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# Transcriptome profile of yeast reveals the essential role of PMA2 and uncharacterized gene YBR056W-A (MNC1) in adaptation to toxic manganese concentration†

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Adaptation of *S. cerevisiae* to toxic concentrations of manganese provides a physiological model of heavy metal homeostasis. Transcriptome analysis of adapted yeast cells reveals upregulation of cell wall and plasma membrane proteins including membrane transporters. The gene expression in adapted cells differs from that of cells under short-term toxic metal stress. Among the most significantly upregulated genes are *PMA2*, encoding an ortholog of Pma1 H<sup>+</sup>-ATPase of the plasma membrane, and *YBR056W-A*, encoding a putative membrane protein Mnc1 that belongs to the CYSTM family and presumably chelates manganese at the cell surface. We demonstrate that these genes are essential for the adaptation to toxic manganese concentration and propose an extended scheme of manganese detoxification in yeast.

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## Significance to metallomics

Yeast provides a unique model to study manganese detoxication, which is important to understand heavy metal ion homeostasis in eukaryotes. A whole-transcriptome analysis of manganese-adapted yeast revealed multiple upregulated genes. Among the top activated genes were *PMA2*, a rarely expressed H<sup>+</sup>-ATPase of the cytoplasmic membrane, and *YBR056W-A*, encoding an uncharacterized protein, a putative member of the CYSTM family. The null mutants demonstrated reduced viability at high manganese ion concentrations. We hypothesize that Pma2 is necessary for cytoplasmic membrane energization and YBR056W-A (Mnc1) chelates manganese at the cell surface and thus participates in manganese detoxication.

## Introduction

Metal cations are common components of the environment and are involved in multiple processes in living cells as enzyme cofactors and metabolism regulators. For example, magnesium fluxes across the plasma membrane regulate timekeeping and energy balance<sup>1</sup> and potassium and sodium gradients are essential for cell viability.<sup>2</sup> Yeast is an exclusive model to study the adaptation of eukaryotic cells to toxic heavy metals.<sup>3–6</sup> Multiple signaling pathways were found to be responsible for yeast viability under an excess of heavy metals. Many genes essential for survival in the presence of copper, silver, zinc, cadmium, mercury, and chromium were previously identified in *Saccharomyces cerevisiae*.<sup>7</sup> The mechanisms of heavy metal tolerance in yeast include environmental sensing, sulfur and glutathione metabolism, vacuolar and endosomal transport and sorting.<sup>4</sup> The pathways involved in the Cd<sup>2+</sup> tolerance of *Schizosaccharomyces pombe* included sulfate assimilation, phytochelatin synthesis and transport, ubiquinone biosynthesis, stress signaling, cell wall

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biosynthesis and cell morphology, gene expression and chromatin remodeling, vacuole function, and intracellular transport of macromolecules.<sup>8</sup>

Manganese is an essential trace element in living cells; its ions are cofactors of many enzymes including oxidases, dehydrogenases, DNA and RNA polymerases, and sugar transferases.<sup>9</sup> In humans, disorder of manganese homeostasis is known for neurotoxicity.<sup>10,11</sup> High concentrations of manganese are toxic for yeast. Manganese homeostasis in yeast involves transport proteins for  $Mn^{2+}$  uptake, sequestration and excretion; they are located in the plasma membrane, Golgi, vacuolar and mitochondrial membranes.<sup>3,12,13</sup> Yeast possesses transporter Smf1, which is localized at the cell surface, and intracellular transporter Smf2, which is localized mainly in intracellular Golgi like vesicles.<sup>13</sup> Smf1 also participates in the oxidative stress response.<sup>13</sup> The phosphate transporter of the yeast plasma membrane, *PHO84*, is responsible for  $Mn^{2+}$  uptake via manganese-phosphate complexes.<sup>13</sup> Disruption of the *PHO84* gene results in a manganese-resistant phenotype.<sup>14</sup> This resistance is associated with the inability of yeast cells to take up large amounts of  $Mn^{2+}$ . The *PHO80* mutants of *S. cerevisiae* are defective in phosphate uptake, storage and metabolism, and exhibit a wide range of defects in metal homeostasis.<sup>15</sup>

The Golgi manganese transporter Pmr1p is a P-type calcium and manganese transporting ATPase that transports these ions from the cytoplasm into Golgi lumen.<sup>13</sup> This protein participates in detoxification of excess manganese via excretion by the secretory pathway. The vacuolar manganese transporter Ccc1 is responsible for manganese sequestration in vacuoles.<sup>16</sup>

The sufficient manganese concentration for *S. cerevisiae* growth is in the range of 0.01–0.1 mM.<sup>3,14</sup> Inhibition of the growth of *S. cerevisiae* was observed at 0.5 mM  $Mn^{2+}$ .<sup>17</sup> Recently, we have demonstrated the ability of *S. cerevisiae* to adapt to toxic  $Mn^{2+}$  concentrations (2–5 mM) after an unusually long lag phase (4–5 days).<sup>18</sup> The adaptation was accompanied by the enlargement of vacuoles and whole cells, and with the drastic increase in the content and chain length of acid soluble polyphosphate, which probably forms complexes with manganese ions.<sup>18,19</sup> If re-inoculated into a fresh medium with  $Mn^{2+}$ , the adapted cells had no growth delay.<sup>18</sup> Such adaptation is a useful model to study yeast tolerance to toxic manganese concentrations.

In this study, we have used RNA-Seq to analyze the whole transcriptome of *S. cerevisiae* adapted to the toxic concentration of  $Mn^{2+}$ .

