1R}$ (compare lanes 7–9 of *MtnB* with lanes 10–12, and with lanes 13 and 14).

manipulation, even though many aspects of homologous recombination were described before (Engels *et al*., 1990; Gloor *et al*., 1991; Hagmann *et al*., 1998). Using an 'ends-in' recombination approach, we observed a targeting frequency of one targeting event in 1700 flies, a number that compares quite well with the best values obtained so far by Golic and co-workers at the *yellow* (*y*) gene locus (Rong and Golic, 2000). Our findings thus underline the usefulness of homologous gene targeting in *Drosophila*.

Unlike the *MTF-1* mutant mice, which die from embryonic liver degeneration *in utero*, *MTF-1* mutant flies are viable yet sensitive to heavy metals and copper depletion. This phenotype is very similar for combinations of a variety of different alleles including an initiator triplet mutation, a 1.1 kb deletion, a 4.1 kb deletion, and tandem or single mutant genes in combination with a chromosomal deficiency. Subtle differences exist amongst different allelic combinations in their sensitivity to either heavy metal load or copper chelators. The allele *MTF-1*$^{140\text{-}1R}$ carrying a 4.1 kb deletion of the coding region possesses the strongest phenotype and is most probably a null mutation.

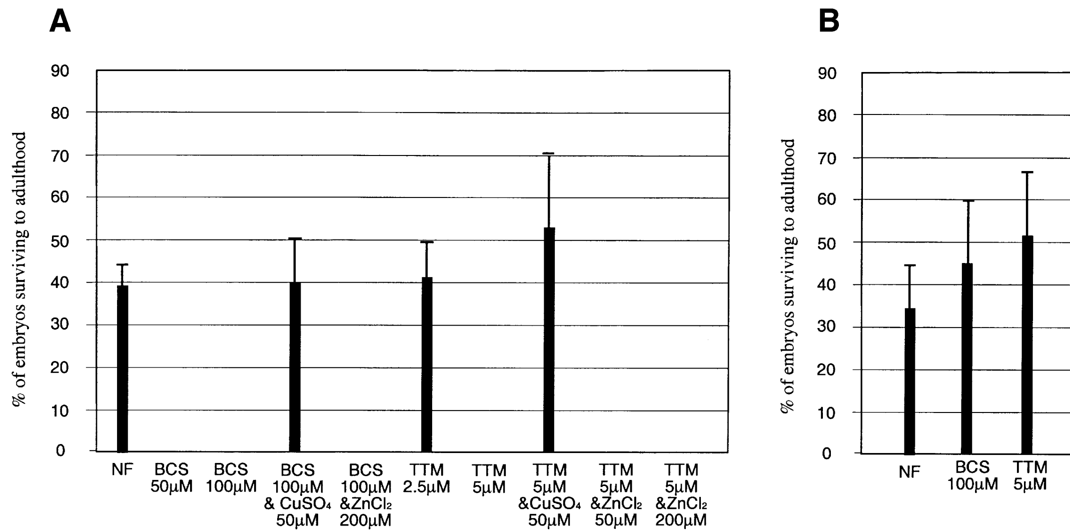

Fig. 6. *MTF-1* mutants are highly sensitive to copper chelators. The bar diagrams depict the percentage survival of mutant and wild-type embryos to adulthood. $MTF\text{-}1^{-/-}$ mutant (allele $MTF\text{-}1^{140\text{-}1R}$) (**A**) or y w control flies (**B**) were allowed to deposit 150–300 eggs on food containing the indicated concentrations of chelator, and eclosing adults were counted. NF = normal food. Error bars represent standard deviations calculated from the number of flies in at least three different tubes.

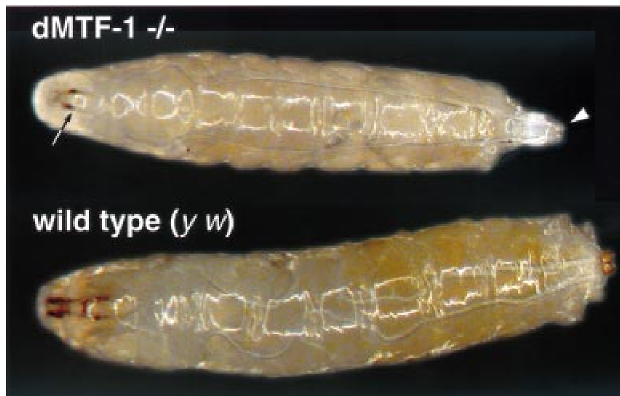

Fig. 7. Treatment with the copper chelator TTM results in extreme prolongation of the larval period. Shown are an *MTF-1* mutant larva (allele $MTF\text{-}1^{140\text{-}1}$) at 22 days kept in 10 µM TTM and a wild-type (y w) control larva at the wandering stage. Note that in the mutant larva, although its size approaches that of a wandering third instar larva, several anatomical deviations, including tracheal system (arrowhead points to posterior spiracles) and mouth hooks (arrow) correspond to those of second instar larvae. Anterior spiracles, not visible here, also resemble second instar larvae. Note that the diameter of the tracheal tubes is smaller in the *MTF-1* knockout than in y w controls. Such larvae cannot enter pupation and die between 20 and 32 days.

