# Metallomics

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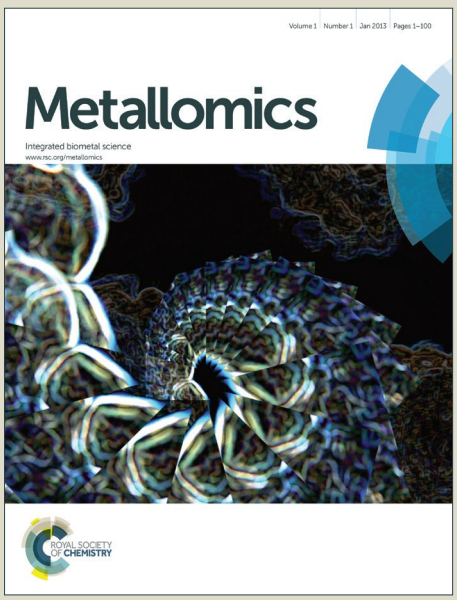





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ARTICLE

# Zinc sensing by metal-responsive transcription factor 1 (MTF1) controls metallothionein and ZnT1 expression to buffer the sensitivity of the transcriptome response to zinc.

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Only a small number of genes are known direct targets of the zinc-responsive transcription factor MTF1; therefore, the aim of this study was to gain a more complete understanding of the MTF-1 regulated zinc-responsive component of the transcriptome. A targeted siRNA was used to deplete MTF1 expression in the human intestinal cell line Caco-2. We predicted that the response to zinc of direct MTF1 target genes would be abrogated by MTF1 knockdown. Surprisingly, a greater number of genes were regulated by zinc following MFT1 knockdown, and most genes that responded to zinc under both control and MTF1-depleted conditions had an augmented response in the latter condition. Exceptions were the zinc effluxer ZnT1 and a suite of metallothionein genes, suggesting that responses of other genes to zinc are usually buffered by increases in these proteins. We propose that MTF1 heads a hierarchy of zinc sensors, and through controlling the expression of a raft of metallothioneins and other key proteins involved in controlling intracellular zinc levels (e.g. ZnT1) alters zinc buffering capacity and total cellular zinc content. We tested and validated this model by overexpressing metallothionein and observing the predicted curtailment in response of the zinc-repressed SLC30A5 (ZnT5) promoter. The model provides the framework for an integrated understanding of cellular zinc homeostasis. Because MTs can bind metals other than zinc, this framework links with overall cellular metal homeostasis.

## Introduction

In environments where availability fluctuates, metal sensing is essential for organisms to fulfil their requirement for these species, which can be toxic in excess and are required for a plethora of cellular functions. Moreover, the sensing of different metals must be exquisitely orchestrated to balance and coordinate the cellular response such that the availabilities of different metals, which can compete for occupancy of metal binding sites in proteins, are controlled appropriately. Although numerous metal-responsive proteins are known, most do not fulfil the requirements of a metal sensor; rather, they respond downstream to the initial metal sensing event. A metal sensor should head a hierarchy of metal-responsive events that ramify throughout the cell. Factors with this role have been uncovered in unicellular organisms, specifically bacteria [1] and yeast [2], but have been identified with less clarity in metazoans [3, 4]. Metal-responsive transcription factor 1 (MTF1) senses zinc through stabilisation of two of its six zinc fingers [5] and thus binds to the metal response element (MRE; sequence TGCRCNC) in target genes. However, despite its discovery two decades ago, only a very limited number of genes, including *SLC30A1*, which encodes the zinc efflux protein ZnT1, and the metallothionein (MT) family of small cysteine-rich cytosolic zinc-binding proteins, have been found to be directly regulated in response to zinc by MTF1 [6]. Therefore, the aim of this study was to generate a more complete picture of the MTF1-regulated transcriptome.

