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ORIGINAL PAPER

Characterization of MtnE, the fifth metallothionein member in *Drosophila*

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Abstract Metallothioneins (MTs) constitute a family of cysteine-rich, low molecular weight metal-binding proteins which occur in almost all forms of life. They bind physiological metals, such as zinc and copper, as well as non-essential, toxic heavy metals, such as cadmium, mercury, and silver. MT expression is regulated at the transcriptional level by metal-regulatory transcription factor 1 (MTF-1), which binds to the metal-response elements (MREs) in the enhancer/promoter regions of MT genes. *Drosophila* was thought to have four MT genes, namely, *MtnA*, *MtnB*, *MtnC*, and *MtnD*. Here we characterize a new fifth member of *Drosophila* MT gene family, coding for metallothionein E (MtnE). The *MtnE* transcription unit is located head-to-head with the one of *MtnD*. The intervening sequence contains four MREs which bind, with different affinities, to MTF-1. Both of the divergently transcribed MT genes are completely dependent on MTF-1, whereby *MtnE* is consistently more strongly transcribed. *MtnE* expression is induced in response to heavy metals, notably copper, mercury, and silver, and is upregulated in a genetic background where the other four MTs are missing.

Keywords Metallothionein E · *Drosophila* · Metal-regulatory transcription factor 1 · Metal responsive transcription factor · Metal-response element · Cadmium toxicity

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Introduction

Metallothioneins (MTs) are cysteine-rich, low molecular weight proteins that are able to bind a wide range of metals, including cadmium, zinc, mercury, copper, and silver [1–5]. MTs were first described in 1957 by Vallee and Margoshes [6] as cadmium-binding proteins in the horse renal cortex. MTs play an essential role in heavy metal detoxification and in maintaining the homeostasis of essential trace metals. They are also involved in the protection against free radicals and oxidative stress [7–9]. MTs are ubiquitously found in eukaryotes and also in some prokaryotes [10]. Humans have at least one dozen MT genes, whereas the mouse has four. *Drosophila* was thought to have four MT genes (*MtnA*, *MtnB*, *MtnC*, *MtnD*) expressed mainly in the digestive tract [11]. *Saccharomyces cerevisiae* has two MT genes, *CUP1* and *CRS5* [12, 13].

Metal-regulatory transcription factor 1 (MTF-1) is a zinc-finger-containing transcription factor conserved from insects to humans and is the main regulator of the expression of MTs (reviewed in [14–16]). MTF-1 plays a role not only in heavy metal stress but also in other cell stress conditions, such as oxidative stress and hypoxia [17–20]. MTF-1 was discovered in 1988 and was shown to be a protein which requires elevated zinc concentrations for optimal DNA binding [21]. Subsequently the mouse gene

was cloned and characterized as a ubiquitously expressed zinc finger transcription factor essential for basal and heavy-metal-induced expression of MTs [22, 23]. The cloning of *MTF1* genes of human, fish, and *Drosophila* followed [24–27]. *MTF1* null mutant mouse embryos develop severe liver degeneration and die in utero at approximately day 14 of gestation [28]. Conditional *MTF1* knockout mice in which the gene is disrupted in the liver and hematopoietic tissues are viable, but are highly sensitive to cadmium exposure and display reduced leukocyte counts [29]. In contrast to the lethal phenotype of the mouse *MTF1* null mutant, *MTF1* knockout *Drosophila* are viable and fertile under standard laboratory conditions, but are sensitive to heavy metal load and to copper starvation [30]. *Drosophila* MTF-1 is essential for the basal and metal-induced expression of the MT genes [30]. Moreover, it is also active under copper starvation, where it mediates expression of the intestinal copper importer *Ctr1B* [31]. Transcriptome analysis also revealed *ferritins*, the ABC transporter CG10505, the zinc transporter *ZnT35C*, and glutathione *S*-transferase genes (*GstD2* and *GstD5*) as target genes of *Drosophila* MTF-1 [32]. The involvement of the latter two genes is in agreement with findings in mammals, in that both MTF-1/MTs and glutathione play a role in counteracting metal stress [33].

Mammalian MTF-1 has a molecular mass of approximately 75 kDa (mouse and human MTF-1 are 675 and 753 amino acids long, respectively). The N-terminal domain is followed by six Cys2-His2-type zinc fingers. Mammalian MTF-1, besides nuclear localization and nuclear export signals, contains three activation domains: an acidic one, a proline-rich one, and a serine/threonine-rich one [34]. A conspicuous cysteine cluster (CQCQCAC) near the C-terminus is also required for transcriptional activity [35] and mediates homodimerization of human MTF-1 (V. Günther and W. Schaffner, unpublished data). Recently, it was shown that the nuclear localization signal of human MTF-1 is a nonconventional one, spanning zinc fingers 1–3 within the DNA-binding domain [36]. *Drosophila MTF1* complementary DNA (cDNA) encodes a protein of 791 amino acids; it shares 39% amino acid similarity with human MTF-1 and has a particularly striking similarity in the region of all six zinc fingers to its vertebrate homologs [27]. However, whereas human *MTF1* shows a strong induction in response to zinc and cadmium, *Drosophila MTF1* is best activated by copper and cadmium [27].