In general, *MTF-1* mutants are sensitive to distortions of heavy metal balance. One aspect, namely the sensitivity to high concentrations of cadmium but also to copper and zinc, is in agreement with earlier findings with cultured cells of $MTF\text{-}1^{-/-}$ mice (Günes *et al.*, 1998) and *Drosophila* (Zhang *et al.*, 2001). The failure of our *MTF-1* mutants to induce metallothionein genes provides the most likely explanation for their sensitivity to heavy metal load.

Quite unexpected, however, is the exquisite sensitivity of *MTF-1* mutants to copper depletion. This is particularly interesting because scarcity of trace heavy metals is probably encountered more often under natural conditions than heavy metal load. It is worth mentioning that another *Drosophila* mutant displays a similar phenotype: flies with a deletion of a copper transporter (*Ctr1B*) are also sensitive to both excess copper and copper depletion (D.J.Thiele, personal communication). The mechanism for this dual sensitivity may be a translocation of the protein from the outer membrane to vacuoles/lysosomes under limiting and excess copper concentrations, respectively (see also Petris *et al.*, 1996; Schaefer *et al.*, 1999; Borrelly *et al.*, 2002). So far, it is unclear how MTF-1 enables a cell to cope with metal depletion. Our results suggest that MTF-1 regulates either import or efficient usage of copper, since wild-type flies may be grown continuously on copper chelator food, whereas *MTF-1* mutants are able to do so under sublethal conditions for just a single generation. We speculate that MTF-1, upon copper depletion, activates a copper import pump and/or inactivates an export pump or, alternatively, regulates the expression of a copper chaperone. *Drosophila* metallothioneins themselves, which preferentially bind copper (Valls *et al.*, 2000), may act not only as heavy metal scavengers upon heavy metal load, but also, under limiting copper concentrations, as copper chaperones similar to Cox17 and Atx1 (reviewed in Harrison *et al.*, 1999). Alternatively, *Drosophila* copper-loaded metallothioneins may act as a storage pool for copper (see also Dalton *et al.*, 1996). This scenario is compatible with our finding that MTF-1 is also required for the basal transcription of metallothionein genes, as seen in Figure 5. Thus, the lack of metallothioneins in *MTF-1* mutants may also be responsible for their sensitivity to copper depletion.

Another enigma is the extreme extension of larval development in the presence of copper chelator in the food. It is not clear at present whether this prolonged larval period involves a genuine longevity effect. Several hypotheses can be envisaged to explain this phenomenon.

(i) Extension of larval life could be due to a decrease in cytochrome *c* oxidase (COX) activity, a key copper-containing enzyme in the respiratory chain. In the fungus *Podospora anserina*, elimination of *grisea*, a copper-modulated transcription factor, affects the expression of a copper transporter and leads to impaired copper uptake. This correlates with a reduced activity of the COX complex and is associated with delayed growth and an extended lifespan. The same phenotype is produced by the direct elimination of COX5, a subunit of the cytochrome *c* oxidase (Begel *et al*., 1999; Dufour *et al*., 2000; Borghouts *et al*., 2002; reviewed in Osiewacz and Borghouts, 2000; see also Carr *et al*., 2002). Extension of lifespan in *P.anserina* can also be achieved by the mere addition of the copper chelator BCS to the medium (Borghouts *et al*., 2001). In our case, a decrease in cytochrome *c* oxidase activity due to insufficient copper supply could reduce ATP production, slow growth and, consequently, prolong the larval period. (ii) Copper on the one hand is an essential component of enzymes, including tyrosinase/phenol oxidase (Asada *et al*., 1999) and superoxide dismutase for radical scavenging, but on the other hand contributes directly to the formation of oxygen radicals in the Fenton reaction (Zhou *et al*., 2001); for the latter reason, copper depletion may result in less oxidative damage. This by itself would not explain stalled development, but rather why larvae survive that long. (iii) Mutant larvae raised with a copper chelator grow but retain features of second instar larvae including thinner tracheal ducts (Figure 7); thus insufficient oxygen supply could restrict growth and prolong the larval period, perhaps again in combination with less oxidative damage of tissues. (iv) Larvae raised in chelator-containing food also have smaller mouth hooks, which may prevent them from using the food efficiently. Thus extended larval life could be the result of a caloric restriction, which is known to delay growth and extend lifespan in a large variety of organisms from yeast to mammals (Lin *et al*., 2002; reviewed in Roth *et al*., 1995; Sohal and Weindruch, 1996; Masoro, 2000). We consider this unlikely, because starvation due to a 10-fold dilution of the food cake did not reveal any differences between wild-type and *MTF-1* mutant larvae. (v) The gene for SHC adaptor protein (*shc*) involved in tyrosine kinase receptor signaling is located quite close to the *MTF-1* transcription unit (two genes upstream, at 3.3 kb distance), and in the mouse, knockout of *shc* has been found to extend lifespan (Migliaccio *et al*., 1999). Thus the knockout of *MTF-1* might adversely affect regulatory sequences of the *shc* gene. However, our simple mutation of three bases at the *MTF-1* translation initiation codon is unlikely to have such an effect. Furthermore, sequencing of the genomic region in the mutant flies revealed no difference from wild-type in the *shc* or *Rps17* region, showing that the targeting process has not affected these two neighboring genes (data not shown).

