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Chromatin repositioning activity and transcription machinery are both recruited by Ace1p in yeast *CUP1* activation

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ABSTRACT

The relationship among transcriptional activators, nucleosome repositioning activity and transcription machinery at the yeast *CUP1* gene was addressed. *CUP1* encodes a cysteine-rich, copper-binding metallothionein that protects cells against copper toxicity through its ability to sequester copper. The induction of *CUP1* requires the presence of Ace1p and the binding of Ace1p at the *CUP1* promoter during activation provides evidence that Ace1p is directly involved in *CUP1* induction. Furthermore, transcriptional activation of *CUP1* resulted in nucleosome repositioning at the *CUP1* promoter and sequences further downstream in the coding region, suggesting a gene-wide chromatin remodeling activity. Such remodeling activity depends on the presence of transcription activator Ace1p. The recruitment of RNA polymerase II also requires the presence of Ace1p. Therefore, these observations provide insight into the molecular mechanism of *CUP1* activation.

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1. Introduction

Copper homeostasis is a tightly regulated pathway and it plays an important role in regulating the response of cells to copper. Two different types of homeostatic pathways might control the levels of copper in cells. The first pathway is the posttranslational mechanism, including intracellular trafficking of copper transporters, copper-stimulated endocytosis, and degradation of proteins involved in copper uptake [1–3]. The second pathway is mediated by the transcriptional control of copper homeostatic genes. These genes are responsible for copper acquisition, mobilization, and sequestration [4]. Loss of copper homeostasis in humans leads to diseases, such as Wilson disease and Menkes disease [5,6]. Therefore, the study of copper homeostasis at the molecular levels can provide insight in treating such disorders.

The regulation of copper homeostatic genes is conserved throughout eukaryotes. This high degree of conservation makes *Saccharomyces cerevisiae* an excellent model organism to utilize in order to gain insight into mammalian copper metabolism. In yeast, resistance to copper is primarily mediated by the Ace1p-dependent induction of the *CUP1* gene [7,8]. *CUP1* encodes a cysteine-rich, copper-binding metallothionein that protects cells against copper toxicity through its ability to sequester copper [9–12]. In the cell, copper ions bind to the N-terminal domain of Ace1p, a transcription activator, which activates *CUP1* transcription through its C-terminal acidic activation domain. When enough metallothionein is produced, it will bind free copper in the cytoplasm and this will lead to the departure of Ace1p from the nucleus, which will shut off *CUP1* transcription [13–17].

Transcriptional coactivators, including chromatin remodelers and histone modifiers contribute to transcriptional activation. Previously, we have shown that the induction of *CUP1* with copper results in nucleosome repositioning across the entire *CUP1* gene, which requires the transcriptional activator but not the TATA boxes [18]. This result suggests that chromatin remodelers are recruited by Ace1p. Induction of *CUP1* with copper also resulted in targeted acetylation of both H3 and H4 at the *CUP1* promoter. Targeted acetylation of H3 and H4 required the transcriptional activator and the TATA boxes [19]. This suggests that targeted acetylation occurs when the TATA-binding protein binds to the TATA box or at a later stage in initiation. Taken together, the movement of nucleosomes occurring on *CUP1* during induction is independent of targeted acetylation. However, the mechanism of *CUP1* expression with respect to the recruitment of chromatin remodeling activity and transcriptional machinery in *CUP1* induction still remains unclear.

Here, biochemical analyses were used to examine the role of Ace1p in recruiting chromatin remodeling activity and transcrip-

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tional machinery during *CUP1* activation. We demonstrated that *CUP1* exhibits Ace1p-dependent expression through survival analysis and RNA analysis. Subsequently, chromatin immunoprecipitation (ChIP) coupled with real-time PCR (qPCR) analysis was employed to show that Ace1p is directly involved in regulating *CUP1* induction and it is required in the recruitment of RNA polymerase II (Pol II). These observations provide direct evidence of the recruitment of chromatin remodeling activity and transcriptional machinery in *CUP1* induction.

2. Materials and methods

2.1. Yeast strains and growth conditions

WT (BJ5459; MATa ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL cir+), ace1Δ (*MATa ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL cir+ ace1ΔURA 3*), and *ace1Δ-ACE1*HA (*MATa ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL cir+.ace1ΔURA 3:: ACE1HA*) were used in this study. Yeast cells were grown at 30 °C in SC media (synthetic complete media) containing 2% glucose (wt/vol) without copper. When the optical density (O.D.) reached 0.9–1.1, copper was added to a final concentration of 1.5 mM as required for inducing conditions, and incubated for 1 h at 30 °C.

