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Genome plasticity in response to stress in *Tetrahymena thermophila*: selective and reversible chromosome amplification and paralogous expansion of metallothionein genes

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Summary

Extreme stress situations can induce genetic variations including genome reorganization. In ciliates like *Tetrahymena thermophila*, the ~45-fold ploidy of the somatic macronucleus may enable adaptive responses that depend on genome plasticity. To identify potential genome-level adaptations related to metal toxicity, we isolated three *Tetrahymena thermophila* strains after an extended adaptation period to extreme metal concentrations (Cd^{2+} , Cu^{2+} or Pb^{2+}). In the Cd-adapted strain, we found a ~5-fold copy number increase of three genes located in the same macronuclear chromosome, including two metallothionein genes, *MTT1* and *MTT3*. The apparent amplification of this macronuclear chromosome was reversible and reproducible, depending on the presence of environmental metal. We also analysed three knockout (KO) and/or knockdown (KD) strains for *MTT1* and/or *MTT5*. In the *MTT5*KD strain, we found at least two new genes arising from paralogous expansion of *MTT1*, which encode truncated variants of *MTT1*. The expansion, can be explained by a model based on somatic recombination between *MTT1* genes on pairs of macronuclear chromosomes. At least two of the new paralogs are transcribed and upregulated in response to Cd^{2+} . Altogether, we have thus identified two distinct mechanisms, both involving genomic plasticity in the polyploid macronucleus, that may represent adaptive responses to metal-related stress.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Availability of data and materials

The sequence data generated are publicly available in the GeneBank database under the accession numbers KY206888 (*MTT1a*) and KY206889 (*MTT1b*).

Conflict of Interest

The authors declare no conflict of interests.

Introduction

Environmental pollutants can exert selective stresses on biological organisms. Persistent stresses can result in directed changes of organisms by micro-evolutionary processes to acquire compensatory protective mechanisms (Busvine, 1954; Kettlewell, 1955; Kültz, 2005). Because such selection can be strong, favourable genetic variants can rise to high frequency within affected populations (Van Straalen *et al.*, 2011). Examples of environmental stressors are extreme temperatures, high salinity, or hypoxia, the presence of antibiotics or other toxic xenobiotics, or the absence of readily metabolizable substrates. Stress-adapted organisms have been isolated from natural environments (Khan *et al.*, 2015), but also generated in the laboratory by culture under particular stress conditions that favour the development of new metabolic abilities or other phenotypes (Sauer, 2001).

Genetic changes in response to stress can occur at many levels. Intense stress can induce “genomic chaos” or genetic instability involving chromosome shattering and reorganization, i.e., karyotypic alterations (Heng, 2007; Liu *et al.*, 2014). Such genomic re-structuring can result in increased transcription of selected genes (Stevens *et al.*, 2014). More generally, organisms that survive prolonged stress conditions acquire altered genomes. Those alterations can include isolated mutations (Voordeckers *et al.*, 2015), epigenetic modifications (Ansell *et al.*, 2015; Meyer, 2015), gene duplication and other gene copy number variation (Hittinger and Carroll, 2007; Adamo *et al.*, 2012a; Tang and Amon, 2013; Hong and Gresham, 2014), increased copies of the nuclear genome (endopolyploidy) (Scholes and Paige, 2015), and aneuploidy or chromosome loss (Pavelka *et al.*, 2010). Many of these changes, and most obviously those resulting in altered gene copy numbers, can affect gene expression patterns and result in an altered cellular stress response (Tang and Amon, 2013; Berman, 2016).

Among environmental stressors, metals are of special relevance as some of the most abundant, toxic and persistent pollutants (Fairbrother *et al.*, 2007). For that reason, the protective strategies acquired by metal-adapted organisms are highly significant. Characterized strategies include constitutive upregulation of genes encoding metal-chelating proteins, like metallothioneins (Timmermans *et al.*, 2005; Costa *et al.*, 2012) or other proteins related to the stress response (Van Straalen *et al.*, 2011; Adamo *et al.*, 2012b; Costa *et al.*, 2012). A second mechanism is the differential amplification of genes that are essential to the metal stress response (Adamo *et al.*, 2012a; Zhang, 2003). For example, in strains of *Saccharomyces cerevisiae* that were selected for resistance to toxic Cu-salts, the *CUP1-1* gene encoding a Cu-metalllothionein was amplified to dozens of copies (Adamo *et al.*, 2012a). Other characterized mechanisms serve to reduce the quantity of metal entering the cell (White and Gadd, 1989) and/or increase the cell's capacity to bind metals extracellularly (biosorption) (Sundar *et al.*, 2011).