## Materials and methods

### CRY strain and growth conditions

The strain CRY MATa ( $\Delta ade2 \Delta his3 \Delta leu2 \Delta trp1 \Delta ura3$ ) was kindly provided by N. Rao and A. Kornberg.<sup>20</sup> This strain was used earlier as a model for manganese adaptation.<sup>18</sup> The cells of this strain were used for transcriptome analysis (two technical replicates of the same cultivation), enzyme activity assay, and qPCR verification of differential gene expression.

The cells were cultivated in YPD (2% glucose, 2% peptone, 1% yeast extract) at 29 °C and 145 rpm for 18 hours in the control medium and for 120 hours in manganese-rich medium (stationary growth stage). Growth curves for CRY are shown in the ESI,† Fig. S1. The CRY strain growth curve and cell morphology change in the presence of 2.5 to 5.0 mM  $Mn^{2+}$ .<sup>18</sup>  $MnSO_4$  was added to a final concentration of 2.5 mM. In the control YPD medium, the  $Mn^{2+}$  concentration was estimated to be 0.0036 mM. The cells were harvested at 5000g for 20 min, washed with sterile distilled water, and used for analysis.

### Mutant strains

Mutant strains were used to assess the involvement of particular genes in manganese adaptation. The yeast parental strain BY4743, Yeast HomDip Knock Out Strain YBR056W-A ( $\Delta ybr056-a$ ), Yeast HomDip Knock Out Strain YPL036W ( $\Delta pma2$ ), and Yeast HomDip Knock Out Strain YMR303C ( $\Delta adh2$ ) were obtained from Yeast Knockout Collection (YKO, Dharmacon) and grown in standard YPD medium or with the addition of 2.5 mM  $MnSO_4$ .

### RNA extraction and sequencing

After growing to A600 of 16 (control) and 12 (manganese, see the ESI,† Fig. S1), the cells were washed twice with distilled water at 0 °C and centrifuged after each washing. This step was applied to both control and manganese-adapted cells. Washing was necessary because without it RNA degradation in yeast samples grown in manganese medium prevented sequencing library construction. The resulting samples were placed in RNeasy Lysis Buffer, frozen and used for RNA isolation. Total RNA was extracted using the RNeasy Mini kit (Qiagen), with the addition of the Plant RNA Isolation aid reagent (Ambion) to lysis buffer RLT. Libraries were prepared using the TruSeq RNA sample preparation kit (Illumina) and sequenced using a HiSeq2000 instrument with 50 nt read length.

### Enzyme activity assay

For assay of enzyme activities, each sample of biomass was frozen at –70 °C, and the cells were broken by French-press (three biological replicates). The homogenate was suspended in 10 mM Tris-HCl, pH 7.0, with 0.5 mM PMSF. The homogenate was centrifuged at 3000g for 5 min and then the supernatant was centrifuged at 13 000g for 60 min. The supernatant (cellular extract) was used for the assay of alcohol dehydrogenase activity; the pellet (crude membrane fraction) was suspended in 50 mM MES-Na, pH 6.0, and used for the assay of the plasma membrane and mitochondrial ATPase activities. The alcohol dehydrogenase activity was measured as described in ref. 21. The activities of plasma membrane ATPase and mitochondrial ATPase were estimated by Pi release as described in ref. 22. The reaction was carried out in 50 mM MES-Na, pH 6.0, containing 6 mM ATP and 4 mM  $MgSO_4$  or  $MnSO_4$  at 30 °C in the case of plasma membrane ATPase. The activity was measured in the absence and presence of 0.1 mM orthovanadate, a specific inhibitor of the plasma membrane ATPase of yeast.<sup>22</sup> The vanadate-sensitive activity was taken as the activity of plasma membrane ATPase. The reaction was carried out in 50 mM Tris-HCl, pH 8.5, containing 2.5 mM ATP and 2.5 mM  $MgSO_4$  at 30 °C in the case of mitochondrial ATPase.



As a specific inhibitor, 10 mM of  $\text{NaN}_3$  was added. In both cases, the reagent for the Pi assay was added as a stop solution.<sup>23</sup> All assays were repeated in triplicate and the average values with standard deviations were computed with MS Excel.

### Fluorescence and light microscopy

Living and dead cells were revealed using the LIVE/DEAD Fungal Light Yeast Viability Kit (Molecular Probes Inc., USA) according to the official manual. Yeast cultures were stained without washing and incubated with a stain reagent for 15 min at 37 °C. The samples were examined under the fluorescence microscope AXIO Imager A1 ZEISS (Germany) with a filter kit 56 (ZEISS) at a wavelength of 450–500 nm (excitation) and 600–650 nm (emission). The cell concentration was estimated by light microscopy in a standard counting chamber. All assays were repeated in triplicate and the average values with standard deviations were computed with MS Excel.

### Processing of RNA-Seq data

The raw reads were processed as follows: trimming was performed with cutadapt<sup>24</sup> and sickle,<sup>25</sup> mapping to the S288C reference genome<sup>26</sup> was performed with TopHat,<sup>27</sup> HTSeq<sup>28</sup> was used for read counting, and R environment and edgeR<sup>29</sup> were used to estimate differential gene expression. Overall statistics and the complete data on differential gene expression are provided in the ESI,† Table S1.

The raw and processed data are deposited in GEO (accession number GSE85109).