The response of the human transcriptome to zinc was examined in the human intestinal cell line Caco-2 under control conditions and following siRNA-mediated knockdown of MTF1. We hypothesized that genes regulated by MTF1 in response to zinc would become refractory to changes in zinc availability following knockdown of MTF1. However, knockdown of MTF1 abolished the zinc-induced regulation of only one quarter of zinc-responsive genes in Caco-2 cells. Furthermore, a number of additional genes became responsive to changes in zinc availability following depletion of MTF1. With the exception of a raft of metallothionein genes and *SLC30A1*, the zinc-induced fold changes in the expression levels of zinc-responsive genes were generally increased following MTF1 depletion. These transcriptomic data and other findings are consistent with a model in which zinc sensing by MTF1 heads a hierarchy of gene responses to zinc. The fact that MTs have the capacity to bind metals other than

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zinc, including copper, suggests that MTF1 may play a key role in orchestrating the cellular response to net metal balance.

## Experimental

### Culture and transfection of Caco-2 cells

Caco-2 cells were cultured under standard conditions as described previously [7].

For knockdown of MTF1, cells were seeded into 6-well plates at a density of 3.5 x $10^5$ cells/well and then transfected with one of two different siRNAs: sequence $_{1070}$UGAAAGGUCAUGAUAACAA$_{1088}$ (used in cells subsequently analysed by hybridisation of RNA to a DNA microarray; ON-TARGETplus, Thermo Scientific) or sequence $_{1061}$AAAGUCACAUGAAAGGUCAUGAUAA$_{1085}$ (used in cells subsequently analysed by RT-qPCR; Stealth, Thermo Scientific) (both sequences numbered according to the sequence deposited under Genbank accession number NM_005955.2). Comparative negative control cells were transfected with a negative control siRNA (ON-TARGETplus Non-targeting siRNA #1; Thermo Scientific).Transfection with siRNA was achieved using Lipofectamine RNAiMAX (Invitrogen), following the manufacturer's instructions and using 4 µl and 100 pmol siRNA per well. Cells were exposed to zinc, which was added at 3 µM or 150 µM to serum-free culture medium, at 24 h post-transfection and RNA was prepared after a further 24 h.

For stable expression of MT2A, a plasmid construct was generated by RT-PCR amplification of a 197 bp product that included the full MT2A coding sequence. Reverse transcription was performed using Moloney Murine Leukaemia Virus Reverse Transcriptase (Promega). PCR was performed using Expand High Fidelity polymerase (Roche) with the following primers: $_{41}$CGCCTCTTCAGCACGCCATG GATCCCAAC$_{69}$ and $_{245}$CATCAGGCGCAGCAGCTGCAC$_{225}$ (numbered according to the sequence deposited under GenBank accession number NM_005953) (Eurofins MWG Operon). The thermal cycling parameters were as follows: 94°C for 2 min; followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s; and then a final extension step of 72°C for 5 min. The 197 bp product was subcloned into the mammalian expression vector pcDNA3.1/V5-His-TOPO (Invitrogen). Sense orientation of the insert and fidelity of the PCR product were confirmed by DNA sequencing.

To generate Caco-2 cells overexpressing MT2A stably an endotoxin-free preparation of the plasmid (prepared using the Qiagen EndoFree Plasmid Maxi kit, according to the manufacturer's instructions) was used withGeneJammer reagent (Stratagene), according to the manufacturer's instructions. A stable pool of transfectants was generated by growth of the cells in 400 µg/ml G418 (determined empirically to be the optimum concentration to kill non-transfected Caco-2 cells) for three weeks.

Transfection of Caco-2 cells overexpressing MT2A with an *SLC30A5* promoter-reporter construct (pBlueSLC30A5prom) and subsequent treatment with zinc was performed as described previously [8].

### Preparation and analysis of RNA from Caco-2 cells

RNA was prepared from Caco-2 cells using Trizol reagent (Invitrogen), following the manufacturer's instructions, and then its integrity was confirmed (RIN = 10) using an Agilent 2100 Bioanalyzer.

For analysis by RT-qPCR, RNA was treated with DNAase (Roche) and then first-strand cDNA synthesis was performed using Superscript III RNase H- Reverse Transcriptase (Invitrogen) and random hexamer primers (Promega), following the manufacturer's instructions.