2.2. RNA preparation and qRT-PCR analysis

The total RNA preparation and qRT-PCR analysis was performed as described previously [20,21]. Briefly, 250 ml cells were harvested and resuspended in 400 μl lysis solution (10 mM Tris HCl pH 7.5, 10 mM EDTA, 0.5% SDS). Subsequently, an equal volume of acid phenol (pH 4.3) was added to the cells suspension. After 1 h incubation at 65 °C, the mixture was subjected to centrifugation, and the aqueous phase was mixed with an equal volume of acid phenol again. After 5 min of incubation on ice, the aqueous phase was subjected to chloroform extraction, ethanol precipitation, and was resuspended in 50 μl DEPC-treated H_2O.

Equal amounts (10 μg) of total RNA were treated with RNase-free DNase (Qiagen Cat#79254) at 37 °C for 1 h, and purified by phenol/chloroform (3:1) extraction and ethanol precipitation. 1 μg of pure RNA was used in SYBR GreenER Two-Step qRT-PCR kit (Invitrogen Cat#11765-100) for the first strand cDNA synthesis and real-time PCR reaction preparation as described in manufacture's manual.

The real-time PCR primers are described in the Supplementary materials. All experiments were repeated twice, and in each experiment, PCRs were done in triplicate in a 7500 sequence detection system (Applied Biosystems). Target DNA sequence quantities were estimated as described previously [20,21]. Briefly, target DNA sequence quantities were estimated from the threshold amplification cycle number (C_T) using Sequence Detection System software (Applied Biosystems). Each DNA quantity was normalized to the *ACT1* DNA quantity by taking the difference between each gene's C_T and *ACT1*'s C_T value which is a ΔC_T value. Each relative RNA fold change was calculated with the following formula: $2^{(-\Delta c_T)}$ [21]. The expression ratio was then calculated by taking the ratio of inducing relative RNA fold change to repressing relative RNA fold change.

2.3. ChIP and real-time PCR analysis

The preparation of cross-linked chromatin, immunoprecipitation procedures, and real-time PCR analyses were performed as described previously [20,21]. All real-time PCR primers, TaqMan MGB probes, and the procedures to prepare cross-linked chromatin and to immunoprecipitate target DNA are listed in the Supplementary Materials. Briefly, 1 μg of ChIP-derived DNA, except for input DNA, which was 10 ng, were used as the template in 50 μl singleplex reactions containing 25 μl 2× TaqMan Universal Master Mix (Applied Biosystems), 0.9 μM of each real-time primer and 0.25 μM of each TaqMan MGB probe. The reaction was performed in a 7500 sequence detection system. Target DNA sequence quantities were estimated from the threshold amplification cycle number (C_T) using Sequence Detection System software (Applied Biosystems). A ΔC_T value was calculated for each sample by subtracting the C_T value for the IP sample from the C_T value for the corresponding input DNA to normalize the differences in ChIP aliquots. Each IP quantity was then calculated with the following formula: $2^{(-\Delta c_T)}$. For the Ace1p occupancy experiments, the IP DNA quantity of the upstream activation sequences (*UAS*; normalized to the *CUP1 UAS* input) or of the *TATA* region (*TATA*; normalized to the *CUP1 TATA* input) were normalized to the *CUP1* open reading frame acetylation quantity (ORF; normalized to the *CUP1* ORF input) by taking the ratio of *CUP1 UAS* IP DNA to *CUP1 ORF* IP DNA (*UAS*/*ORF*) or the ratio of *CUP1 TATA* IP DNA to *CUP1 ORF* IP DNA (*TATA*/*ORF*) [20,21]. For the Pol II and H4 occupancy experiments, the IP DNA quantity of the *UAS* (normalized to the *CUP1 UAS* input) or of the *TATA* (normalized to the *CUP1 TATA* input) was normalized to the *POL1* open reading frame acetylation quantity (normalized to the *POL1* input) by taking the ratio of *CUP1 UAS* IP DNA to *POL1* IP DNA (*UAS*/*POL*) or the ratio of *CUP1 TATA* IP DNA to *POL1* IP DNA (*TATA*/*POL*) [20,21]. All experiments were repeated twice, and in each experiment, PCRs were done in triplicate.

3. Results

3.1. CUP1 gene exhibits Ace1p-dependent expression

It has been shown that *CUP1* is expressed in the presence of copper sulfate during the logarithmic phase of growth. To demonstrate that *CUP1* expression is regulated by the transcriptional activator Ace1p, both WT and *ace1Δ* cells were grown in SC–Cu media. When the O.D. reached 0.2, copper was added to a final concentration of 1.5 mM as required for inducing conditions. Aliquots of culture were removed for O.D. measurements following copper addition. The results showed that the WT cells grew well in the absence of copper. In the presence of copper, the WT cells also grew well but with a doubling time about 60% longer (Fig. 1A). The *ace1Δ* cells grew well in the absence of copper, but were unviable in the presence of copper. The introduction of the engineered *ACE1*-HA tag into *ace1Δ* cells (*ace1Δ-ACE1*HA strain) restored growth in 1.5 mM of copper. As a result, Ace1p is required for *CUP1* expression in the presence of copper and the integrated *ACE1*HA is functional in the *ace1Δ* cells.