Metallothioneins (MTs) constitute a superfamily of cytosolic proteins (Gutiérrez *et al.*, 2011) that can bind metal cations through numerous cysteine (Cys) residues organized in conserved domains (Vasák and Meloni, 2011; Sakulsak, 2012; De Francisco *et al.*, 2016). While the full functional significance of these proteins is still debated, MTs are not essential for most organisms that have been studied (Masters *et al.*, 1994; Hughes and Sturzenbaum,

2007), but may provide advantages under stress situations. For instance, MT knockout (KO) mice are generally healthy and fertile, but are more vulnerable to oxidative stress (Michalska and Choo, 1993; Klaassen and Liu, 1998). Likewise, KO strains for all four *Drosophila melanogaster* MT genes are viable but very sensitive to Cu^{2+} , Cd^{2+} or Zn^{2+} (Egli *et al.*, 2006). The one reported exception to this pattern is the ciliate *Tetrahymena thermophila*, one of whose MT encoding genes (*MTT5*) is essential (De Francisco *et al.*, 2017). *T. thermophila* have five MT genes: three cadmium-MTs (*MTT1*, *MTT3*, *MTT5*) and two copper-MTs (*MTT2*, *MTT4*) (Díaz *et al.*, 2007). The genes are differentially expressed in a coordinated fashion, and the individual proteins may play distinctive functions (De Francisco *et al.*, 2017). Likewise, these genes present different induction patterns under diverse environmental stressors (Gutiérrez *et al.*, 2011; De Francisco *et al.*, 2016; De Francisco *et al.*, 2017). *MTT1* protein has binding preference for Cd^{2+} , after analysing by electrospray mass spectrometry (ESI-MS), circular dichroism (CD), and UV-vis spectrophotometry (Espart *et al.*, 2015) and seems important both for metal homeostasis and also Cd^{2+} and Pb^{2+} detoxification (De Francisco *et al.*, 2017). *MTT3* isoform has an ambiguous binding preference for Cd^{2+} and Cu^{+} (Espart *et al.*, 2015) but may be key for homeostasis of essential metals like Zn^{2+} (Díaz *et al.*, 2007; Gutiérrez *et al.*, 2011). *MTT3* may also provide resistance to diverse stressors (Gutiérrez *et al.*, 2011; Atrian and Capdevila, 2013; De Francisco *et al.*, 2017). The nearly-identical *MTT2* and *MTT4* genes encode CuMTs with highest binding preference for Cu^{+} (Espart *et al.*, 2015). Finally, *MTT5* may function as an “alarm” in response to stress, and up-regulate transcription of other MT genes (De Francisco *et al.*, 2017).

T. thermophila, like other ciliates, are unicellular eukaryotes in which each cell has two functionally distinct nuclei: a germ line diploid micronucleus (Mic) and a somatic polyploid macronucleus (Mac). The two nuclei are related: whenever the cells mate, the Macs in the conjugating pair are degraded, and new Macs are generated from meiotic, cross-fertilized products of the Mics in the paired cells. While the Mic contains 5 pairs of standard chromosomes, these are fragmented and edited during the process of Mac formation. The Mac genome therefore consists of about 187 centromere-less chromosomes (Yao *et al.*, 2014). The plasticity of the Mac genome, in *T. thermophila* and other ciliates, has attracted much recent attention (Betermier and Duharcourt, 2014; Yao *et al.*, 2014; Yerlici and Landweber, 2014). Genome rearrangements, which can be guided by epigenetic modifications and non-coding RNAs (Pilling *et al.*, 2017), have been analysed in the context of ribosomal DNA somatic recombination (Lovlie *et al.*, 1988), Mac development during conjugation (Howard and Blackburn, 1985; Yao *et al.*, 2014), mating type determination (Cervantes *et al.*, 2013), and copy number variation (Xu *et al.*, 2012). In work described in this paper, we extend the analysis of Mac genome plasticity to include environmental stress.

The specific aim of this study was to analyse potential genome-level changes in *T. thermophila* strains that were adapted to growth in high metal-containing medium (De Francisco *et al.*, 2017). We also examined KO or knockdown (KD) strains for *MTT1* and/or *MTT5* genes, since the absence of a specific MT could generate stress due to impaired metal responsiveness and therefore reveal genetic compensation mechanisms (El-Brolosy and Stainier, 2017). Interestingly, we detected amplification of MT genes by two different mechanisms. The first involved a fast and reversible specific copy number increase or

differential amplification of a macronuclear chromosome, while the second involved paralogous expansion. It constitutes the first time that an over-amplification of a specific macronuclear chromosome, induced by an environmental stress, is reported in ciliates. Until what we know, this is also the first time that the creation of new paralog genes is reported as a genetic strategy to compensate the copy number reduction of an essential MT gene.

Results

MTT gene copy number increases in a strain following prolonged cadmium exposure

To see whether acquiring resistance to high metal concentrations involved changes in key metal tolerance genes in *T. thermophila*, we determined the copy number of the five MT genes (*MTT1*, *MTT3*, *MTT5* and the pair *MTT2/4*) in the metal-adapted strains Cd-adap, Cu-adap and Pb-adap. In all these analyses, we included two additional genes. The β -actin gene (*ACT*) served as a loading control. A second gene, *CNBDP* (cyclic nucleotide-binding domain protein, TTHERM_00241620), was chosen because it is located 2.7 Kb upstream of *MTT1* on a macronuclear chromosome (Fig. 1B). Results are shown in Supporting Information Table S1 and Fig. 1A.