Whatever the reason for this greatly expanded larval period, our results firmly establish for MTF-1 a central role in the heavy metal metabolism of the fly. The exquisite sensitivity of *MTF-1* mutants to copper depletion points to a new role for this protein that waits to be explored also in mammals.

Materials and methods

DNA constructs

Targeting construct. The clone aj271817 containing the three genes *shc*, *RpS17* and *MTF-1* was cloned as a *Not*I fragment into pTV2 vector (Rong *et al*., 2002).

Changes were introduced by PCR with the following oligos (altered bases are underlined). The primers used to change the ATG→TTA were 5′-GCGAATAACAAATAATACGACTTAAGCGACCAAGAGAAACAACACCAGC-3′ and 5′-GCTGGTGTTGTTTCTCTTGGTCGCTTAAGTCGTATTATTTGTTATTCGC-3′. Mutation of CATGAAC to CTTAAGC generates a new restriction site (*Afl*II) for easy identification of mutant DNA.

Oligos used to introduce the I-*Sce*I cleavage site were 5′-TATTCCTAGGGATAACAGGGTAATACGGATAACTCAAGCGCGGAG-3′ and 5′-TATTCCTAGGCTCGCTAAGGTATTCCTCCTCG-3′. The reason for mutating the ATG was that there are no other nearby in-frame ATGs further downstream that could give rise to a truncated, yet possibly functional protein. The next downstream in-frame initiation codon occurs at amino acid position 222 at the end of the fourth zinc finger; DNA binding of MTF-1 has been shown to be completely dependent on zinc fingers 1–4 (Chen *et al*., 1998, 1999).

Genomic rescue construct. The clone aj271817 containing the three genes *shc*, *RpS17* and *MTF-1* was cloned as a *Not*I fragment into a P-element vector and injected into y w flies.

Fly stocks and genetics

The stocks *y w (v); P[ry$^+$, 70FLP]4 P[v$^+$, 70I-SceI]2B Sco/S^2 CyO* were provided by Y.Rong and K.Golic. Targeting was done from a single donor on the second chromosome. To maximize the efficiency of donor excision, three heat shocks (38°C, 60 min) were performed on days 2, 3 and 4 after egg laying. Heat-shocked virgins were crossed to y w, ey-Flp; *D gl/TM3,y$^+$* males, and only females were screened for the presence of the *white$^+$* (*w$^+$*) gene. *w$^+$* flies represent homologous or non-homologous integrations, respectively, since the ey-Flp completely removes any unexcised donor. Successful targeting events into the resident *MTF-1* locus on chromosome 3 were verified by Southern analysis (of 24 independent recombination events, just one was due to non-homologous insertion). Of the 23 targeted events, all except one retained the ATG→TTA mutation. A low frequency of gene conversion was to be expected, since the I-*Sce*I recognition site is located 4.3 kb away from the TTA mutation in the targeting construct and it is known that gene conversion frequencies decrease with the distance from the double strand break (Gloor *et al*., 1991; Rong *et al*., 2002). For the reduction to a single copy allele by homologous recombination, the targeted alleles *MTF-1$^{140-1}$* and *MTF-1$^{61-2}$* were chosen, both of which carry a deletion in one copy and an ATG point mutation in the other. Reduction by I-*Cre*I was performed by crossing the targeted alleles (class III event; Rong and Golic, 2000) to y w; *P[I-CreI]/TM3,Sb Ser*, a P-element insertion kindly provided by Y.Rong and K.Golic. The offspring were given a single heat shock (36°C, 60 min) at the third larval stage. Males were re-crossed to *y w*; *P[I-CreI]/TM3,Sb Ser* females to select for *w$^-$* flies. These were crossed individually to *y w*; *TM3,y$^+$/D gl* to make stocks which were tested for the presence of either the introduced mutation or the spontaneous deletion. Since I-*Cre*I cutting may result in elimination of a functional *w$^+$* gene either by homologous recombination or by exonuclease resection and DNA end joining, the correct removal of the duplication was checked by PCR and by Southern blotting (see below). The new single copy alleles were named as follows: *MTF-1^{140-1R}* for the 4.1 kb deletion, *MTF-1^{61-2R}* for the 1.1 kb deletion and *MTF-1^{53-1R}* for the ATG point mutation. These alleles are identified by the PCR primers listed below. The reduction process by I-*Cre*I worked with ~10% efficiency even with our very long duplication, where >17 kb of DNA of the *MTF-1* locus plus the *w$^+$* marker gene had to be removed, a total of 26 kb.