Quantitative PCR was performed using the Roche Lightcycler 480 system and SYBR Green I Master Mix (Roche). The 20 µl reactions were set up in 96-well format and contained 0.5 µM of each primer. Primers and thermal cycling parameters are listed in Table 1. Amplification of the expected products was confirmed by DNA sequencing. $C_t$ values for the target and reference transcripts were converted to relative levels of target transcripts using the $2^{-\Delta Ct}$ method.

To confirm overexpression of MT2A in cells transfected with the expression construct cDNA was measured by semi-quantitative RT-PCR using the same primers used to generate the MT2A product cloned into the expression vector (see above). GAPDH was used as a reference gene using the same primers as for RT-qPCR (Supplementary Table S1). PCRs were performed using 0.5 µM each primer and ThermoStart Taq DNA polymerase (Abgene), according to the manufacturer's instructions. The thermal cycling parameters for both reactions were as follows: 95°C for 15 min; followed by 25 cycles (within the linear range of product amplification) of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and then a final extension step of 72°C for 5 min. The products were analysed by agarose gel electrophoresis and identity to the expected sequence was confirmed by DNA sequencing.

Analysis of RNA by microarray hybridisation was carried out by Service XS (Leiden, The Netherlands). Following first and second strand cDNA synthesis (reverse transcription), biotin-labelled RNA was generated in vitro, hybridised to the HumanHT-12 v4 Expression Bead Chip (Illumina), and detected using streptavidin Cy3. Data were imported into GeneSpring GX 11 (Agilent) for visualisation. Hierarchical clustering was used to assess the similarity of biological replicates. Probes were examined further if the detection P-value was <0.6 in all samples in a replicate group. To determine differential expression between groups, the RankProd package from BioConductor [8]was used to perform a RankProducts analysis. This analysis was used to identify probes with a p.f.p of <0.05 over 100 permutations of the class labels with a resulting fold change of >1.2. The microarray data have been deposited in the GEO database under accession number GSE76510.

**Measurement of *SLC30A5* promoter activity using a promoter-reporter construct**

The activity of the *SLC30A5* promoter was measured as β-galactosidase activity expressed from the promoter-reporter plasmid pBlueSLC30A5prom, as described previously [9].

## Results

**Depletion of MTF1 enhances the transcriptomic response to zinc**

To identify the components of the cellular transcriptome that are under the control of MTF1, we used siRNA to reduce MTF1 expression in human Caco-2 cells. This cell line is a zinc-responsive intestinal model that we have used as the focus for much of our previous research on zinc homeostasis in mammalian cells [7, 9-12]. Cells transfected with control or MTF1-specific siRNA were maintained for 24 h at an extracellular zinc concentration of either 3 µM or 150 µM, which we use routinely to test gene responses to zinc in this cell line and which are both sufficient for normal growth and are non-toxic [9]. The siRNA-induced reduction of MFT1 mRNA (relative to GAPDH mRNA) was confirmed by RT-qPCR in cells exposed to both zinc concentrations. At 3 µM zinc, the relative MTF1 mRNA expression was 1 ± 0.1 and 0.43 ± 0.03 in control and MTF1-specific siRNA-treated cells, respectively (mean ± SEM, n = 3; P < 0.01 by Student's unpaired two-tailed t-test). At 150 µM zinc, the relative MTF1 mRNA expression was 1 ± 0.1 and 0.12 ± 0.001 in control and MTF1-specific siRNA-treated cells, respectively (mean ± SEM, n = 3; P < 0.001 by Student's unpaired two-tailed t-test).