### qPCR assay

The biomass samples were frozen at –70 °C and broken by French press (two biological replicates). Total RNA was isolated by TRIzol LS Reagent (Thermo Scientific). Genomic DNA was removed by treating RNA samples with the RNase-free DNase I (Thermo Scientific). cDNA was obtained using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). qPCR was performed using the qPCRmix-HS SYBR + LowROX kit (Evrogen) and the DTLite Real-Time PCR System (DNA Technology).

### Spot test assay

A spot test was performed on YPD medium supplemented with 4% agar. 0.005 ml of cell suspension was applied per spot with the cell concentrations from  $2 \times 10^6$  to  $2 \times 10^4$  cell  $\text{ml}^{-1}$ . Petri dishes

were incubated at 28 °C for 24 h (control) and 44 h (5 mM  $\text{Mn}^{2+}$ , 2.5 mM used in liquid medium were not toxic in solid medium).

## Results

The cells of *S. cerevisiae* adapted to 2.5 mM of manganese after a prolonged lag-phase are characterized by specific changes in morphology.<sup>18</sup> To find out the genes involved in manganese adaptation we performed transcriptome analysis of the control and adapted cells in the early stationary growth stage.

Using the arbitrary threshold of  $\text{FDR} < 0.05$  and requiring a two fold difference ( $|\log_2 \text{fold change}| > 1$ ) for gene expression between the experiment and the control, we identified 609 (537) upregulated (downregulated) genes. The GO-enrichment analysis performed with YeastMine<sup>30</sup> revealed downregulation of various cellular biosynthesis processes (including translation and ribosome assembly), which agrees with the observed decrease in the growth rate (see the ESI,† Fig. S1). Upregulated genes were enriched in GO-terms associated with the cell wall and membrane assembly and various transporter activities (including ion transporters, see the ESI,† Table S2).

### Manganese adaptation upregulates genes not involved in known metal stress responses

We took a closer look at the top 10 upregulated genes with the lowest FDR and 10–20-fold increase in expression under manganese adaptation (Table 1). Some genes (such as *PHO89* encoding the plasma membrane  $\text{Na}^+/\text{Pi}$  cotransporter<sup>31</sup>) are consistently upregulated in various metal stresses.<sup>7,32</sup> Upregulation of other genes is detected specifically in manganese adaptation: *HXT2* (a high-affinity glucose transporter), *YNR064C* (epoxide hydrolase), *POX1* (Fatty-acyl coenzyme A oxidase), *PMA2* (the ortholog of *PMA1* encoding the plasma membrane  $\text{H}^+$ -ATPase that is rarely expressed, e.g. in diauxic growth<sup>33</sup>), and *YBR056W-A* gene encoding an uncharacterized protein. Adaptation-specific genes *YBR056W-A* and *PMA2* were selected for detailed analysis and verification. Additionally, in further analysis we considered *ADH2* encoding glucose-repressed alcohol dehydrogenase, characteristic of the stationary growth stage<sup>34</sup> and upregulated in other metal stresses.

First of all we focused particular attention on the *YBR056W-A* gene encoding the previously uncharacterized protein. This gene

**Table 1** Top 10 genes upregulated in toxic manganese-adapted yeast cells in comparison to short-term manganese exposure<sup>7,32</sup>

Gene systematic name (according to the Saccharomyces Genome database)	Gene name	Upregulated in		
		manganese adaptation	Short-term manganese exposure	Other metal stresses
YMR011W	<i>HXT2</i>	+	–	–
YMR303C	<i>ADH2</i>	+	–	+
YBR056W-A	(Uncharacterized protein) <i>MNC1</i>	+	–	–
YNR064C	(Epoxide hydrolase)	+	–	–
YOR385W	(DIA1 homolog)	+	+	+
YMR316W	<i>DIA1</i>	+	+	+
YBR296C	<i>PHO89</i>	+	+	+
YPL036W	<i>PMA2</i>	+	–	–
YGL205W	<i>POX1</i>	+	–	–



is translated<sup>35</sup> and encodes a peptide (66 a.a.) that bears a notable similarity to cysteine-rich TM module stress tolerance domain<sup>36</sup> (CDD<sup>37</sup> Blast *E*-value  $\sim 2 \times 10^{-15}$ , see also the ESI,† Fig. S2 for a multiple sequence alignment). The members of the CYSTM family were reported to play a role in stress responses, in particular, these proteins prevent metal uptake into plant cells.<sup>38</sup> We verified the YBR056W-A differential expression by qPCR (see the ESI,† Fig. S3), which confirmed the upregulation in the manganese-adapted yeast. Based on this evidence, we suggest *MNC1* (manganese-chelating protein 1) as a gene symbol for YBR056W-A.

### Enzyme activity assay confirms differential gene expression

To provide an independent validation of RNA-Seq differential expression, we compared the activities of alcohol dehydrogenase and H<sup>+</sup>-ATPases in control and manganese-adapted cells.

The activity of mitochondrial ATPase in manganese-adapted cells was lower compared to the control cells (Table 2). It agrees with the down-regulation (FDR < 0.05) of most of the genes encoding F<sub>1</sub>F<sub>0</sub>-ATPase subunits (ESI,† Table S1). The gene expression of *PMA1* encoding the major H<sup>+</sup>-ATPase was unchanged under adaptation, while the *PMA2* ortholog was significantly upregulated. However, the activity of plasma-membrane ATPase in manganese-adapted cells was lower compared to the control cells (Table 2). It was shown that Pma2 is more active in the presence of manganese than Pma1.<sup>39</sup> Probably, the upregulation of Pma2 maintains the transmembrane potential at the plasma membrane under manganese excess.