As already mentioned, MTF-1 regulates the expression of MTs at the transcriptional level [21–23]. Upon metal stress, MTF-1 binds to short DNA sequences in the enhancer/promoter regions of MT genes and other target genes, termed metal-response elements (MREs). MREs consist of a highly conserved core consensus sequence, (t)TGCRCNC (R is A or G, and N is any nucleotide), followed by less conserved GC-rich sequences [37–41]. A synthetic promoter composed of several MRE sequences is sufficient for MTF-1 to drive expression of a gene upon metal induction [42]. Mutations of single nucleotides in MRE sequences can reduce or abolish its function [22].

Here we describe the fifth *Drosophila* MT gene, annotated as CG42872 in FlyBase (http://www.flybase.org). It encodes a protein that is highly similar to the other *Drosophila* MTs, especially MtnB, MtnC, and MtnD [11, 30], and was named metallothionein (MT) E (MtnE) (Fig. 1a, b). CG42872 (*MtnE*) was discovered by targeted cDNA screening of gene predictions as part of the modENCODE transcriptome project [43]. The captured cDNA MIP01063 provided evidence for 5′ and 3′ untranslated regions and allowed characterization of the upstream regulatory region of the gene (Fig. 2a). Similar to other MT genes, *MtnE* possesses upstream MRE sequence motifs which are apparently shared with *MtnD* (Fig. 2b, c). We cloned the genomic region containing the *MtnE* and *MtnD* genes, which are adjacent to each other in a head-to-head orientation. In order to study the expression profiles, *MtnD* and *MtnE* were differentially tagged with green fluorescent protein (GFP) and/or mCherry fluorescent tags. *MtnE* is induced by several heavy metals and its expression, like that of the divergently transcribed *MtnD*, is completely dependent on MTF-1.

Materials and methods

Annotation of MT genes in insects

MT-type genes in the different insect genomes were identified by standard BLAST searches (NCBI BLAST and FlyBase BLAST) against the sequenced genomes and against protein databases. Sequence alignments were generated with CLC Sequence Viewer, version 6.3, with standard parameters.

Fly culture

One liter of fly food was composed of 55 g cornmeal (Maisgriess 54.401.025, Meyerhans Hotz), 100 g yeast (Hefe Schweiz), 75 g sugar (dextrose monohydrate), 8 g agar (Insectagar type ZN5) and 15 ml antifungal nipagin (nipagin 33 g/l, nipasol 66 g/l in 96% ethanol). For experiments, food was supplemented with $CuSO_4$, $HgCl_2$, $AgNO_3$, or $ZnSO_4$ to the concentrations indicated in the figures. Flies were raised at 25 °C in 65% humidity.

Tissue preparation and microscopy

For the whole-larvae imaging, wandering third instar larvae were placed on glass slides and immobilized by incubating

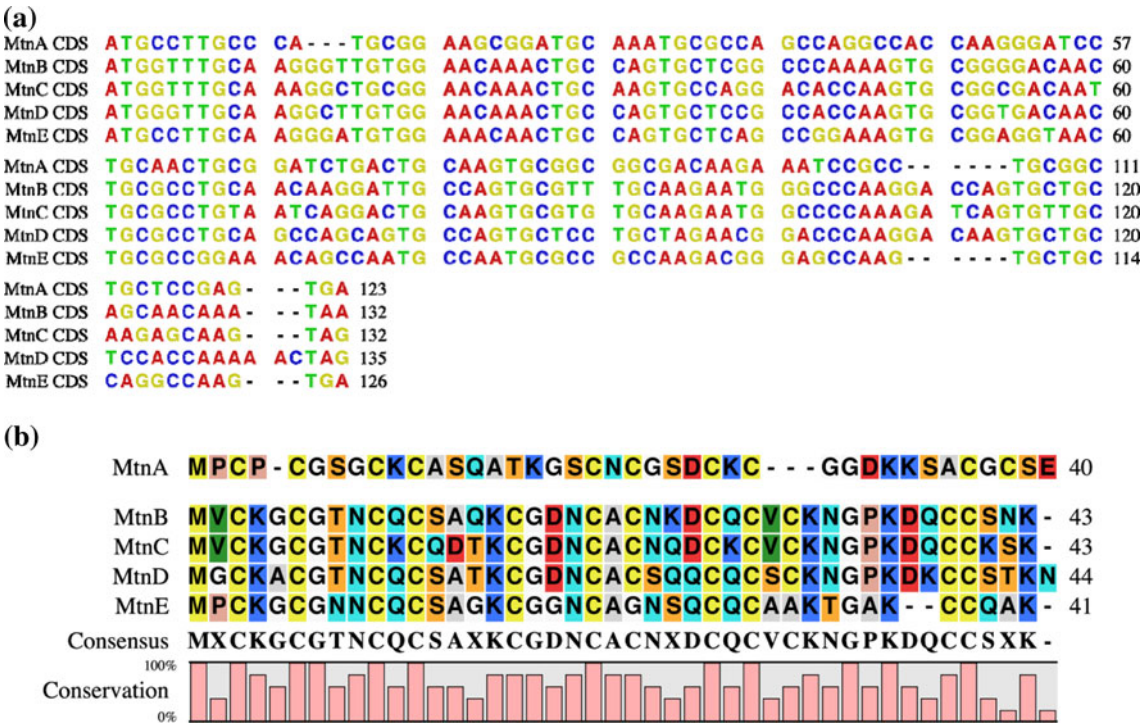

Fig. 1 *Drosophila melanogaster* metallothioneins (*MtnA–MtnE*). **a** Sequence alignment of protein-coding complementary DNA (cDNA) regions of metallothioneins, which are all located on the *D. melanogaster* R arm of chromosome 3. **b** Protein sequence alignment of MtnA-MntE. Note that MtnB, MntC, MntD, and MntE sequences are most closely related to each other

them briefly (approximately 1 min) at 4 °C. The pictures were taken using a Leica DMRB microscope and a Zeiss Axiocam color camera with ×2.5 magnification.