We predicted that, compared with control cells, a smaller number of genes would be expressed differentially in response to changes in zinc availability under conditions of MTF1 knockdown. These genes would comprise the set regulated in response to zinc by transcription factors other than MTF1. However, we unexpectedly discovered that the number of genes regulated by zinc, as defined by the cut off criteria applied in our analysis of the transcriptome data, increased from 186 in control cells (Supplementary Table S2 column B (n=48) plus column H (n=138)) to 337 in cells with MTF1 knockdown (Supplementary Table S2 column E (n=199) plus column H (n=138)), suggesting an increased sensitivity of the transcriptome to zinc in the absence of MTF1. Of these genes, 138 were differentially expressed in response to zinc in both control and MTF1-depleted cells. However, the fold-changes were generally higher in the MTF1-depleted cells than the control cells (Supplementary Table S2, column H). Using these 138 genes, a plot of the fold-changes in expression in control cells (on the x-axis) versus those in MTF1-depleted cells (on the y-axis) had a positive slope, with some obvious outliers (Fig. 1). Excluding the outliers, the slope was 1.36; the fact that this value is greater than 1 suggests an increase in the sensitivity of these genes to zinc under conditions of MTF1 knockdown. Three MT genes (*MT1A*, *2A* and *1X*) followed this relationship. Notable exceptions to this trend were multiple other MT genes (specifically *MT1B, E, F, G, H* and *M*) and *SLC30A1*, which encodes the major plasma membrane zinc efflux protein ZnT1 and is known to be regulated by MTF1 [13]. These genes were all positioned below the trend line (Fig. 1), suggesting that, compared with the other zinc-responsive genes, the effect of changes in zinc availability on expression levels is curtailed under conditions of MTF1 knockdown.

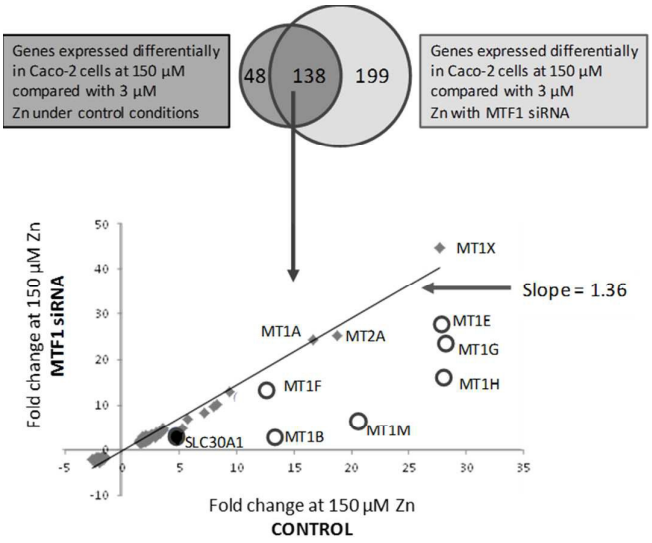


**Fig. 1**. Genes expressed differentially at 3 µM compared with 150 µM zinc in Caco-2 cells under control conditions and following siRNA-mediated knockdown of MTF. The Venn diagram represents the number of genes with a fold-change of 1.2 or greater, as detected by hybridisation of RNA to a DNA oligonucleotide microarray. The plot below shows the fold-changes in expression of the 138 genes that responded under both conditions. The solid line is the best fit to the data represented by the square symbols; the positive slope( >1) demonstrates that, for most genes in this group, the response to zinc was enhanced when MTF1 expression was reduced. Genes lying below this line, represented using circular symbols, are those for which the response to zinc was markedly curtailed with MTF1 knockdown, and which are proposed direct targets of MTF1.

To validate the findings of the microarray-based analysis we selected 3 genes (*ATP7B, EMP3* and *SLC39A10*) that, on the basis of this analysis, showed an augmented response to zinc when MTF1 expression was reduced. We also selected *TTR* as an example of a gene that showed a repressed response to zinc when MTF1 expression was reduced. We measured the effect of knockdown of MTF1 by siRNA on the response of these genes to the addition of 100 µM zinc to the extracellular medium using RT-qPCR. We used *BUB3* as a negative control; the microarray analysis indicated that this gene was not regulated by zinc under control conditions or when MTF1 expression was reduced. The siRNA used for these experiments was different from the siRNAs used for the microarray analysis, to provide an independent, more robust, validation of the data, and all comparisons were made with cells transfected with the control siRNA. We confirmed by RT-qPCR that the siRNA was effective in reducing MTF1 mRNA. Measured relative levels of MTF1 mRNA using GAPDH as the reference gene in cells maintained in 3 µM extracellular zinc

were: control 1.00 ± 0.05; siRNA 0.55 ± 0.03 (mean ± SEM, n=6; P<0.001 by Student's unpaired t-test).