The activity of alcohol dehydrogenase in the cell-free extract of manganese-adapted cells was higher compared to the control cells (Table 2). However, this increase was less marked compared to the gene expression data (2 times *versus* more than 10 times). Probably, this difference is due to a specific property of Adh2: the enzyme contains a Zn<sup>2+</sup> ion in the active center, which is replaced by a Mn<sup>2+</sup> ion under manganese excess lowering the stability of the enzyme.<sup>40</sup>

### Mutant strains exhibit different adaptability to toxic manganese concentrations

The adaptation to manganese was studied in three BY4743 mutant strains: *Δybr056w-a*, *Δpma2*, and *Δadh2*. All strains grew similarly in YPD medium; in the presence of 2.5 mM manganese, the growth of all strains was inhibited (ESI,† Fig. S4). The adaptation of BY4743 cells to manganese was similar to that of CRY cells with decreased growth rate and reduced optical density at the stationary phase compared to growth in YPD medium (ESI,† Fig. S1 and S4). The increase in the cells' sizes in

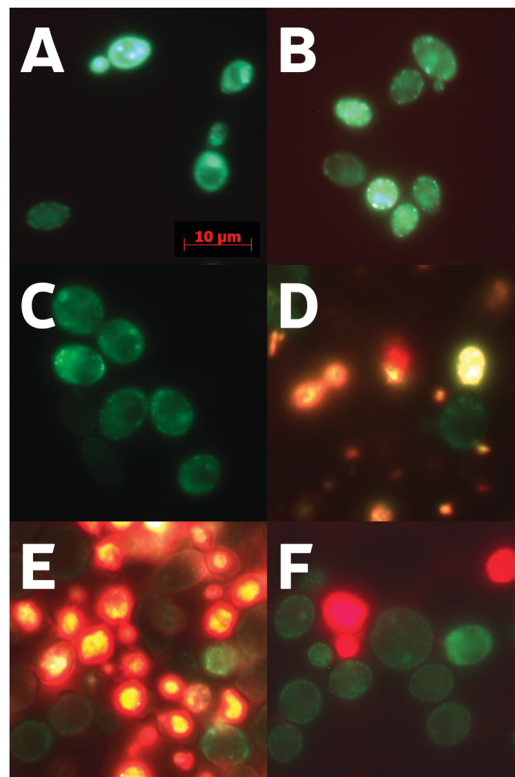


Fig. 1 The micrographs of LIVE/DEAD Yeast Viability Kit-stained cells (Living cells – green, dead cells – orange and red) of *S. cerevisiae* in the stationary growth stage. Control growth: (A) parent strain BY4743; (B) *Δybr056w-a* strain. Growth in the presence of 2.5 mM MnSO<sub>4</sub>: (C) parent strain BY4743; (D) *Δybr056w-a*; (E) *Δpma2*; (F) *Δadh2*.

manganese-medium compared to the control medium was also observed (see Fig. 1A and C).

The BY4743 and all the mutant strains had similar cell concentrations and culture densities in control growth (ESI,† Fig. S4). However, notable differences were found for growth in manganese-rich medium: (1) the culture density of the *Δadh2* mutant was notably higher than that of the three other strains including BY4743, but (2) the cell concentrations of the *Δybr056w-a* and *Δpma2* mutants were notably lower than of the parent strain and of the *Δadh2* mutant (ESI,† Fig. S4).

The light microscopy of the *Δybr056w-a* and *Δpma2* cultures revealed large-scale cell lysis in the stationary phase in manganese-rich medium (Fig. 2). Indeed, the staining using the LIVE/DEAD Yeast Viability Kit showed that the cell cultures of *Δybr056w-a* and *Δpma2* contained multiple dead and lysed cells (Fig. 1, 2 and Table 3). Probably, the cell lysis products enhance light scattering resulting in comparably high optical density of these cell cultures. The presence of living cells in the cultures of these mutants is probably due the fact that the cells, which have not yet accumulated a toxic amount of manganese, preserved the budding ability (Fig. 1).

The *Δadh2* mutant demonstrated the most resistance to manganese (ESI,† Fig. S4 and Table 3); in particular, it did not demonstrate cell lysis in the manganese-rich medium (Fig. 2). The stability of the Adh2 enzyme drastically decreases under

Table 2 The activity of alcohol dehydrogenase, mitochondrial ATPase and plasma membrane ATPase in *S. cerevisiae* cells grown up to the stationary phase in the absence and presence of 2.5 mM of MnSO<sub>4</sub>

Enzyme activity, E/mg protein	Control cells	Mn <sup>2+</sup> adapted cells
Alcohol dehydrogenase	4.9 ± 2.0	11.7 ± 4.0
Mitochondrial ATPase	0.64 ± 0.2	0.2 ± 0.05
Plasma membrane ATPase	0.32 ± 0.02	0.21 ± 0.09





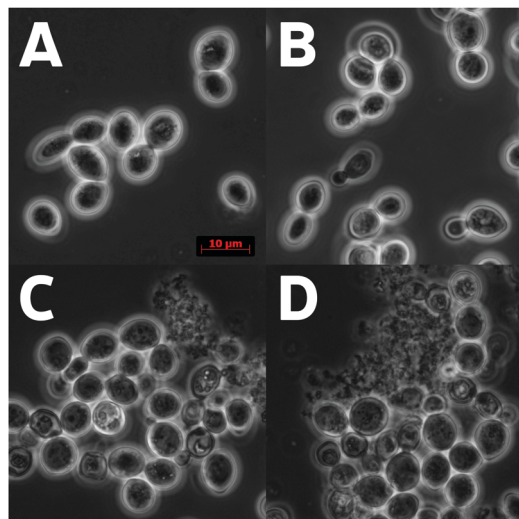


Fig. 2 The phase contrast microscopy of *S. cerevisiae* cells grown in the presence of 2.5 mM  $\text{MnSO}_4$  for 45 h: (A) parent strain BY4743; (B)  $\Delta adh2$ ; (C)  $\Delta pma2$ ; (D)  $\Delta ybr056w-a$ .  $\Delta pma2$  and  $\Delta ybr056w-a$  mutants exhibit cell lysis.

