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Metal-sensing transcription factors Mac1p and Aft1p coordinately regulate vacuolar copper transporter *CTR2* in *Saccharomyces cerevisiae*

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ABSTRACT

CTR2 encodes a low-affinity copper transporter that mediates the mobilization of vacuolar copper stores in yeast. We previously reported that *CTR2* can be upregulated by copper deficiency via copper-sensing transcription factor Mac1p. In the present study, we found that iron depletion also induces the transcription of *CTR2*. The upregulation of *CTR2* induced by iron depletion was abrogated by the genetic deletion of either Mac1p or iron-sensing transcription factor Aft1p. The ablation of either *MAC1* or *AFT1* also abrogated *CTR2* expression induced by copper depletion. Our further study revealed that exogenous Aft1p upregulates *CTR2* transcription only in the presence of Mac1p, whereas exogenous Mac1p upregulates *CTR2* transcription only in the presence of Aft1p. Exogenous Mac1p and Aft1p form a stable complex and synergistically enhance *CTR2* transcription. These data suggest that Aft1p and Mac1p might corporately regulate transcription of *CTR2*.

1. Introduction

Copper is an essential metal element that functions as crucial prosthetic groups of enzymes involved in mitochondrial respiration, reactive oxygen species (ROS) scavenging, and iron incorporation. However, high concentrations of cytosolic copper ions are toxic, and copper homeostasis is tightly controlled by sophisticated regulatory systems in organisms, ranging from bacteria and plants to mammals. As a model eukaryote that shares similar metal homeostasis pathways with mammalian cells, *Saccharomyces cerevisiae*, commonly known as baker's yeast, is widely used for the investigation of copper homeostasis, and most knowledge in this field originated from yeast studies [1–4].

Metal-sensing transcription factors are vital for the regulation of genes involved in metal homeostasis in yeast. Mac1p is a copper-responsive transcription factor. It has two nuclear localization signals and is found in the nucleus in both copper-starved and copper-replete cells [5–7]. Mac1p is inactivated and unstable at high levels of copper ions. Under copper-deficient conditions, however, Mac1p is prone to dimerize to form ternary activation complexes on the promoters of target genes, including the high-affinity copper transporters Ctr1p and Ctr3p [7]. Aft1p and Aft2p are transcription factors involved in iron utilization and homeostasis. In response to iron deficiency, Aft1p and Aft2p activate a group of genes collectively known as the iron-regulon. Genes in this group encode proteins responsible for increasing bio-available iron [8–10]. Aft1p has been reported to be modulated by monothiol glutaredoxins Grx3p and Grx4p through physical interactions [11,12]. Recently, Aft1p was also found to interact with kinetochore protein Iml3p and promote pericentromeric cohesion, which plays important roles in chromosome stability and transmission [13].

Ctr2p is a low-affinity copper transporter located at the vacuolar membrane that mediates the mobilization of copper stored in yeast vacuoles under copper-deficient conditions [14–16]. In mammalian cells, Ctr2 is localized at the membrane of late endosomes and lysosomes and mediates the mobilization of lysosomal copper stores [17]. We previously reported that *CTR2* gene transcription can be regulated by Mac1p in yeast. Copper depletion can upregulate *CTR2* transcription, and this regulation can be abrogated by the genetic ablation of *MAC1*. Chromatin immunoprecipitation (ChIP) experiments revealed that Mac1p can form a complex with the *CTR2* promoter. This regulation may promote copper mobilization, thus allowing organisms to survive copper starvation [18].

In the present study, we found that Aft1p can also mediate the transcriptional regulation of *CTR2*. Iron depletion upregulates *CTR2* transcription, which can be abrogated by ablating either *AFT1* or *MAC1*. The genetic deletion of Mac1p or Aft1p can also eliminate the upregulation of *CTR2* induced by copper deficiency. Further experimentation confirmed that Aft1p and Mac1p depend on each other for the upregulation of *CTR2* transcription, in which exogenous Aft1p can upregulate *CTR2* transcription only in the presence of Mac1p, and exogenous Mac1p can upregulate *CTR2* transcription

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only in the presence of Aft1p. Exogenous Mac1p and Aft1p form a stable complex and synergistically enhance *CTR2* transcription. These data suggest that Aft1p and Mac1p coordinately regulate *CTR2* transcription. Our study is helpful to elucidate the regulation of *CTR2* and metal homeostasis.

2. Materials and methods

2.1. Yeast strains and constructs

Wildtype BY4742 (genotype: *MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ), aft1Δ* (BY4742 background; genotype: *MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, aft1Δ::KanMX4),* and *aft2Δ* (BY4742 background; genotype: *MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, aft2Δ::KanMX4)* were obtained from the laboratory of Bing Zhou (Tsinghua University, Beijing, China).

To generate the *mac1Δ* yeast strain (BY4742 background; genotype: *MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, mac1Δ*::*LEU2*), we designed polymerase chain reaction (PCR) primers to amplify the *LEU2* cassette flanked by 40–50 bases that correspond to the immediately downstream and upstream region of *MAC1* ORF. Yeast cells were transformed with the PCR product, and integrants were selected on an SD-Leu plate (Genmed, USA). The genetic deletion of *MAC1* was verified by PCR.

pADH1-Mac1-Flag was constructed previously [18]. The coding region of the yeast *AFT1* gene was cloned into a modified pYes2 vector (Invitrogen), pYes2-ADH1-Myc, in which Aft1p was tagged with a Myc epitope at the *C*-terminus and controlled by an ADH1 promoter. The resulting construct, pYes2-ADH1-Aft1-Myc, was verified by sequencing. Plasmids were amplified in *Escherichia coli* DH5α and extracted with a Plasmid DNA mini-prep kit (Sangon, Shanghai, China). pADH1-Mac1-Flag and/or pYes2-ADH1-Aft1-Myc or their vector controls were transformed into yeast of the desired background using a standard lithium acetate method [19].