To harvest guts, wandering third instar larvae were dissected in ice-cold phosphate-buffered saline, pH 7.4. Tissues were directly mounted in *Drosophila* Ringer's solution [3 mM $CaCl_2$, 46 mM NaCl, 182 mM KCl, 10 mM tris(hydroxymethyl)aminomethane, pH 7.2] and microphotographed. Fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate filtered epifluorescence pictures from larval guts were taken using a Zeiss Axioplan 2 microscope and a Zeiss Axiocam MRm camera with ×5 magnification.

Plasmid construction and transgenic fly strains

Transgenic fly lines were generated using φC31-mediated transgenesis [44, 45]. In all MT expression experiments the *86Fb* (R arm of chromosome 3) landing site was used for plasmid integration. Flies carrying the transgene integration site also expressed the φC31 integrase, which was removed by crossing out after the integration event had occurred. To generate *MtnD* and *MtnE* transgenes, a DNA segment containing the two transcription units and flanking sequences corresponding to the segment of *Drosophila melanogaster* of coordinates 3R, complement (16360941…16363020), was cloned into a vector containing an *attB* site for integration into the specific genomic locus, and a *miniwhite* marker gene. For the overexpression of *Drosophila* MTF-1, we used the constitutively active *Drosophila* tubulin promoter (*Tub*). The VSV-tagged transgene (*Tub-dMTF1-VSV*) was integrated into the *51D* locus on the second chromosome. The null allele for *dMTF1* is termed *D4*.

For bacterial expression, first the coding region of *MtnE* was obtained using coupled reverse transcriptase PCR technology (QIAGEN). Then, the PCR product was introduced to pET-24a (Novagen), where *MtnE* is expressed under the T7 bacteriophage promoter. Plasmid sequences and detailed cloning strategies are available upon request.

Cadmium toxicity assay in *Escherichia coli*

Escherichia coli BL21 (DE3) bacteria were transformed with the *MtnE*-expression or pET-24a control vector. Liquid cultures (LB medium plus kanamycin) were grown to an optical density at 600 nm (OD_{600}) between 0.4 and 0.5, and expression of *MtnE* was induced by 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h. Cadmium was added to the indicated concentrations and bacteria were grown at 37 °C. After 21 h the OD_{600} was determined.