Table 3 Cell viability of *S. cerevisiae* mutants after 45 h of cultivation

Strain	Dead cells, %	
	Control YPD	YPD, 2.5 mM $\text{MnSO}_4$
BY4743, parent strain	0	12
$\Delta ybr056w-a$	0	52
$\Delta ypl036w$ ( $\Delta pma2$ )	0	62
$\Delta ymr303c$ ( $\Delta adh2$ )	0	18

excess manganese.<sup>40</sup> Probably, observed upregulation of *ADH2* can be a result of the feedback mechanism to compensate for the rapid inactivation of the enzyme. It is not clear why the  $\Delta adh2$  mutant is more manganese-resistant than the parent strain, but this was confirmed by the Spot test (ESI,† Fig. S5).

However, the same test failed to distinguish  $\Delta ybr056w-a$  and  $\Delta pma2$  from the parent strain when grown on manganese-rich medium; this was also the case for the culture density test (ESI,† Fig. S4).

All in all, it seems  $\text{Mn}^{2+}$  excess does not prevent budding of  $\Delta ybr056w-a$  and  $\Delta pma2$ , but induces cell lysis in later stages of the cell cycle. In particular, this explains the limitation of the culture density test and the Spot test<sup>41</sup> to determine the growth differences in manganese-rich medium.

## Discussion

We compared the set of genes upregulated in manganese adaptation with the sets of genes upregulated upon various metal stresses found in previous microarray experiments.<sup>7,32</sup> There was only a small overlap between sets of upregulated genes comparing adaptation and short-term exposure<sup>32</sup> to  $\text{Mn}^{2+}$ : 570 of the 609 upregulated genes were specific to adaptation (not upregulated in short-term manganese exposure) and 419 of the 609 adaptation-upregulated genes were not upregulated in any other metal stresses.<sup>7,32</sup> In particular, YeastMine<sup>30</sup> analysis of gene ontology

for genes unique to short-term manganese stress shows activation of general biosynthesis pathways (primarily amino-acid and organic acid synthesis). In contrast, genes unique for adaptation are enriched with sugar transporters and cell wall and membrane proteins. Thus, yeast manganese adaptation drastically differs from short-term stress response in terms of gene expression patterns. This highlights the specific state of adapted cells, which pertain morphology highly deviating both from control cells and cells under stress response.<sup>18</sup>

Multiple proteins participate in manganese homeostasis in *S. cerevisiae*.<sup>5,13</sup> Based on our findings we propose several key additions to the existing model of manganese detoxication in yeast cells (Fig. 3). The adaptation of yeast cells to toxic concentrations of manganese ions involves, in particular, transport systems responsible for manganese compartmentalization, systems of phosphorus transport and polyphosphate accumulation, as well as other membrane proteins.

In particular, Smf1 is a known manganese transport protein localized in the plasma membrane and endosomes<sup>13</sup> and responsible for manganese ion uptake. On the one hand, its expression does not change upon adaptation (Table 4). This agrees with reported Smf1 stability under manganese exposure<sup>13</sup> and high level of manganese uptake in adapted cells.<sup>18</sup> On the other hand, the genes of transporters providing  $\text{Mn}^{2+}$  compartmentation (Smf2, Ccc1 and Pmr1) are slightly upregulated (passing FDR < 0.05) in manganese adapted cells (Table 4) enhancing manganese sequestration in the endoplasmic reticulum, vacuoles, and Golgi, respectively. Interestingly, Spf1, an important regulator of the manganese transport in ER,<sup>42</sup> demonstrates stable expression between growth in control and  $\text{Mn}^{2+}$  excess conditions (ESI,† Table S1). Thus, low differential expression of manganese transporters suggests limited involvement of these proteins in adaptation to toxic manganese concentration.

Variable upregulation of multiple genes encoding PHO-proteins is also observed in manganese-adapted cells, in particular, there are cytoplasmic membrane phosphate transporters Pho84, Pho87, Pho89 and Pho90. Previously it was reported that Pho84 is responsible for phosphate and manganese uptake under manganese excess.<sup>15</sup> It is not clear whether upregulation of Pho84 has any adaptive significance in our conditions or whether it is a side-effect of disturbed phosphorus metabolism. However, upregulation of phosphate transporters explains the increase of phosphate accumulation by manganese adapted cells.<sup>18</sup> The  $\text{P}_i$  excess may increase the synthesis of inorganic polyphosphate by Vtc4 polyphosphate synthase, the activity of which is stimulated by manganese ions. As for polyphosphate hydrolases, no up-regulated genes were found, while Ppx1 polyphosphatase was downregulated. Thus, the enhanced  $\text{P}_i$  uptake and polyphosphate synthesis and the decreased polyphosphate degradation explain the polyphosphate accumulation in manganese adapted cells.<sup>18</sup> Probably, the polymers accumulated in all cellular compartments bind manganese and provide an additional way of detoxication.