2.2. Yeast culture and growth conditions

Wildtype BY4742, *mac1Δ*, *aft1Δ*, or *aft2Δ* yeast cells grown in liquid YPD (1% [w/v] yeast extract, 2% [w/v] peptone, 2% [w/v] dextrose) to midlog phase were 1:1 inoculated into fresh liquid YPD supplemented with the indicated chemicals [20]. For the strains transformed with pYes2-ADH1-Aft1-Myc (with a *URA3* selection marker), pADH1-Mac1-Flag (with a *LEU2* selection marker), or their vector controls, the medium was substituted with liquid SD-Ura, SD-Leu, or SD-Ura-Leu (Genmed). The experiments were performed in triplicate. Five hours later, the cells were harvested and subjected to quantitative reverse-transcription PCR (qRT-PCR).

2.3. Quantitative reverse transcription-polymerase chain reaction

RNAs were extracted using the hot phenol method [20] and digested with RNase-free DNaseI (TaKaRa) to remove genomic DNA contamination. Reverse transcription with Superscript III Reverse Transcriptase (Invitrogen) was performed as described in the manufacturer's instructions. Quantitative PCR experiments were performed using a 7300 ABI instrument (Invitrogen) and a standard PCR protocol (denaturation at 95 °C and annealing/extension at 60 °C) with the addition of a final dissociation step to ensure amplicon-specific detection by SYBR Green. Samples were prepared by adding cDNA to SYBR Green PCR Master Mix (Applied Biosystems) using the following primers: GGTGCACACGCGT GGCTTTT and CTGTGGTCGTGGCCCGCATT for *CTR2*; ATTGCTGTTG CCGATACCACTTC and GTCGCGCTCTATGTTTGCTTGAT for *CTR1*; ACAAGCGGAGACGCACACG and CTGCACACGGTCACCTTTG for *FET3*; TTTGGTTCCGGTGTTATTGTTGC and CCCCAGTTACCGGTTTG TCCTAC for *ZRT1*. We chose *ACT1* as a reference gene because it has a very low variation coefficient under different conditions. Its primers were as follows: TCCGGTGATGGTGTTACTCA and GGCC AAATCGATTCTCAAAA.

2.4. Coimmunoprecipitation and Western blot

Yeast cells co-transformed with pADH1-Mac1-Flag and pYes2-ADH1-Aft1-Myc were grown to midlog phase in SD-Leu-Ura medium (Genmed), and cell lysates were prepared by glass beading in a lysis buffer containing 50 mm Tris–Cl, pH 7.5, 150 mm sodium chloride, 0.1% Nonidet P-40, 0.05% sodium deoxycholate, and a protease inhibitor mixture [20]. The supernatant was subjected to coimmunoprecipitation (CoIP) as previously described [21]. Briefly, approximately 1 mg of total protein was divided into two equal halves for immunoprecipitation with protein A-agarose (GE Healthcare) plus 3 μg of M2 Flag mouse monoclonal antibody (Sigma) or normal mouse immunoglobulin G (IgG; as a negative control), respectively. The beads were collected by centrifugation, washed three times, and boiled in sodium dodecyl sulfate (SDS) sample buffer. The immunoprecipitated protein was resolved by SDS-10% polyacrylamide gel electrophoresis and transferred to nitrocellulose. The membranes were blocked and probed with either mouse monoclonal anti-Flag or rabbit polyclonal anti-Myc (Santa Cruz Biotechnology). Detection was performed by enhanced chemiluminescence (ECL; Pierce Technology) after incubation with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology).

2.5. Statistical analysis

Data were expressed as mean value ± standard deviation (SD). *P* values were calculated using Student's *t*-test.

3. Results

3.1. Iron depletion upregulates CTR2 transcription, which can be abrogated by ablating AFT1 or MAC1

We previously reported that *CTR2* transcription can be regulated by copper levels [18,20]. However, a report from another group indicated that *CTR2* mRNA levels are increased by treatment with the hydrophilic iron chelator bathophenanthroline disulfonate (BPS) [22]. To verify the *CTR2* upregulation induced by iron depletion, we treated the wildtype yeast strain BY4742 with 100 μM BPS. The qRT-PCR results (Fig. 1A) showed that *CTR2,* similar to the well-known iron-responsive gene *FET3*, was upregulated by BPS [1]. As an iron-unresponsive control, the mRNA levels of the high-affinity zinc transporter *ZRT1* were not significantly changed by BPS. These results indicate that *CTR2*, similar to *FET3*, can also be upregulated by iron depletion. This is consistent with a previous report [22].

Two transcription factors, Aft1p and Aft2p, have been reported to sense intracellular iron levels and regulate the transcription of genes involved in iron homeostasis [9]. To identify the factor that mediates the upregulation of *CTR2* induced by iron deficiency, we used yeast strains with genetic ablation of these two transcription factors, *aft1Δ* and *aft2Δ*, respectively. The qRT-PCR results (Fig. 1B) showed that the genetic deletion of *AFT1*, but not *AFT2*, abrogated the BPS-induced upregulation of *CTR2*. This suggests that Aft1p, but not Aft2p, is involved in *CTR2* expression induced by iron depletion. Unexpectedly, the genetic deletion of copper-sensing transcription factor Mac1p, which can remove the upregulation of *CTR2* induced by copper deficiency [18], also abrogated *CTR2* transcription enhanced by iron depletion (Fig. 1B). These data suggest that both