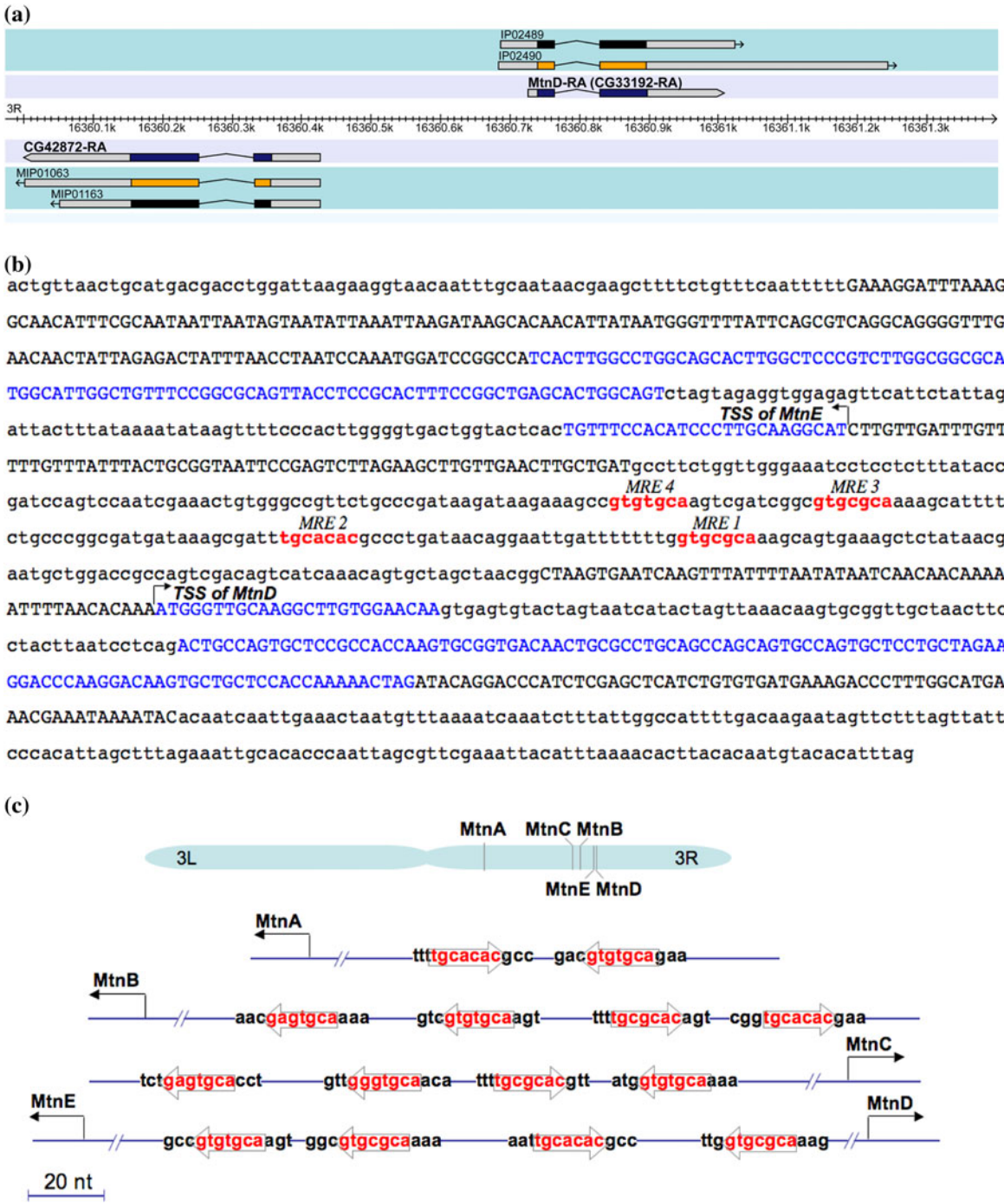

Fig. 2 Metal-response elements (MREs) of *MtnA-MtnE* and genomic organization of *MtnD* and *MtnE*. **a** *MtnD* and *MtnE* genomic region, including messenger RNA untranslated regions (*gray*) and protein coding sequences (*colored*). The CG42872 (*MtnE*) model is based on the cDNA MIP01063. There are alternative 3′ untranslated regions for *MtnD* and *MtnE*. **b** Sequence of the *MtnD* and *MtnE* genomic region, including transcription units and the regulatory regions. Four MREs are located between the transcription start sites (*TSS*). MREs are indicated in *red* and named *MRE1–MRE4*; nucleotides in *capital letters* are sequences found in cDNA isolates, *blue letters* indicate the protein-coding region. **c** Schematic view of all five *Drosophila* metallothionein genes located on the R arm of chromosome 3. For each gene the transcription direction is indicated (*black arrow*). Core MRE nucleotides are depicted in *red*, with an *open arrow* indicating the MRE's orientation. The *scale bar* of 20 nucleotides (*nt*) is valid only for *solid lines* between the MREs, but not for the lettered DNA sequences themselves

Electrophoretic mobility shift assay

Drosophila Schneider S2 cells were transiently transfected with the respective constructs and collected 48 h later. Transfected cells were incubated in medium containing 400 μM $CuSO_4$ prior to harvesting. Electrophoretic mobility shift assays (EMSAs) were performed as described by Radtke et al. [22] and Zhang et al. [27]. Binding reactions were performed by incubating 20 fmol of [γ-^{32}P]ATP end-labeled, 24-bp-long double-stranded DNA oligonucleotides containing the MRE sequences with nuclear extracts prepared according to Schreiber et al. [46]. The following MRE oligonucleotides (core consensus sequences TCGRCNC indicated in capital letters) were used for EMSA:

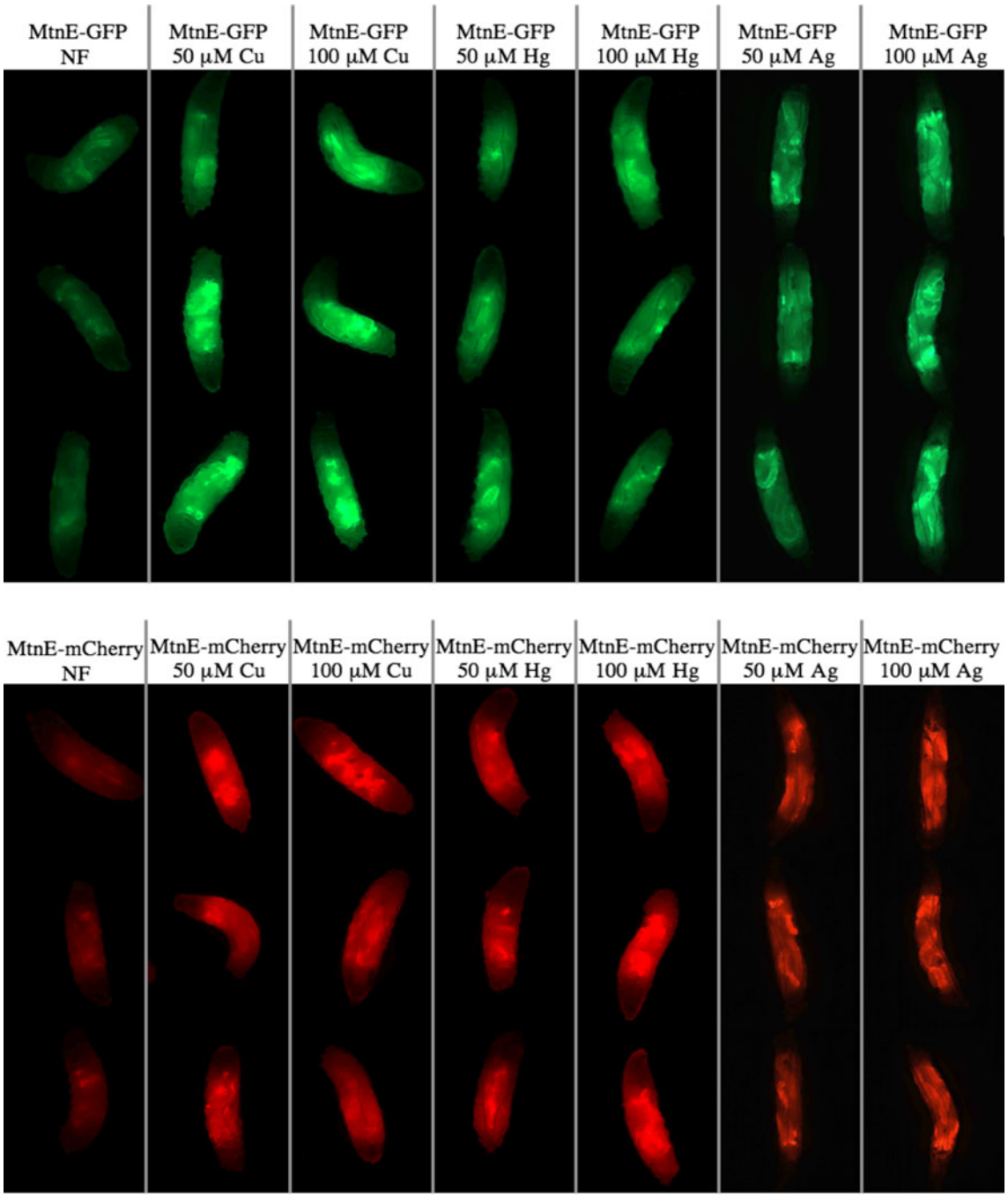

Fig. 3 *MtnE-GFP* and *MtnE-mCherry* transgenes are expressed mostly in the larval digestive tract. Transgenic larvae were raised on normal food (*NF*), or food containing copper, mercury, or silver. Pictures with three representative larvae each were taken with 175- and 100-ms exposure time for mCherry- and green fluorescent protein (GFP)-tagged metallothioneins, respectively. The concentration of metal ions in fly food is indicated at the *top of each panel*

MRE1: 5′-gatttttttgGTGCGCAaagcagt-3′
3′-aaaaaacCACGCGTttcgtcactt-5′
MRE2: 5′-taaagcgattTGCACACgccctga-3′
3′-tcgctaaACGTGTGcgggactatt-5′
MRE3: 5′-gtcgatcggcGTGCGCAaaagcat-3′
3′-ctagccgCACGCGTtttcgtaaaa-5′
MRE4: 5′-taagaaagccGTGTGCAagtcgat-3′
3′-ctttcggCACACGTtcagctagcc-5′
MRE-s: 5′-cgagggagctcTGCACACggcccgaaaagtg-3′
3′-tcgagctccctcgagACGTGTGccgggcttttcac
agct-5′

Results

MtnE transcription is induced by several heavy metals and is dependent on MTF-1

Transgenic larvae carrying a genomic construct encoding a GFP- or mCherry-tagged *MtnE* gene showed stronger fluorescence in the presence of heavy metals, namely, copper, mercury, and silver (Fig. 3). An elevated expression of *MtnE* was also observed when the larvae were raised on food supplemented with 1 mM zinc, whereas iron had at most a marginal effect on *MtnE* expression (data not shown).

The expression of *MtnE*'s partner gene *MtnD* was also metal-responsive, whereby the *MtnE* fusion transgene yielded an even stronger fluorescence than *MtnD* raised both on normal food and on copper-containing food (Fig. 4).