The direction of response to zinc measured using the DNA microarray (up-regulation for *EMP3* and repression for *ATP7B*, *SLC39A10* and *TTR*) was confirmed by RT-qPCR for all four genes (Table 1). *BUB3*, did not respond to zinc under control conditions or when MTF1 expression was reduced (Table 1). The analysis of the microarray data determined that *TTR* responded to zinc only when MTF1 was expressed at endogenous levels (control siRNA). However, the measurements made by RT-qPCR revealed a response also with MTF1 knockdown, suggesting that the generally augmented response of the transcriptome to zinc under conditions of MTF1 knockdown may be greater than indicated by the microarray data. For *ATP7B* and *EMP3*, which showed smaller responses than *SLC39A10*, the response to zinc was observed only under conditions of MTF1 knockdown, commensurate with the augmented response to zinc under these conditions measured using the DNA microarray (Table 1). In contrast to *EMP3* and *ATP7B*, however, the magnitude of the response of *SLC39A10* to zinc was not affected by this siRNA (Table 1). The reason for the discrepancies between the microarray data and data derived by RT-qPCR is unknown. However, many published studies that measure responses of a selected gene panel by RT-qPCR to validate DNA microarray data report discrepancies, and systematic comparison confirms the occurrence of gene-specific discrepancies [14]. The fact that agreement tends to be better when microarray and PCR probes are in close proximity [14] indicates that discrepancies may often be due to the different probes measuring different slice variants. For *SLC39A10* there are 2 NCBI Reference Sequences in assembly GRCh38.p2 of the human genome (NM_001127257.1 (transcript variant 1) and NM_020342.2 (transcript variant 2)). The region targeted by the primers used for RT-PCR (bases 1425-1636 of NM_001127257.1) is common to both variants. However, it is possible that the microarray probe set for *SLC39A10* is biased towards additional, unidentified variants, providing a plausible though unproven explanation for the discrepancy in this case. Another formal possibility, though again an explanation for which there is no direct evidence, is that the 2 different siRNAs used to knock down MTF1 for the microarray experiment and for measurement of effects by RT-qPCR affect different MTF1 variants, which in turn affect the response of *SLC39A10* to zinc differentially.

Measurement of different variants by DNA microarray versus RT-qPCR could potentially account also for the fact that *SLC30A5*, a zinc transporter gene that we have shown to be regulated by zinc in Caco-2 cells using RT-qPCR [15, 16], was not found to be regulated by zinc using the cut-off criteria applied for the microarray analysis in the current work.

The responses of *SLC39A4*, *ATP7B* and *TTR* also served to validate the microarray data with respect to measures of

**Table 1.** Validation by RT-qPCR of effects of MTF1 knockdown on responses of selected genes to zinc detected using the DNA microarray. Data were calculated using the $2^{\Delta\Delta Ct}$ method using GAPDH and TOP1 as reference genes and are stated as mean ± SD (n=6). *P<0.05, **P<0.01, ***P<0.001 for 100 µM Zn compared with control for the same siRNA by Student's unpaired t-test. #P<0.05, ##P<0.01 for the MTF1-targeted siRNA compared with the control siRNA at 100 µM Zn by Student's unpaired t-test.

| Gene | Predicted effect of MTF1 knockdown on response to zinc based on microarray data (Fold change with control siRNA vs fold change with MTF1-targeted siRNA) | Treatment condition | Control siRNA | MTF1-targeted siRNA |
|---|---|---|---|---|
| *ATP7B* | Enhanced down-regulation (-1.86 vs -2.25) | Control | 1.02 ± 0.12 | 1.03 ± 0.27 |
| | | + 100 µM Zn | 0.90 ± 0.19 | 0.64 ± 0.05***## |
| *EMP3* | Enhanced up-regulation (no change vs +2.30) | Control | 1.13 ± 0.49 | 1.02 ± 0.30 |
| | | + 100 µM Zn | 1.16 ± 0.65 | 1.79 ± 0.88*# |
| *SLC39A10* | Enhanced down-regulation (no change vs -2.01) | Control | 1.01 ± 0.10 | 1.03 ± 0.25 |
| | | + 100 µM Zn | 0.44 ± 0.22*** | 0.44 ± 0.13*** |
| *TTR* | Repressed down-regulation (-2.26 vs no change) | Control | 1.02 ± 0.13 | 1.04 ± 0.14 |
| | | + 100 µM Zn | 0.74 ± 0.18** | 0.60 ± 0.23** |
| *BUB3* | N/A (No response to zinc) | Control | 1.03 ± 0.25 | 1.01 ± 0.11 |
| | | + 100 µM Zn | 1.01 ± 0.12 | 0.97 ± 0.27 |