As judged by quantitative PCR of genomic DNA, all three wild-type strains (SB1969, CU428 and SB210), as well as GFPM_{TT1}, Cu-adap and Pb-adap contained 1 copy/ μ l of *MTT1*, *MTT3* and *MTT5*, and 2 copies/ μ l for the pair *MTT2/4*. While the genomic DNA includes both the micronuclear and macronuclear content, the much higher ploidy of the Mac (~45C, compared to 2C for the Mic) means that we are in essence measuring gene copy number in the Mac. *MTT2* and *MTT4* are too closely related in sequence to be distinguished as amplicons (De Francisco *et al.*, 2017). In contrast, the Cd-adap strain contained roughly 5 copies/ μ l of *MTT1*, *MTT3* and *CNBDP*.

The increase in the chromosome copy numbers reversible

To ask whether the change in gene copy number were reversible, we analysed MT gene copy number after returning the Cd-adapted strain to normal growth medium (i.e., without cadmium) for 1, 7, or 10 months (called -1M, -7M, and -10M). By 1 month, the copy number of *MTT1*, *MTT3* and *CNBDP* was already decreased ~2-fold (~2.5 copies/ μ l) compared to ~5 copies/ μ l when the same strain was maintained in Cd. After an additional period of maintenance for a total of 7 or 10 months in the absence of Cd, the cells regained the wild-type number of gene copies (1 copy/ μ l) (Fig. 1A and Supporting Information Table S1).

The increase in the chromosome copy numbers depends on the cadmium exposure

To ask whether the copy number increase in *MTT1* and *MTT3* was reproducible and dependent on Cd²⁺ exposure, we returned the -10M sample to cadmium-rich medium for 1 week (-10M + 1week) and again measured gene copy numbers. Notably, the copy numbers for *MTT1*, *MTT3* and *CNBDP* genes increased rapidly to 3 copies/ μ l, while the copy numbers of the other MT genes remained at wild-type levels (Fig. 1A and Supporting Information Table S1).

MT gene knockouts can be balanced by copy number increases

Since MT gene copy number can vary under conditions of metal stress, we asked whether selective amplification of MT genes might also occur in strains lacking the full wild-type complement of the five MT genes in this species. That is, the knockout or knockdown of individual MT genes might represent a stress that could be compensated by amplification at other *MT* loci. This idea assumes that different MT proteins provide at least partially overlapping functions. We therefore determined the MT gene copy number in strains in which the *MTT1* and/or *MTT5* genes were disrupted or knocked down. We also asked whether gene copy number might be adjusted if one of the MT genes were over-expressed by analysing strain GFPMTT1, in which a GFP-tagged copy of *MTT1* is expressed from a multi-copy plasmid. These analyses also include the three wild-type strains described above.

To begin, we measured MT gene copy numbers for the previously-reported KO and/or KD strains (Fig. 2 and Supporting Information Table S1). This confirmed that both MTT1KO and MTT1KO+MTT5KD lack *MTT1*. We also confirmed that MTT5KD and MTT1KO +MTT5KD are knockdowns rather than knockouts for *MTT5*, consistent with our previous conclusion that *MTT5* is an essential gene (Fig. 2) (De Francisco *et al.*, 2017). The *MTT5* copy number was 0.012 copies/ μ L in the MTT5KD and 0.071 copies/ μ L in the MTT1KO +MTT5KD strain (Supporting Information Table S1). Since 1 copy/ μ L corresponds to a macronuclear ploidy of ~45C, 0.012 copies/ μ L corresponds to ~1C and 0.071 copies/ μ L corresponds to ~3C. Maintaining the low copy number of *MTT5* in these strains depended, as expected, on the constant presence of the drug that selects for the gene knockout cassette. That is, when the strains were transferred to medium without selective drug and passaged for just one week, the *MTT5* gene copy number increased rapidly to 0.55–0.61 copies/ μ L (~23–27C) (Fig. 2).

The *MTT1* but not *MTT3* gene shows increased copy number in MTT5KD cells

We found no potential compensatory changes in copy number of any MT gene in the MTT1KO+MTT5KD or GFPMTT1 strains. In contrast, we found that the copy number of *MTT1* increased in the MTT5KD strain, in this case to ~2x (Fig. 2 and Supporting Information Table S1). This was similar, though smaller in magnitude, to results reported above for the Cd-adap strain. However, unlike in Cd-adap, neither the *MTT3* nor *CNBDP* genes in MTT5KD showed any parallel increase in copy number (Fig. 2). This striking difference between the Cd-adap and MTT5KD strains suggested that different mechanisms were responsible for the amplification of *MTT1*.