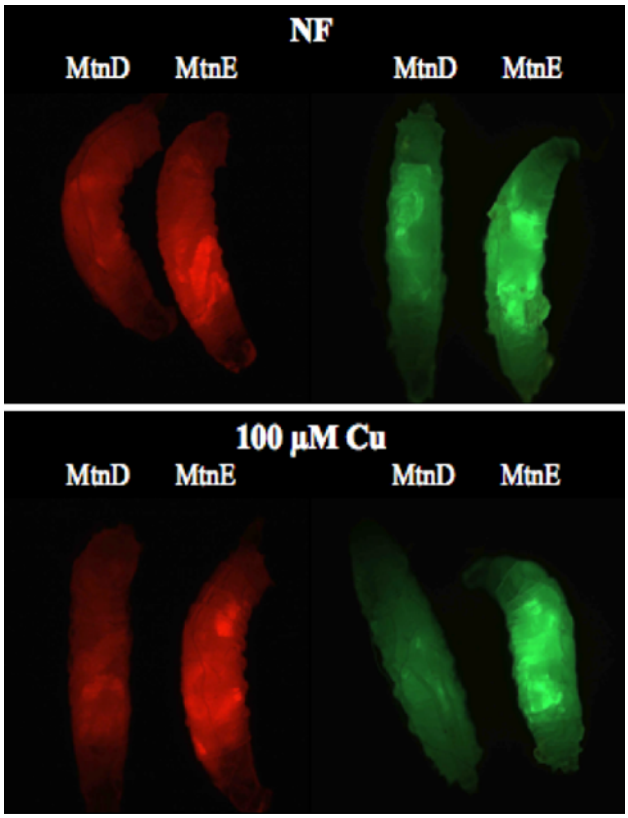

Fig. 4 *MtnE* is more strongly transcribed than *MtnD*. In each picture, the larva on the left has the *MtnD* transgene tagged with mCherry or GFP, whereas the larva on the right carries *MtnE-mCherry* or *MtnE-GFP* transgenes. A longer exposure time (350 ms) was chosen for the larvae grown on NF (*upper panel*) than for the larvae raised on copper-containing food (150 ms) to demonstrate better the relative difference between *MtnD* and *MtnE* expression levels

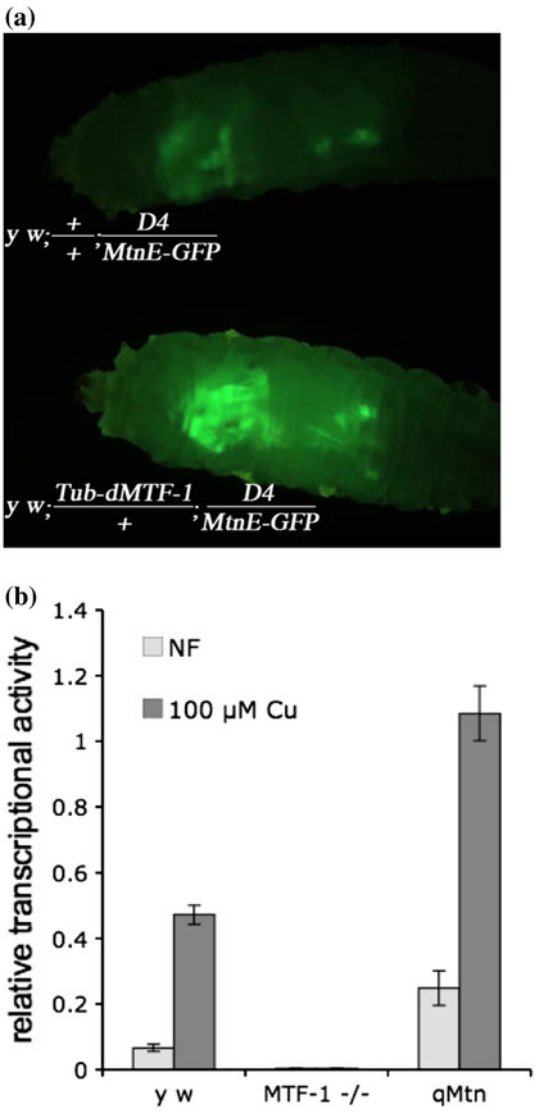

Fig. 5 Expression of *MtnE* is dependent on metal-responsive transcription factor 1 (*MTF1*). **a** Expression of the *MtnE-GFP* transgene is stronger when MTF-1 is overexpressed (on an *MTF1* heterozygous background; *D4* denotes the disrupted *MTF1* locus) in comparison with the control without an *MTF1* transgene. **b** Expression of *MtnE* is completely dependent on MTF-1. *y w*, *MTF1* −/−, or *qMtn* (quadruple knockout of *MtnA–MtnD*) larvae were raised on NF or copper-containing food and transcript levels of endogenous *MtnE* was measured by S1 nuclease protection assay. Signals were normalized to *Actin 5c* levels and *error bars* indicate standard deviations of three experiments