repressed expression in response to zinc. *SLC39A4* was ranked 10th of 104 genes repressed at the higher zinc concentration under conditions of MTF1 knockdown; *ATP7B* was ranked 14th of 42 genes that showed enhanced down-regulation under conditions of MTF1 knockdown, and *TTR* was ranked top of 32 genes that showed down-regulation at endogenous levels of MTF1 expression (control siRNA) (see Supplementary Table S2),

The microarray data were further validated by measuring the magnitude of the response to zinc of the metallothionien isoforms MT1A, MT1B, MT1E, MT1F, MT1G, MT1H and MT1X by RT-qPCR and comparing the data with the response size measured using the DNA microarray (Table 2). Analysis by Spearman's rank correlation of the response size to zinc of all isoforms measured showed a significant agreement between the two data sets (r value = 0.8524; P<0.05).

**Table 2.** Comparison of responses of metallothionein mRNAs to zinc measured using the DNA microarray and by RT-qPCR. r= 0.8524; P<0.05 by Spearman's rank correlation. For the data derived by RT-qPCR the values in parentheses are SD (n=6) for the control (=1) then zinc-supplemented condition, respectively.

| | Measured fold-increase in mRNA | |
|---|---|---|
| | Microarray | RT-qPCR |
| MT1A | 16.6 | 2.39 (0.16, 0.42) |
| MT1B | 13.6 | 3.19 (0.81, 1.63) |
| MT1E | 28.1 | 77.8 (0.88, 29.7) |
| MT1F | 12.9 | 6.17 (0.06, 2.53) |
| MT1G | 28.6 | 162 (0.83, 53.9) |
| MT1H | 28.2 | 123 (0.84, 58.8) |
| MT1X | 27.7 | 13.5 (0.11, 9.08) |

**Classification of zinc-responsive genes into two groups, according to the effect of MTF1 depletion**

Based on our microarray data, genes can be classified into two groups depending on whether MTF1 knockdown attenuates or augments their response to zinc. The group of genes for which knockdown of MTF1 attenuated the response to zinc includes most of the MT genes, the *SLC30A1* gene encoding the zinc efflux protein ZnT1, the 48 genes for which we observed a response only in cells transfected with the control siRNA and 29 of the group of 138 genes that responded to zinc in cells transfected both with control or MTF1 siRNA. These are the genes shaded red in Supplementary Table S2 and for which the fold change ratio (column K) is >1. We propose that these genes are direct targets of MTF1 or genes that are components of zinc-regulated networks that include direct MTF1 targets.

The group of genes for which knockdown of MTF1 augmented the response to zinc is represented by the 199 genes for which we observed a response in cells with MTF-1 knockdown but not in control cells plus 103 of the group of 138 genes that responded to zinc in cells transfected both with control or MTF1 siRNA. These are the genes shaded green in Supplementary Table S2, and for which the fold change ratio (column K) is <1). We propose that these genes are regulated by zinc through mechanisms independent of MTF1 and that the increase in their responsiveness to zinc when MTF1 is depleted is due to reduced MTF1-induced buffering of regulatory responses of MTs and other genes (e.g. *SLC30A1*).