To further examine the role of Ace1p on *CUP1* expression, *CUP1* mRNA levels were measured by qRT-PCR. The expression ratios of *CUP1*/*ACT1* were 3.8 ± 0.2 and 7.5 ± 0.5 for WT cells after a ½ and 1 h addition of copper, respectively (Fig. 1B). In the absence of copper, the expression ratios were about 0.8 ± 0.1 and 0.7 ± 0.2 for WT cells after a ½ hour and 1 h incubation, respectively. Thus, *CUP1* is expressed within ½ h of induction under the given conditions. It reached the maximum level of expression after 1 h of induction. For *ace1Δ* cells, *CUP1* mRNA did not significantly increase in either repressing or inducing conditions. The ratios of *CUP1*/*ACT1* were approximately 1 under both conditions (Fig. 1B). For *ace1Δ-ACE1*-HA cells, the expression ratios of *CUP1*/*ACT1* were 5.2 ± 0.5 and 8.0 ± 0.7 after a ½ and 1 h addition of copper, respectively. In the absence of copper, the expression ratios were about 1.0 ± 0.2 and 1.0 ± 0.2 for after a ½ and 1 h incubation, respectively. Therefore,

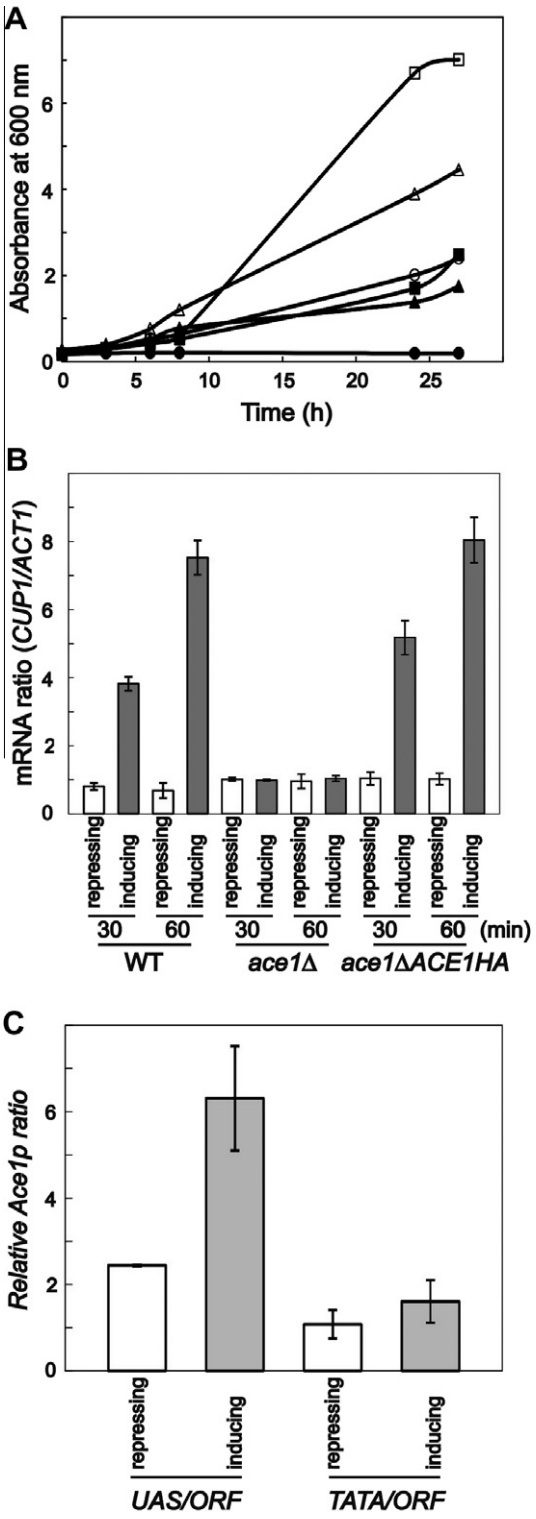

Fig. 1. *CUP1* expression is regulated by Ace1p. (A) Growth of yeast cells in the presence or absence of copper. WT cell: (□) SC, (■) SC + 1.5 mM $CuSO_4$ *ace1Δ* cell: (○) SC, (●) SC + 1.5 mM $CuSO_4$ *ace1Δ-ACE1*HA cell: (Δ) SC, (▲) SC + 1.5 mM $CuSO_4$. (B) *CUP1* mRNA was examined by qRT-PCR analysis. The expression ratio for *CUP1* mRNA/*ACT1* mRNA after 30 and 60 min repression or induction is graphed as mean ± standard deviation. All experiments were repeated at least twice, and in each experiment PCR reactions were done in triplicate. (C) Real-time PCR analysis of Ace1p at the *CUP1* promoter and *ORF*. Real-time PCR of DNA immunoprecipitated with antibodies against HA tag (α-HA) at the *CUP1* upstream activation sequences *(UAS), CUP1 TATA* sequences (*TATA*), and part of the coding region of *CUP1* gene (*ORF*) for *ace1Δ-ACE1*HA cells. All experiments were repeated at least twice and, in each experiment, PCRs were done in triplicate. The relative Ace1p ratios for the *UAS/ORF* and *TATA/ORF* are graphed as mean ± standard deviation.

the deletion of *ACE1* led to copper sensitivity due to an inability to express *CUP1* at the transcriptional level.

3.2. Ace1p directly participates in CUP1 activation

Subsequently, ChIP coupled with qPCR analysis was performed to prove that Ace1p directly participates in the induction of *CUP1*. We have demonstrated that *ace1Δ-ACE1*HA cells exhibit similar growth pattern as WT cells in the presence of copper (Fig. 1A and B), the HA antibody was used in *ace1Δ-ACE1*HA strain to examine the presence of Ace1p at *CUP1* promoter.