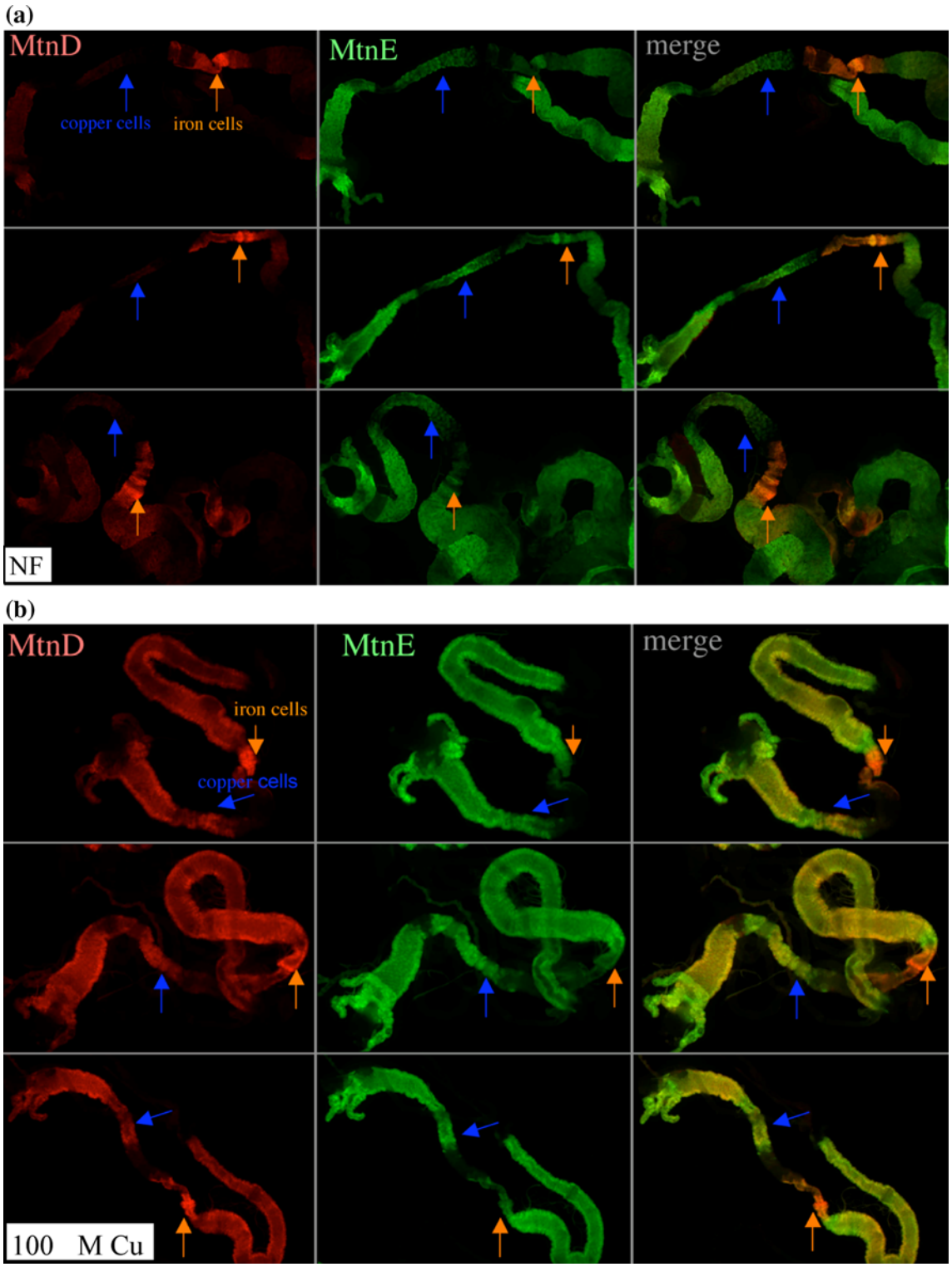

Fig. 6 Expression pattern of metallothionein reporter constructs. Larvae were raised on normal food (**a**) or food containing 100 μM copper (**b**). Fluorescence intensities are only comparable within **a** or **b** and there only within the same (*red* or *green*) fluorescent tag. Copper (cuprophilic) cells are indicated with *blue arrows*; *orange arrows* indicate the region of iron cells/midgut constriction. Three representative gut dissections are shown for each condition

Table 1 Expression profiles of metallothionein genes according to RNA-seq data

Gene	0–2-h embryos	14–16-h embryos	L1 larvae	Pupae	Adult male	Adult female
MtnA	4.3	318.9	1,939.1	166.2	746.1	151.6
MtnB	0.1	0.0	235.3	133.7	2.1	17.9
MtnC	0.0	0.1	13.2	12.9	20.8	10.0
MtnD	0.1	0.2	94.1	15.5	4.7	10.6
MtnE	0.1	0.7	239.8	9.0	4.4	6.0

Overexpression of *MTF1* (in a heterozygous *MTF1* mutant background) greatly enhanced the expression of *MtnE* (Fig. 5a). In a homozygous *MTF1* mutant background, basal and copper-induced expression of *MtnE* is undetectable (Fig. 5b). From these results we conclude that the expression of *MtnE*, like the other members of the *Drosophila* MT family, is regulated by MTF-1. Knockout of the other four members of the MT family enhances basal as well as induced expression of *MtnE*, indicating a compensatory upregulation (Fig. 5b).

Expression patterns of *MtnE* and *MtnD* genes largely overlap

We further investigated the expression of MtnE-GFP and MtnD-mCherry proteins in *Drosophila* larval tissues. Like that of other *Drosophila* MTs, expression of *MtnE* was observed in the intestine of larvae raised on normal food (Fig. 6a) and was boosted by copper-containing food (Figs. 3, 6). The expression patterns of *MtnD* and *MtnE* mostly overlap in the *Drosophila* larval gut, but there are some differences: *MtnE* is more widely expressed than *MtnD* and *MtnB*, the latter of which is strongly expressed in a subregion of the middle midgut that contains "copper cells" [11]. Copper cells are known to accumulate metal ions following copper or cadmium intoxication [11, 47, 48]. As indicated by blue arrows in Fig. 6, copper also boosts the expression of *MtnD* in copper cells, whereas *MtnE* remains less induced. *MtnD* is also constantly expressed in the so-called iron cell region [49], independent of heavy metal load (Fig. 6).

According to RNA-seq data obtained with standard food [43], basal expression of *MtnE* essentially follows the pattern of *MtnB*, *MtnC*, and *MtnD*, whereas expression of *MtnA* in the absence of metals is always higher (Table 1).