MTT1 amplification in MTT5KD reflects a paralogous expansion

To examine the additional copies of *MTT1* in the MTT5KD strain, we used PCR to amplify the complete *MTT1* ORF from both genomic and cDNA samples. Compared to wild-type, two additional species were detected in MTT5KD, subsequently called *MTT1a* (321 bp) and *MTT1b* (150 bp) (Supporting Information Figs. S1 and S2). These amplicons were both smaller and less abundant than the original *MTT1* 489 bp ORF (Fig. 3). The abundance of *MTT1a* and *MTT1b* increased notably when cDNA was prepared from Cd-treated MTT5KD cultures (44.5 or 50 μ M, 1h treatment). The *MTT1a* and *MTT1b* bands were gel-extracted, cloned and sequenced. cDNA sequences from both new MT genes with their 3'UTRs and

polyA tails are shown in Supporting Information Fig. S3B. Two additional larger bands were also detected in MTT5KD, particularly in cDNA samples (Fig. 3, lanes 7 and 8), and called *MTT1c* (~650 bp) and *MTT1d* (~800 bp). While the sizes of these products could be inferred based on their migration, the low level of these products precluded our successfully extracting them for further analysis.

Discussion

Metallothioneins (MTs) have historically been discussed as metal-chelating proteins that are involved in metal detoxification (Sakulsak, 2012). More recently, MTs have been recognized as dynamic players in basic cellular processes including regulation of both gene expression and enzymatic activities, signaling, storage of essential metals, and transport. These activities can be accounted for in part by interactions between MTs and Rab3A GTPase (Knipp *et al.*, 2005), LPR receptors (Ambjørn *et al.*, 2008), bovine serum albumin (Quiming *et al.*, 2005) and p53 and NF- κ B transcription factors (Ostrakhovitch *et al.*, 2006).

Reversible and specific MT gene copy number increase as a genetic strategy in *T. thermophila* Cd-adapted strain

Variations in the copy number of specific genes, or the absence of whole chromosomes (aneuploidy) or subchromosomal regions (partial aneuploidy) can have profound impact on the physiology of an organism (Tang and Amon, 2013). Many studies in bacteria, yeasts and mammals have reported that variation in gene copy number can be selected under environmental stress situations, and may result in cells with increased fitness under specific conditions (Berman, 2016; Mishra and Whetstone, 2016). For instance, amplification of a metallothionein locus has been detected in a cyanobacterium Cd-tolerant strain (Gupta *et al.*, 1992). Also, gene amplification is involved in the development of antibiotic resistance in *Salmonella enterica* (Sun *et al.*, 2009). Gene copy number variation has been also associated with antifungal drug resistance (Ford *et al.*, 2015). In *Candida albicans*, a segmental aneuploidy confers resistance to antifungal drugs by amplifying the *ERG11* gene (encoding the drug target) and *TAC1* (encoding a transcriptional activator of the ABC transporter efflux pumps) (Selmecki *et al.*, 2008). Such mechanisms involving genome plasticity can therefore underlie adaptations to environmental stressors by changing the expression of critical response genes. Likewise, genome plasticity and copy number variation can also play an adaptive role in non-stress conditions. For example, the human salivary amylase gene is increased in populations that have a higher starch content in their diets (Perry *et al.*, 2007).

Ciliates represent an interesting case for gene copy number variation, since the somatic macronucleus already contains multiple copies of each gene. Moreover, in *T. thermophila* the macronuclear chromosomes are partitioned amitotically at each vegetative cell division, so the daughters will, in general, inherit unequal copy numbers of each chromosome and its resident genes. Thus, stochastic copy number variation is inherent in this organism, though balanced by mechanisms for restoring 45C macronuclear content in each cell cycle (Brunk and Navas, 1992).

In this paper, we report a remarkable ~5-fold increase of the copy number of three genes (*MTT1*, *MTT3* and *CNBDP*) in a *T. thermophila* strain that was adapted to high Cd²⁺

concentrations. These three genes are closely spaced on a single macronuclear chromosome. *MTT1* is separated from *MTT3* and CNBDP by 1.7 Kb and 2.7 Kb, respectively (Fig. 1B). This chromosome is just one of the ~187 in the macronucleus, ranging in size from 100 to over 1,500 Kbp (Yao *et al.*, 2014). The simplest hypothesis to explain the parallel amplification of *MTT1*, *MTT3* and CNBDP in the Cd-adap strain, is that the entire chromosome is present at ~5x the wild-type complement, or ~225 copies.

The amplification of *MTT1* in the Cd-adap strain must contribute to the high basal *MTT1* expression in this strain, ~28x that in wild-type cells (De Francisco *et al.*, 2017). Interestingly, we saw no similarly high basal expression of *MTT3* in Cd-adap (De Francisco *et al.*, 2017), even though the *MTT3* gene is also highly amplified as shown above. The relative transcript abundance for *MTT1/MTT3* is ~30 in Cd-adap, compared to ~1.6 in control strains (De Francisco *et al.*, 2017). Taken together, our results indicate that *MTT3* basal expression is relatively insensitive to gene copy number under the experimental conditions sampled. This may be due to a negative regulatory mechanism similar to gene dosage-compensation, as described in sex chromosomes of *Drosophila*, *Caenorhabditis* and mammals (Nguyen and Disteché, 2006; Stenberg and Larsson, 2011; Farslow *et al.*, 2015).