Molecular characterization of the targeted events

Southern blots were performed with a probe derived from a PCR product using genomic DNA with the primers 5′-CCTAATAGCATTGTTATACTCG-3′ and 5′-AGGGAGCACATACGAATCAG-3′. Allele-specific PCR was used to check for the presence of the mutations: the two primers 5′-AAAGCGAATTGCGCGACGAG-3′ and 5′-GAATAACAAATAATACGACTTAAG-3′ amplify a 158 bp fragment only from the mutant allele with the ATG→TTA mutation.

The two primers 5′-CGTTCCGCCCATGGTCACACTGGTTC-3′ and 5′-TGCCGGGTGTAACCGTAGACAGCCAG-3′ amplify a 545 bp fragment from the *MTF-1*$^{140-1}$ and *MTF-1*$^{140-1R}$ deletion allele and a 4.7 kb fragment from wild-type, and the primer pair 5′-CGCAAAAGCC-CGCTGCCACAAGGAGC-3′ and 5′-GAGCCCTCCAGGAAACGGC-TG-3′ amplifies a 0.9 kb fragment from the *MTF-1*$^{61-2}$ and *MTF-1*$^{61-2R}$ deletion allele instead of a 1.6 kb fragment in wild-type.

Cloning of MtnC and MtnD

The two primer pairs 5′-ACTGGCAAACACAGTATTCAG-3′/5′-ACAGGGGCTATCATTTATTGG-3′ and 5′-GTATTTTATTTCGTTG-TCATG-3′/5′-TTTTAACACAAAATGGGTTGC-3′ were used to amplify *Mtn C* and *Mtn D* by RT–PCR from total RNA, and cDNAs were cloned into pBluescript. *MtnC* is annotated at FlyBase (http://flybase.bio.indiana.edu/) as CG5097.

The fly strains used for sequencing the *MtnD* gene were OregonR, CantonS and a wild catch from Küsnacht, Switzerland, tentatively designated KantonZ. This sequence has been submitted to the DDBJ/EMBL/GenBank database under accession No. AF546903.

Fly food

Flies were raised on standard cornmeal molasses-based food supplemented with either $CdCl_2$, $CuSO_4$, $ZnCl_2$, TTM (Sigma-Aldrich 32,344-6), BCS disodium salt hydrate (Sigma-Aldrich 14,662-5) or BPS disodium salt hydrate (Sigma-Aldrich 14,661-7). The concentrations of trace metals, based on the content of the individual ingredients, are ~5 µM for copper and 150 µM for zinc.

RNA extraction and S1 nuclease protection assay

Third instar larvae were transferred for 6 h to food containing 50 µM $CdCl_2$, 500 µM $CuSO_4$, or to non-supplemented food. Total RNA was extracted using the TRIzol reagent (Life Technologies). Nuclease S1 mapping of transcripts with 50 µg of total RNA was performed as described previously (Weaver and Weissmann, 1979). The gels were developed using PhosphorImager (Molecular Dynamics) and bands were quantified.

Database searches and computer analysis of the sequences

Blast searches were performed using the BDGP BLAST service http://www.fruitfly.org/blast/. MREs were mapped using Sequencer™ 4.1 software. Sequence alignments were performed using the CLUSTAL_W and Boxshade programs.

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