We have not classified 6 of the genes that responded to zinc according to the cut-off criteria we applied to our analysis of the microarray data into either group because the difference in the size of the response to zinc under control conditions and with MTF1 knockdown was smaller than (an arbitrary) 1%. It may be that the balance of direct or indirect actions of MTF1 and of zinc buffering by metallothioneins on the responses of these genes to zinc was completely balanced under our experimental conditions. However, precision of the microarray data is unlikely to be sufficient to draw this conclusions definitively. Likewise data precision may have led us to 'misclassify' other genes for which the effect of MTF1 knockdown on the magnitude of the response to zinc was marginal.

**Overexpression of MT curtails the transcriptional response to zinc.**

A prediction based on our findings is that increasing the level of expression of MT in the cell would curtail the transcriptional response of other genes to zinc. To test this prediction, we overexpressed MT2A from a transgene introduced stably into Caco-2 cells and measured the response of the *SLC30A5* promoter, which is a robust response that we have characterised and studied extensively in this cell line [9, 15]. As predicted, the characteristic zinc-induced repression of *SLC30A5* promoter activity and mRNA levels was abrogated under conditions of MT2A overexpression (Fig. 2).

**The MRE does not cluster in genes for which depletion of**

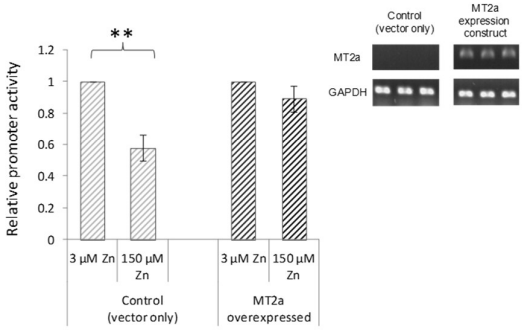


**Fig 2.** Response of the *SLC30A5* promoter and mRNA levels to elevated extracellular zinc concentrations in Caco-2 cells stably expressing MT2A. Control cells or cells stably overexpressing MT2A were transiently transfected with a promoter-reporter construct containing the β-galactosidase gene immediately downstream of the -950 to +50 region of the *SLC30A5* promoter. The graph shows promoter activity measured as reporter gene activity in cell lysates following incubation of the cells with serum-free medium supplemented with 3 µM $ZnCl_2$ or 150 µM $ZnCl_2$ for 24 h. Data are represented as the mean ± SEM for n = 6 and were normalised to reporter gene activity at 3 µM $ZnCl_2$. ** *P* <0.01 by Student's unpaired t-test. The additional panel shows confirmation by RT-PCR of MT2a overexpression in cells transfected with the MT2a expression plasmid construct compared with cells transfected with vector only following selection with G418. GAPDH was used as a reference gene.

**MTF1 curtails the response to zinc.**

We also predicted that the MRE, which is the target sequence for MTF1 binding, would appear more frequently in the groups of genes that we proposed to include direct targets of MTF1 (i.e., the genes with a response to zinc that was curtailed following MTF1 knockdown) than in the group that showed an enhanced response to zinc following MTF1 depletion. However, we found that the frequency of occurrence of the MRE sequence (TGCRCNC) in the region comprising 1000 bp upstream of the transcription start site (TSS) plus the mRNA sequence did not differ significantly (P = 0.50 by Kruskall-Wallis test) between the genes that responded to zinc only under control conditions (median 1.5, minimum 0, maximum 8) and those that showed an augmented response to zinc following MTF1 knockdown (median 1.0, minimum 0, maximum 13). Moreover, no differences of likely biological relevance were revealed by analysing separately the regions upstream and downstream of the TSS. In the genes that responded to zinc only under conditions of MTF1 knockdown, there were significantly more occurrences (P < 0.001 by Kruskall-Wallis test followed by Dunn's multiple comparisons test) of the MRE in the mRNA sequences (median 2, minimum 0, maximum 13) than the 1000 bp regions upstream of the TSS (median 1, minimum 0, maximum 9). In addition, there were more copies of the MRE in the mRNAs of this same group (median 2, minimum 0, maximum 13) than in the 1000 bp regions upstream of the genes that responded to zinc under both control conditions and following MTF1 knockdown (median 1, minimum 0, maximum 9). However, these statistically-significant differences are likely a reflection of the fact that the mRNA sequences are generally longer than 1000 bp, and thus would include more copies of the MRE purely by chance, and of the fact that the group of genes that responded to zinc only following MTF1 knockdown is the largest group.