MREs shared by *MtnD* and *MtnE* bind MTF-1 in vitro

As mentioned before, there are four MRE sequence motifs in the DNA segment between the divergently oriented *MtnD* and *MtnE* transcription units that are most likely shared functionally by these two genes. We studied the MTF-1 binding properties of single MREs in EMSA to elucidate their potential for metal-induced expression of *MtnD* and *MtnE*. MRE2, MRE3 and MRE4 clearly bind MTF-1, although not as strongly as the synthetic consensus sequence (MRE-s) (Fig. 7).

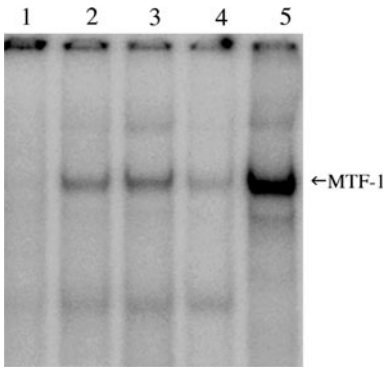

Fig. 7 MTF-1 binds to the MREs of *MtnD*/*MtnE* genes. *Drosophila* S2 cells were transfected with mouse MTF-1 expression plasmids, and 10 μg of nuclear protein extract was used for each bandshift reaction. *Lanes 1–4* bandshift (electrophoretic mobility shift assay) with [γ-^{32}P]-labeled oligonucleotides representing MRE1–MRE4, respectively; *lane 5* bandshift with a strong consensus binding site called MRE-s. For oligonucleotide sequences see "Materials and methods"

Evidence for enhanced cadmium resistance of *MtnE*-expressing bacteria

To test whether MtnE could act as a metal scavenger in a heterologous system, we produced recombinant *E. coli* expressing *MtnE* from an IPTG-inducible T7 bacteriophage promoter. All clones were adjusted to the same optical density, the lac promoter driving MtnE was induced, and bacteria were grown in liquid culture of standard medium containing different concentrations of $CdCl_2$. The next day, OD_{600} was determined for each sample. Upon *MtnE* induction, bacteria displayed a relatively higher cadmium resistance than controls (Fig. 8).

Discussion

The family of *Drosophila* MT genes is completed with a new member, *MtnE*. All five MT genes in *Drosophila* are

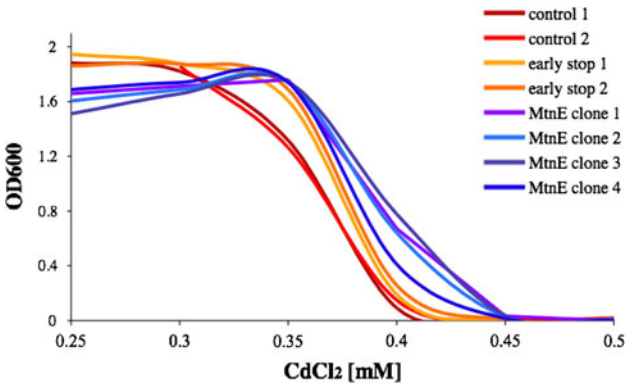

Fig. 8 Survival of recombinant bacteria expressing *Drosophila MtnE*. Separate bacterial colonies were tested for growth in cadmium-containing LB medium. *Control 1* and *control 2* recombinant clones carrying "empty" plasmid vector (pET-24a); *early stop 1* and *early stop 2* clones with a premature stop codon after the 12th amino acid, still including four of ten cysteines and thus expected to produce a mild protective effect; *MtnE clone 1–MtnE clone 4* full-length *MtnE* constructs. Bacteria were grown to the same optical density at 600 nm (OD_{600}) between 0.4 and 0.5; for induction isopropyl β-D-thiogalactopyranoside was added. Two hours later, cadmium was added at the indicated concentration and bacteria were grown for 21 h

located on the same chromosome and they harbor many structural and regulatory similarities. Here we showed that *MtnE*, like the four previously described MT genes, is expressed primarily in the intestine and in response to copper and cadmium. The *MtnE* gene shares a cluster of MREs with the divergently transcribed *MtnD*, and similar to the other MT genes, its expression depends on MTF-1. It was shown before that flies with the quadruple knockout of *MtnA–MtnD* genes are viable and fertile but sensitive to elevated concentrations of copper or cadmium, to a lesser extent to zinc, but not to mercury or silver load or copper depletion. The new member *MtnE* might complement the function of the others by widening the spectrum of metals that can be detoxified. Flies without *MTF1* are much more sensitive to copper load than flies that lack four of its target genes, *MtnA–MtnD*. Our results help to explain this observation, especially since *MtnE* basal and copper-induced expression levels are elevated in the quadruple MT background. This enhanced expression of the last remaining MT gene is most likely a compensatory upregulation.

So far we have tested the resistance to cadmium of recombinant bacteria expressing *Drosophila MtnE* and found them to be more resistant than control bacteria. Interestingly, *MtnE* expressors apparently grew even better in cadmium-loaded medium than in standard medium. The phenomenon where an organism thrives better under a (usually mild) stress condition than in a stress-free standard condition is known as hormesis. The putative hormesis effect observed for MtnE-producing *E. coli* is likely due to the metabolic cost of producing a protein with no function unless the organism is exposed to metal stress, whereupon the protein's presence becomes advantageous.

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