The enhanced expression of phosphate transporters, as well as other transporter proteins (including hexose transporters, see the ESI,† Table S1), requires an increase of cytoplasmic membrane energization under manganese adaptation. The expression of the



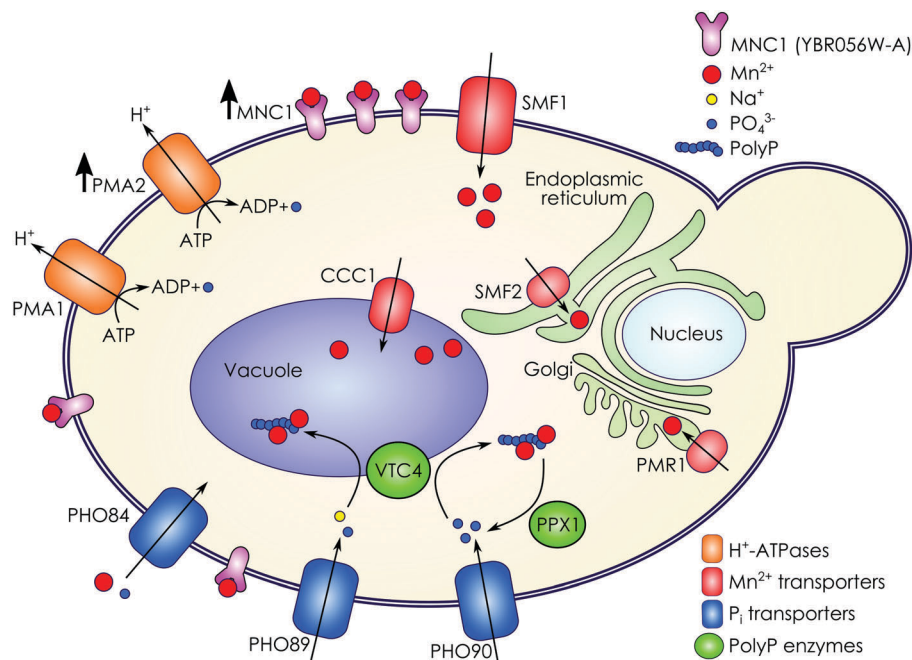


Fig. 3 Scheme of manganese detoxication in *S. cerevisiae* and proposed role of YBR056W (Mnc1) and Pma2.

**Table 4** Differential expression of genes encoding polyphosphate metabolizing enzymes, proteins of the PHO pathway, manganese transporters, and some other transport proteins

Expression fold change (log 2)	FDR	Gene symbol	Gene function
<b>Polyphosphate metabolism enzymes</b>			
0.15	0.25	<i>PPN1</i>	Exo/endopolyphosphatase
↓ −0.89	$6.75 \times 10^{-15}$	<i>PPX1</i>	Exopolyphosphatase
−0.04	0.8	<i>DDP1</i>	Diadenosine and diphosphoinositol polyphosphate phosphohydrolase
−0.14	0.26	<i>VTC4</i>	Polyphosphate synthase
<b>PHO pathway members</b>			
↑ 0.95	$3.92 \times 10^{-16}$	<i>PHO84</i>	High-affinity inorganic phosphate (Pi) transporter; also low-affinity manganese transporter
↑ 0.70	$6.09 \times 10^{-10}$	<i>PHO87</i>	Low-affinity inorganic phosphate (Pi) transporter
↑ 3.35	$1.6 \times 10^{-143}$	<i>PHO89</i>	Plasma membrane Na <sup>+</sup> /Pi cotransporter
↑ 1.01	$2.28 \times 10^{-16}$	<i>PHO90</i>	Low-affinity phosphate transporter
<b>Plasma ATPases</b>			
0.0	1	<i>PMA1</i>	Plasma membrane H <sup>+</sup> -ATPase
↑ 4.72	$2.29 \times 10^{-145}$	<i>PMA2</i>	Plasma membrane H <sup>+</sup> -ATPase
<b>Manganese transporters</b>			
0.15	0.23	<i>SMF1</i>	Cell surface manganese transporter
↑ 0.63	$8.3 \times 10^{-08}$	<i>SMF2</i>	ER membrane manganese transporter
↑ 0.38	0.0006	<i>PMR1</i>	Golgi manganese transporter
↑ 0.60	$3.79 \times 10^{-7}$	<i>CCC1</i>	Vacuolar membrane manganese transporter
<b>Upregulated uncharacterized proteins</b>			
↑ 5.12	$5.4 \times 10^{-185}$	<i>YBR056W-A (MNC1)</i>	CYSTM-family protein
↑ 4.55	$1.08 \times 10^{-181}$	<i>YOR385W</i>	Homologous to DIA1P

major H-ATPase gene, *PMA1*, was unchanged, while the expression of its ortholog, *PMA2*, considerably increased. Probably, the increase in the *PMA2* expression, which is more active in the presence of manganese than *PMA1*,<sup>39</sup> is necessary for cytoplasmic membrane energization. Cell lysis of the  $\Delta pma2$  mutant proves the necessity of *PMA2* for yeast adaptation to high concentrations

of manganese ions. At the same time, the lytic phenotype of the  $\Delta pma2$  and  $\Delta ybr056w-a$  strains (Fig. 2) makes it difficult to study their transcriptomic state and physiological properties under Mn<sup>2+</sup> conditions.