## Discussion

This study demonstrates that reduced levels of MTF1 expression curtail the response to zinc of a relatively small number of genes, including multiple MT genes and *SLC30A1* (zinc efflux protein ZnT1). Notably, the net effect of reduced MTF1 expression in Caco-2 cells was an increase in the sensitivity of the transcriptome to zinc. On the basis of these observations we propose that MTF1 heads a hierarchy of zinc sensors, and through controlling the expression of a raft of MTs and other key proteins involved in controlling intracellular zinc levels (e.g. ZnT1), alters zinc buffering capacity and total cellular zinc content. The concept that MTs, as well as intracellular zinc transporters that sequester and release zinc from intracellular organelles to perform an accessory "muffling" function, are central players in cytosolic zinc buffering is well-established [17].

Inspection of the lists of genes whose responses to zinc were augmented or unmasked by MTF1 knockdown (Supplementary Table S2) reveals some examples worthy of note. The negative response to zinc of *ATP7B*, the gene mutated in Wilson's disease, an inherited copper toxicity resulting from defective hepatic efflux by the ATP7B transporter, was augmented following MTF1 knockdown. We have reported previously that ATP7B can be regulated by zinc in JAR cells [11]. We also reported that the promoter region of this gene contains the zinc transcriptional regulatory element (ZTRE), a sequence responsible for mediating transcriptional repression in response to zinc [15], at position -157 to -135 relative to the transcription start site. It is possible that the as yet unidentified transcription factor that acts at the ZTRE is lower than MTF1 in the hierarchy of zinc control proposed here. The transcription factor KLF4, which is important in the regulation of the zinc transporter Zip4 [18], met our cut-off criteria for zinc responsiveness only after MTF1 knockdown. Again, this result is consistent with KLF4 being a zinc-responsive transcription factor that is positioned below MTF1 in our proposed hierarchy. Zinc-induced repression *of SLC39A5, 8* and *10*, members of the SLC39 zinc uptake transporter family, were also only observed following MTF1 knockdown. MTF1 has been shown to have an atypical repressive role in transcriptional control of *Slc39A10* in both zebrafish [19]and mouse [20]. Assuming that this mode of regulation extends to human cells, our observation may reflect either increased sensitivity to zinc fluctuations of the residual MTF1 under our knockdown conditions, or repression of *SlC39A10* by other mechanisms in response to high zinc levels.

We predicted that the MRE (TGCRCNC), the known binding sequence for MTF1, would be over-represented in the genes for which a response to zinc was observed only when MTF1 was present at control levels. However, there were no differences between the numbers of times this sequence occurred in the different groups of genes we identified. This analysis may reflect a functional association of MTF1 with other regulatory factors, as is known to be the case for the mouse MT-I gene, which is activated by MTF1 in association with the histone acetyltransferase p300/CBP and the transcription factor Sp1 [21]. MREs may therefore be functional only within specific sequence contexts, and many chance occurrences of the sequence may not be sites where MTF1 binds and affects gene expression. Consequently, a more thorough analysis of the sequence context of functional MREs, which could be identified through approaches such as ChIP, is warranted.

## Conclusions

This work uncovers genes whose involvement in zinc homeostasis should be investigated. For the MT genes and *SLC30A1*, there is a clear rationale for their response to zinc being high in the regulatory hierarchy (as direct targets of the proposed master zinc sensor MTF1). A possible role in zinc homeostasis of other genes identified as likely direct targets of MTF1 merits further investigation. These findings form the basis of an integrative understanding of the cellular zinc homeostatic mechanism, including its calibration in relation to

the availability of other metals via the multi-metal-responsive MT pool.

## Acknowledgements

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## Notes

Abbreviations used are: MRE, metal response element; MTF1, metal-responsive transcription factor 1; MT, metallothionein; TSS, transcription start site; ZTRE, zinc responsive transcriptional element.

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