Our data suggest that rapid changes in macronuclear gene copy number represent a mechanism of adaptation in *T. thermophila*. Under the conditions tested, *Tetrahymena* progressively increases the copy number of genes encoding metal-chelating proteins, while copy number reverts to wild-type values after Cd²⁺ is removed from the medium (Fig. 4). The selective amplification of *MTT1* and *MTT3*, rather than the other MTT genes, may suggest that *MTT1* and/or *MTT3* is unique in the ability to confer resistance to elevated Cd. Indeed, the *MTT1* product has the highest binding preference for Cd²⁺ among *T. thermophila* metallothioneins (Espart *et al.*, 2015). Alternatively, or in addition, individual macronuclear chromosomes may differ in their ability to escape the copy number control that maintains the 45-fold ploidy in wild-type cells, and the chromosome bearing the *MTT1* and *MTT3* genes may be more prone to amplification. Interestingly, we saw no indication that this chromosome was amplified in the Cu-adap nor in Pb-adap strains, where adaptation seems to depend on transcriptional up-regulation of *MTT2/4* or *MTT5* (De Francisco *et al.*, 2017).

The fact that *Tetrahymena* could survive exposure to the Cd-MTC (Cd-maximum tolerated concentration), after being maintained for months without metal exposure, could be due to genetic and/or epigenetic mechanisms. The phenomenon itself recalls experiments in which Cu-tolerant yeasts could proliferate under copper stress after 10 cycles of growth/re-inoculation in a medium without copper (Adamo *et al.*, 2012a). The reversibility of these changes in *Tetrahymena* are reminiscent of results in other species, where chromosomal rearrangements have been shown to be unstable when no selective advantages are present (Chang *et al.*, 2013). Likewise, results suggest that a specific macronuclear chromosome can be amplified dramatically, but the underlying mechanism is unknown. Preferential epigenetic recognition by DNA polymerase under particular stress conditions is one possibility.

Several epigenetic mechanisms related to metal toxicity at the level of both DNA methylation and histone modification have been reported (Fragou *et al.*, 2011). Maintenance

of different chromosome copy numbers, in a chromosome-specific fashion, may contribute to baseline transcriptional regulation in some ciliates. In the spirotrich ciliate *Oxytricha trifallax*, copy number differences between 11 macronuclear chromosomes showed a possible correlation with gene expression levels (Xu *et al.*, 2012). Likewise, in *Euplotes raikovi* a correlation between copy number and gene expression for three pheromone genes was reported (La Terza *et al.*, 1995).

Interestingly, recent studies have suggested that parental RNA molecules regulate macronuclear copy number during ciliate conjugation when new Mac are generated (Yao *et al.*, 2014; Yerlici and Landweber, 2014), but the mechanisms are not known. Under conditions of metal stress, a high level of *MTT1* transcript is maintained in *T. thermophila* Cd-adap. High levels of *MTT1* transcript could promote modifications that confer an endoreplication advantage on the corresponding template DNA, an idea similar to that proposed by Heyse *et al.* (2010).

Metallothionein knockout and/or knockdown strains: creation of new *MTT1* paralogs in response to the copy number reduction of an essential MT gene

MTT1 is non-essential, so *MTT1*KO is a stable strain. Although *MTT5* is an essential gene, *MTT5*KD and *MTT1*KO+*MTT5*KD strains with dramatically reduced *MTT5* copy numbers in the macronucleus can be generated by maintaining cells in selective drugs that favor the gene disruption alleles. However, this situation is unstable, and the wild-type *MTT5* gene copy number is restored after removing the drug selective pressure for just one week.

We detected an increase in the *MTT1* copy number (~2.5-fold) in the *MTT5*KD strain compared to the SB1969 control. This was not due to amplification of a macronuclear chromosome, since genes adjoining *MTT1* showed no increase in copy number. Instead, we observed that standard PCR amplification of *MTT1* in genomic DNA produced four species not seen in wild-type cells. This suggested that *MTT1* had undergone one or more duplications in *MTT5*KD. Sequence analysis of the two smaller species clarified their relationship with *MTT1*. *MTT1a* (321 bp) and *MTT1b* (150 bp) are identical in sequence to *MTT1* (Supporting Information in Figs. 1S and 3SA) but represent 168 bp and 339 bp 3' truncations, respectively. The simplest explanation of these bands is that they represent new paralogous copies of *MTT1* in the *MTT5*KD strain. Since they are most abundant in cDNA (Fig. 3) they appear to be expressed, and likely to be under the control of Cd-responsive elements. While it was not possible to clone and analyze the larger species, their sizes (~650 and ~800 bp) suggest they arose from the same process that generated *MTT1a* and *MTT1b*, respectively. We propose a model to explain how four novel *MTT1* paralogs with the observed sizes were generated via homologous recombination between staggered repeated sequences present in two copies of *MTT1* (Fig. 5). This model would predict sizes for *MTT1c* and *d* of 657 and 828bp, respectively.