The important role of *YBR056W-A* in manganese detoxification suggests analysis of other CYSTM family members. For example,



dimers of CYSTM protein Pcc1 of *Arabidopsis thaliana* are anchored in the plasma membrane.<sup>43</sup> A suggested role of CYSTM proteins in metal homeostasis is as follows: the peculiar arrangement of sulfhydryl groups of the protein within the membrane could alter the redox potential of the membrane or directly chelate metal ions.<sup>36</sup>

A yeast paralog of YBR056W-A, YDR034W-B<sup>44,45</sup> also encodes a short peptide (51 a.a.) and has been previously annotated as CYSTM protein and supposed to be localized in the cell periphery.<sup>44,46</sup> However, YDR034W-B did not change its expression under manganese adaptation in this study. Other known members of the CYSTM family were also stably (e.g. YBR016W, YDL012C) or lowly (YDR210W) expressed. Probably they are responsible for chelating other metal ions.

Notably, the microarrays used in the studies<sup>5,32</sup> did not include any probes for the YBR056W-A gene. Thus, it is still possible that YBR056W-A is involved not only in adaptation but in the metal stress response as well. The CYSTM family is not fully annotated and we suppose its significance in stress and pathological processes is yet underestimated. For example, human CYSTM1 was recently found as a biomarker of Huntington's disease.<sup>47</sup>

## Conclusions

In this study for the first time we showed the functional relevance of an uncharacterized protein, YBR056W-A, which is essential for manganese detoxication. The proposed role of this CYSTM protein is to bind manganese at the cell surface, suggesting MNC1 (manganese-chelating protein) as a possible standard gene name. Further studies may elucidate other roles of YBR056W-A (Mnc1) and other CYSTM proteins in stress responses.

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## References

- 1 K. A. Feeney, L. L. Hansen, M. Putker, C. Olivares-Yañez, J. Day, L. J. Eades, L. F. Larrondo, N. P. Hoyle, J. S. O'Neill and G. van Ooijen, *Nature*, 2016, **532**, 375–379.
- 2 D. Canadell and J. Ariño, *Adv. Exp. Med. Biol.*, 2016, vol. 892, pp. 271–289.
- 3 V. C. Culotta, M. Yang and M. D. Hall, *Eukaryotic Cell*, 2005, **4**, 1159–1165.
- 4 M. Thorsen, G. G. Perrone, E. Kristiansson, M. Traini, T. Ye, I. W. Dawes, O. Nerman and M. J. Tamás, *BMC Genomics*, 2009, **10**, 105.
- 5 R. Wysocki and M. J. Tamás, *FEMS Microbiol. Rev.*, 2010, **34**, 925–951.
- 6 S. Rajakumar, C. Ravi and V. Nachiappan, *Metallomics*, 2016, **8**, 453–460.
- 7 Y. H. Jin, P. E. Dunlap, S. J. McBride, H. Al-Refai, P. R. Bushel and J. H. Freedman, *PLoS Genet.*, 2008, **4**, e1000053.
- 8 P. J. Kennedy, A. A. Vashisht, K.-L. Hoe, D.-U. Kim, H.-O. Park, J. Hayles and P. Russell, *Toxicol. Sci.*, 2008, **106**, 124–139.
- 9 J. Crowley, D. Traynor and D. Weatherburn, in *Manganese and its role in biological processes. Met. Ions Biol. Syst.*, ed. A. Siegel and H. Siegel, 1999, pp. 209–257.
- 10 G. F. Kwakye, M. M. B. Paoliello, S. Mukhopadhyay, A. B. Bowman and M. Aschner, *Int. J. Environ. Res. Public Health*, 2015, **12**, 7519–7540.
- 11 S. Bouabid, A. Tinakoua, N. Lakhdar-Ghazal and A. Benazzouz, *J. Neurochem.*, 2016, **136**, 677–691.
- 12 V. C. Culotta and M. J. Daly, *Antioxid. Redox Signaling*, 2013, **19**, 933–944.
- 13 A. R. Reddi, L. T. Jensen and V. C. Culotta, *Chem. Rev.*, 2009, **109**, 4722–4732.
- 14 L. T. Jensen, M. Ajua-Alemanji and V. C. Culotta, *J. Biol. Chem.*, 2003, **278**, 42036–42040.
- 15 L. Rosenfeld, A. R. Reddi, E. Leung, K. Aranda, L. T. Jensen and V. C. Culotta, *J. Biol. Inorg. Chem.*, 2010, **15**, 1051–1062.
- 16 L. Li, O. S. Chen, D. M. Ward and J. Kaplan, *J. Biol. Chem.*, 2001, **276**, 29515–29519.
- 17 K. J. Blackwell, J. M. Tobin and S. V. Avery, *Appl. Microbiol. Biotechnol.*, 1998, **49**, 751–757.
- 18 N. Andreeva, L. Ryazanova, V. Dmitriev, T. Kulakovskaya and I. Kulaev, *FEMS Yeast Res.*, 2013, **13**, 463–470.
- 19 N. Andreeva, L. Ryazanova, V. Dmitriev, T. Kulakovskaya and I. Kulaev, *Folia Microbiol.*, 2014, **59**, 381–389.
- 20 A. Sethuraman, N. N. Rao and A. Kornberg, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 8542–8547.
- 21 J. H. Kagi and B. L. Vallee, *J. Biol. Chem.*, 1960, **235**, 3188–3192.
- 22 B. J. Bowman, S. E. Mainzer, K. E. Allen and C. W. Slayman, *Biochim. Biophys. Acta, Biomembr.*, 1978, **512**, 13–28.
- 23 T. V. Kulakovskaya, N. A. Andreeva, A. V. Karpov, I. A. Sidorov and I. S. Kulaev, *Biochemistry*, 1999, **64**, 990–993.
- 24 M. Martin, *EMBnet.journal*, 2011, **17**, 10.
- 25 N. Joshi and J. Fass, Available at <https://github.com/najoshi/sickle>, 2011.
- 26 S. R. Engel, F. S. Dietrich, D. G. Fisk, G. Binkley, R. Balakrishnan, M. C. Costanzo, S. S. Dwight, B. C. Hitz, K. Karra, R. S. Nash, S. Weng, E. D. Wong, P. Lloyd, M. S. Skrzypek, S. R. Miyasato, M. Simison and J. M. Cherry, *G3*, 2014, **4**, 389–398.
- 27 C. Trapnell, L. Pachter and S. L. Salzberg, *Bioinformatics*, 2009, **25**, 1105–1111.
- 28 S. Anders, P. T. Pyl and W. Huber, *Bioinformatics*, 2014, **31**, 166–169.
- 29 M. D. Robinson, D. J. McCarthy and G. K. Smyth, *Bioinformatics*, 2010, **26**, 139–140.
- 30 R. Balakrishnan, J. Park, K. Karra, B. C. Hitz, G. Binkley, E. L. Hong, J. Sullivan, G. Micklem and J. M. Cherry, *Database*, 2012, **2012**, bar062.
- 31 P. Sengottaiyan, J. Petrlova, J. O. Lagerstedt, L. Ruiz-Pavon, M. S. Budamagunta, J. C. Voss and B. L. Persson, *Biochem. Biophys. Res. Commun.*, 2013, **436**, 551–556.



- 32 D. Hosiner, S. Gerber, H. Lichtenberg-Fraté, W. Glaser, C. Schüller and E. Klipp, *PLoS One*, 2014, **9**, e83330.
- 33 A. R. Fernandes and I. Sá-Correia, *Yeast*, 2003, **20**, 207–219.
- 34 R. C. Vallari, W. J. Cook, D. C. Audino, M. J. Morgan, D. E. Jensen, A. P. Laudano and C. L. Denis, *Mol. Cell. Biol.*, 1992, **12**, 1663–1673.
- 35 A. M. Michel, G. Fox, A. M. Kiran, C. De Bo, P. B. F. O'Connor, S. M. Heaphy, J. P. A. Mullan, C. A. Donohue, D. G. Higgins and P. V. Baranov, *Nucleic Acids Res.*, 2014, **42**, D859–D864.
- 36 T. M. Venancio and L. Aravind, *Bioinformatics*, 2010, **26**, 149–152.
- 37 A. Marchler-Bauer, M. K. Derbyshire, N. R. Gonzales, S. Lu, F. Chitsaz, L. Y. Geer, R. C. Geer, J. He, M. Gwadz, D. I. Hurwitz, C. J. Lanczycki, F. Lu, G. H. Marchler, J. S. Song, N. Thanki, Z. Wang, R. A. Yamashita, D. Zhang, C. Zheng and S. H. Bryant, *Nucleic Acids Res.*, 2015, **43**, D222–D226.
- 38 M. Kuramata, S. Masuya, Y. Takahashi, E. Kitagawa, C. Inoue, S. Ishikawa, S. Youssefian and T. Kusano, *Plant Cell Physiol.*, 2009, **50**, 106–117.
- 39 P. Supply, A. Wach and A. Goffeau, *J. Biol. Chem.*, 1993, **268**, 19753–19759.
- 40 P. L. Coleman and H. Weiner, *Biochemistry*, 1973, **12**, 3466–3472.
- 41 M. Kwolek-Mirek and R. Zadrag-Tecza, *FEMS Yeast Res.*, 2014, **14**, 1068–1079.
- 42 Y. Cohen, M. Megyeri, O. C. W. Chen, G. Condomitti, I. Riezman, U. Loizides-Mangold, A. Abdul-Sada, N. Rimon, H. Riezman, F. M. Platt, A. H. Futerman and M. Schuldiner, *PLoS One*, 2013, **8**, e85519.
- 43 R. Mir and J. León, *PLoS One*, 2014, **9**, e87216.
- 44 J. M. Cherry, E. L. Hong, C. Amundsen, R. Balakrishnan, G. Binkley, E. T. Chan, K. R. Christie, M. C. Costanzo, S. S. Dwight, S. R. Engel, D. G. Fisk, J. E. Hirschman, B. C. Hitz, K. Karra, C. J. Krieger, S. R. Miyasato, R. S. Nash, J. Park, M. S. Skrzypek, M. Simison, S. Weng and E. D. Wong, *Nucleic Acids Res.*, 2012, **40**, D700–D705.
- 45 K. P. Byrne and K. H. Wolfe, *Genome Res.*, 2005, **15**, 1456–1461.
- 46 W.-K. Huh, J. V. Falvo, L. C. Gerke, A. S. Carroll, R. W. Howson, J. S. Weissman and E. K. O'Shea, *Nature*, 2003, **425**, 686–691.
- 47 A. Mastrokolias, Y. Ariyurek, J. J. Goeman, E. van Duijn, R. A. C. Roos, R. C. van der Mast, G. B. van Ommen, J. T. den Dunnen, P. A. C. 't Hoen and W. M. C. van Roon-Mom, *Eur. J. Hum. Genet.*, 2015, **23**, 1349–1356.