Homologous recombination at staggered sites may have played an important role in the evolution of metallothioneins in *Tetrahymena*. Several amino acid motifs in CdMTs show periodicity indicating hierarchical modular organization of these proteins, which is conserved between diverse *Tetrahymena* species (Gutiérrez *et al.*, 2009; Gutiérrez *et al.*, 2011). In general, each *Tetrahymena* CdMT is constituted by 2–3 modules, each formed by

type-1 or -2 submodules (Díaz *et al.*, 2007; De Francisco *et al.*, 2016). In the model, a series of homologous recombination events between *MTT1* or *MTT1*-derived genes on different macronuclear chromosomes is facilitated by the repeated presence of specific sequences in *MTT1*. The *MTT1* ORF contains two pairs of identical 12 bp sequences (Supporting Information in Fig. S2). 5'GGATGTTGCTGTC3' (denoted as SSR1 and located at the beginning of the second type-1 submodule) is found 67 and 406 bases from the start codon, and 5'GGATGCAAATGT3' (denoted as SSR2 and located in the middle of the type-2 submodule), is found 142 and 310 b from the start codon. SSR1 and 2 encode two cysteine-rich motifs (Gly-Cys-Cys-Cys and Gly-Cys-Lys-Cys, respectively, which are conserved among known *Tetrahymena* Cd-metallothioneins (De Francisco *et al.*, 2016). Importantly, recombination between pairs of these sequences can account for the novel *MTT1* paralogs found in the MTT5KD strain, including their sizes, as shown in Fig. 5. Interestingly, *MTT1* also contains a fifth sequence almost identical to SSR1, beginning at position 235, but this sequence does not appear to be a site of recombination. Similarly, we found no evidence for recombination in *MTT3*, though this gene also has two SSR2 and one SSR1 motif.

Recombination events similar to those proposed in Fig. 5 may be responsible for the duplication of submodular motifs in CdMTs, as we previously proposed in an evolutionary model of *Tetrahymena* CdMTs (Gutiérrez *et al.*, 2009). In fact, the sequences of *MTT1c* and *MTT1d* include duplications (Supporting Information in Fig. S2) consisting of two complete SSR1 submodules and half of SSR2 (*MTT1c*) or two complete SSR1 and one complete SSR2 (*MTT1d*). As a result, these paralogs encode increased numbers of Cys residues (Supporting Information in Table S2). Similarly, recombination between copies of the *MTT4* gene in *Tetrahymena malaccensis* could explain the origin of *MTT3* in that species (De Francisco *et al.*, 2016). Thus, our current results suggest that mechanisms of genome plasticity which have been important for diversifying MT genes in *Tetrahymena*, can also contribute to a short-term adaptive stress response. Consistent with this idea, our data indicate that the novel *MTT1* paralogs in MTT5KD are expressed as mRNAs, since they can be amplified from cDNA isolated from Cd-treated cells (Supporting Information in Fig. 3SB).

We have not analyzed protein products of the novel *MTT1* paralogs, but we speculate that these species help to compensate for the drastic reduction in copy number of the essential *MTT5* gene. Genetic compensation is defined as changes in RNA or protein levels that can functionally compensate the loss of another gene function (El-Brolosy and Stainier, 2017). In yeast, strains disrupted in non-essential genes can accumulate compensatory genome alterations including polyploidy, aneuploidy and point mutations, which can help to maintain cellular fitness (Teng *et al.*, 2013; Liu *et al.*, 2015). Likewise, inactivation of essential genes in yeast and bacteria can be balanced by compensatory evolutionary processes (Bergmiller *et al.*, 2012). A revised concept of gene essentiality has recently been proposed (Liu *et al.*, 2015), which takes into account not only cell viability but also “cell evolvability”, i.e., the capacity to adapt to environmental or genetic stresses. In particular, the authors identified a class of essential genes, called “evolvable”, whose loss or reduction could be compensated by changes like adaptive mutations (Liu *et al.*, 2015). We propose that in *Tetrahymena* the accumulation of novel *MTT1* paralogs in MTT5KD represents a mechanism that compensates for the almost complete absence of the essential *MTT5* product.

The main conclusions that we infer from the results are: In *Tetrahymena thermophila*, at least two different mechanisms underlie macronuclear genome plasticity in response to direct or indirect metal stress. Cd-adaptation involves an apparent copy number increase of the macronuclear chromosome containing *MTT1* and *MTT3* genes. This differential amplification process is relatively fast and reversible. As a result, there is increased expression of *MTT1*, a gene that plays a key role in cadmium tolerance. The absence of increased expression of *MTT3*, notwithstanding the copy number increase, may indicate negative regulation by a mechanism similar to gene-dosage compensation. The copy number of *MTT5*, though an essential gene, can be dramatically reduced in the Mac. Such *MTT5* knockdown cells accumulate new paralogs of *MTT1*. The paralogs appear to arise by homologous recombination between two copies of wild-type *MTT1*, based on repeated motifs within the gene. The new genes are transcriptionally active and expressed in response to Cd, and may thus reduce the stress associated with depletion of *MTT5*.

Experimental procedures

Strains and culture conditions

The *Tetrahymena thermophila* strains used were CU428 (*mpr1-1/mpr1-1*; pm-S, mp-S, mtVII), SB210 (*gal1-1/gal1-1*; gal-S, mt VI) and SB1969 (*chx1-1/chx1-1*, *mpr1-1/mpr1-1*; pm-S, cy-S, mtII). The last two were kindly supplied by Dr. E. Orias (University of California, Santa Barbara, USA). CU428 was used to obtain KO and KD strains (MTT1KO, MTT5KD and MTT1KO+MTT5KD), as has been described in de Francisco *et al.*, (2017). GFPMTT1 bears a multi-copy plasmid encoding the *MTT1* gene (Amaro *et al.*, 2014). The three metal-adapted strains (Cd-adap, Cu-adap and Pb-adap) were obtained after extended culture in metal-containing media (De Francisco *et al.*, 2017).

Tetrahymena strains were axenically grown in PP210 medium [2% w/v aqueous solution of proteose peptone (Pronadisa), supplemented with 10 μ M FeCl₃ and 250 μ g/ml of streptomycin sulphate (Calbiochem) and penicillin G (Sigma)] at 30°C. To prevent loss of multi-copy plasmids, the GFPMTT1 strain was maintained in 12 μ g/ml of paromomycin sulphate (Sigma). Similarly, MTT5KD and MTT1KO+MTT5KD strains were maintained in 800 μ g/ml of paromomycin or 60 μ g/ml of cycloheximide, respectively (De Francisco *et al.*, 2017).

For some experiments, metal-adapted strains were grown in PP210 medium to which was added the maximum tolerated concentrations (MTC) of their respective metals: 115 μ M Cd²⁺ (CdCl₂), 4 mM Cu²⁺ (CuSO₄·5 H₂O) and 5.5 mM Pb²⁺ (Pb(NO₃)₂) (Sigma), (De Francisco *et al.*, 2017). To study the reversibility of the metal adaptation process, the Cd-adap strain was maintained for 1, 7 or 10 months in PP210 without cadmium (-1M, -7M and -10M samples). After 10 months, cells were re-exposed to 115 μ M Cd²⁺ for one week (-10M + 1 week).

Total DNA or RNA isolations and cDNA synthesis

Exponential cell cultures (1-3 $\times 10^5$ cells/ml) were harvested by centrifugation at 2,800 rpm for 3 min. Total DNA was isolated using the protocol described in Martín-Platero *et al.*,

(2007) and samples were treated with 10 mg/ml RNase A (Thermo- Scientific) for 2h at 37°C. Total RNA samples were isolated by using the TRIzol Reagent method (Invitrogen). RNA samples were treated with DNase I (Roche) for 30 min at 37°C. Both, DNA and RNA integrities were tested by agarose gel electrophoresis. DNA or RNA sample concentrations were calculated by the NanoDrop 1000 (Thermo Scientific). MultiScribe Reverse Transcriptase 50 units/μl (Life Technologies) and oligo(dT)-adaptor primer (Roche) were used to synthesize cDNAs from 3.5 μg of total RNA.

Obtaining complete cDNAs of two new *MTT1* paralogs

Complete ORF sequences of two new *MTT1* paralogs were isolated from the MTT5KD strain by standard PCR using primers in Supporting Information Table S3. The 3' UTR regions of the cDNAs of these new *MTT1* genes were completed with the 3' RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen), using the adaptor primer and AUAP primers (supplied by the kit) and the *MTT1A-ORF* primer (Supporting Information Table S3). The bands corresponding to the new *MTT1* copies were extracted from agarose gel (QIAquick Gel Extraction Kit, QIAGEN) and re-amplified using the *MTT1A-ORF/MTT1B-ORF* primer set for cloning and sequencing.

Standard PCR reactions, cloning and DNA sequencing

Standard PCR was carried out using the AmpliTaq Gold DNA Polymerase (1.25 U/reaction) of the AmpliTaq Gold PCR Master Mix kit (Applied Biosystems). All primer sequences are in Supporting Information Table S3. The following PCR programme was applied: 7 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 50 ± 3°C and 2 min at 72 °C, and 5 min at 72°C. PCR products were analysed by 1.5% agarose gel electrophoresis in TAE 1x buffer (40 mM Tris, 1 mM EDTA and 5.7% glacial acetic acid) and stained with GelRed (Biotium) (3x in water). Amplicons were cloned using the TOPO TA Cloning Kit and the pCR2.1 vector (Invitrogen).

Quantitative real-time PCR (qPCR)

Genomic DNA samples were adjusted to 1 μg/ml and amplified in duplicate in 96 well plates. Each qPCR reaction (20 μl) contained: 10 μl SBYR Green (Takara), 0.4 μl ROX (Takara), 1 μl each primer (at 400 nM final concentration), 3.6 μl ultrapure sterile water (Roche) and 4 μl of a 10⁻¹ dilution of the genomic DNA. PCR primers (Supporting Information Table S3) were designed using the “Primer Quest and Probe Design” online-application of IDT (Integrated DNA Technologies). Primer specificity was tested, melting curves were obtained and each PCR product was confirmed by gel electrophoresis and sequencing. Real-time PCR reactions were carried out in an iQ5 real-time PCR (Bio-Rad) with the following protocol: 5 min at 95°C, 40 cycles (30 sec at 95°C, 30 sec at 55 °C and 20 sec at 72°C), 1 min at 95°C and 1 min at 55°C. *β-actin* and *CNBDP* [cyclic nucleotide-binding domain protein (TTHERM_00241620)] genes were used as controls. Amplification efficiency (E) was measured by using 10-fold serial dilutions of a positive control PCR template. The efficiency requirement was met for all tested genes in all strains (Supporting Information Table S4). The mean of the number of copies/μl of each gene in each strain was calculated using the following equation: $C_t = \text{slope} \times \log(\text{number of copies}/\mu\text{l}) + y -$

intercept (Gong *et al.*, 2013). Results reflect at least two independent experiments, each performed in duplicate.

In silico analysis of nucleotide and protein sequences and phylogenetic tree construction

The new *MTT1* gene sequences were analysed with the BLAST program (NCBI). Multiple sequence alignments were performed using T-Coffee online application, which uses the Clustal Wallis method (Notredame *et al.*, 2000). The phylogenetic tree was built using the Maximum Likelihood algorithm (MEGA 5.05, using the Poisson model and Bootstrap method with 2000 repetitions).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance Statement

For the first time in ciliates, we report that the response to Cd^{2+} extreme stress involves reversible copy number increase of a specific macronuclear chromosome. This is likely to play an adaptive function. This copy number increase results in over-expression of a specific gene but not others on the same macronuclear chromosome. Besides, for the first time, we report that new metallothionein paralog genes are created by site-specific homologous recombination, and are expressed under metal stress. Genome plasticity appears to function as a compensatory mechanism for the knockdown of an essential metallothionein gene.

Fig. 1.

MT gene copy number in controls and metal-adapted strains. **(A)**: Analysis of gene copy number of *MTT1*, *MTT3*, *MTT5* and the pair *MTT2/4* and two non-MT genes (*β-actin* and *CNBDP*) from *T. thermophila* strains. Each histogram bar represents an average value $\pm$ standard deviation (see Supporting Information Table S1) calculated from two or three independent experiments. Asterisks indicate statistically significant ($p < 0.01$) differences from the control. The dashed line indicates the basal copy number. **(B)**: Schematic representation of micronuclear loci of *CNBDP*, *MTT1* and *MTT3*, and extra copies of the Mac chromosome containing these genes in the Cd-adap strain (see the text for explanation).

Fig. 2.

MT gene copy number in knockout and/or knockdown strains. Analysis of gene copy number of *MTT1*, *MTT3*, *MTT5* and the pair *MTT2/4*) and two non-MT genes (*β-actin* and *CNBDP*) from knockout and/or knockdown *T. thermophila* strains. Each histogram bar represents an average value $\pm$ standard deviation (see Supporting Information Table S1) calculated from two or three independent experiments. Asterisks indicate statistically significant ($p < 0.01$) differences from the control. The dashed line indicates the basal gene copy number.

Fig. 3.

New *MTT*/amplicons from the MTT5KD strain, detected by agarose gel electrophoresis. Lanes 1, 4 and 9: DNA ladder. Lanes 2 and 3: amplicons of the *MTT* ORF, obtained from genomic DNA of MTT5KD. Arrows indicate bands corresponding to wild-type *MTT* (489 bp) as well as two additional species called *MTT1a* (321 bp) and *MTT1b* (150 bp). Lanes 5 and 6: amplicon of the *MTT* ORF, obtained from genomic DNA of control strain SB1969. Only the wild-type species is present. Lanes 7 and 8: amplicons of the *MTT* ORF from cDNA. mRNA was isolated from MTT5KD after 1h treatments with 44.5 (lane 7) or 50 μ M (lane 8) Cd^{2+} . Arrows indicate bands corresponding to the original *MTT* and four additional species. These are *MTT1a* and *MTT1b*, and the larger *MTT1c* (657 bp) and *MTT1d* (828 bp). See also in Supporting Information Fig. S2 and Table S2.

Fig. 4.

Schematic representation of copy number variation in Cd-adap strain. Reversible and specific MT gene copy number increase in Cd-adap strain as a response to the presence or absence of Cd^{2+} . The estimated ploidy (value C) is indicated.

Fig. 5. Recombination events originating new *MTT1* paralogs in MTT5KD strain. **(A)** Novel paralogs *MTT1a* and *MTT1c* production. **(B)** Novel paralogs *MTT1b* and *MTT1d* production. Recombination is proposed to be directed by two different repeated 12 bp motifs, indicated in black or white boxes. See also in Supporting Information Fig.S2.