For repressing and inducing *ace1Δ-ACE1*HA strain, the IP signals of Ace1p-HA was observed at the *CUP1* upstream activation sequences (*UAS*), *CUP1 TATA* sequences (*TATA*), and part of the coding region of the *CUP1* gene (*ORF*). The IP ratio of *UAS*/*ORF* and *TATA*/*ORF* were measured to evaluate the presence of Ace1p. Under repressing conditions, the ratios were 2.44 ± 0.01 and 1.07 ± 0.33 for *UAS*/*ORF* and *TATA*/*ORF*, respectively (Fig. 1C). Under inducing conditions, the ratios were 6.30 ± 1.21 and 1.60 ± 0.49 for *UAS*/*ORF* and *TATA*/*ORF*, respectively. This result suggests the presence of small amounts of Ace1p at the *UAS* region and the absence of Ace1p at the *TATA* region under repressing conditions. Upon induction, more Ace1p was recruited to the *CUP1 UAS* region and no Ace1p was observed at the *TATA* region. As such, ChIP coupled with qPCR analysis proved the presence of Ace1p at the *CUP1 UAS* region and this result provided the direct evidence of the participation of transcriptional activator Ace1p in *CUP1* transcriptional activation.

3.3. Ace1p is required to recruit the transcription machinery

An important function of the transcriptional activator is to recruit chromatin remodeling activity and transcription machinery. Therefore, we first examined whether Ace1p is required to recruit transcription machinery. Again, ChIP coupled with qPCR analysis was used to examine the presence of Pol II. For repressing and inducing WT cells, IP signals of Pol II (Pol-IP) were observed at the *CUP1 TATA* sequences (*TATA*), the *CUP1 ORF* sequences (*ORF*), and part of the coding region of the *POL1* gene (*POL*) [22–24]. Results showed that the relative IP values of *TATA*/*POL* for Pol-IP were 1.16 ± 0.76 and 4.94 ± 0.79 under repressing and inducing conditions, respectively (Fig. 2A). For the *ORF* region, the relative IP values of *ORF*/*POL* for Pol-IP were 0.82 ± 0.04 and 2.06 ± 0.24 under repressing and inducing conditions, respectively (Fig. 2B). These results suggest that Pol II is recruited to the *CUP1 TATA* region and *CUP1 ORF* region under inducing conditions.

Next, *ace1Δ* cells were used to examine whether the recruitment of Pol II is activator-dependent. Our results showed that the relative IP values of *TATA*/*POL* for Pol-IP were 0.69 ± 0.02 and 1.0 ± 0.27 under repressing and inducing conditions, respectively (Fig. 2A). For the *ORF* region, the relative IP values of *ORF*/*POL* for Pol-IP were 0.60 ± 0.20 and 0.99 ± 0.39 under repressing and inducing conditions, respectively (Fig. 2B). These results clearly showed that Pol II is not recruited to the *ace1Δ* cells *CUP1 TATA* region and *ORF* region under inducing conditions. These results suggest that the recruitment of transcription machinery requires the presence of Ace1p. As such, *CUP1* mRNA expression data is accurately reflected in the observed occupancy of Pol II at the *CUP1* promoter, and the recruitment of transcription machinery depends on the presence of the transcriptional activator Ace1p under inducing conditions.

3.4. Chromatin remodeling is an Ace1p-dependent activity

Previous studies observed chromatin remodeling activity at the *CUP1* promoter upon induction [18]. We next wanted to examine

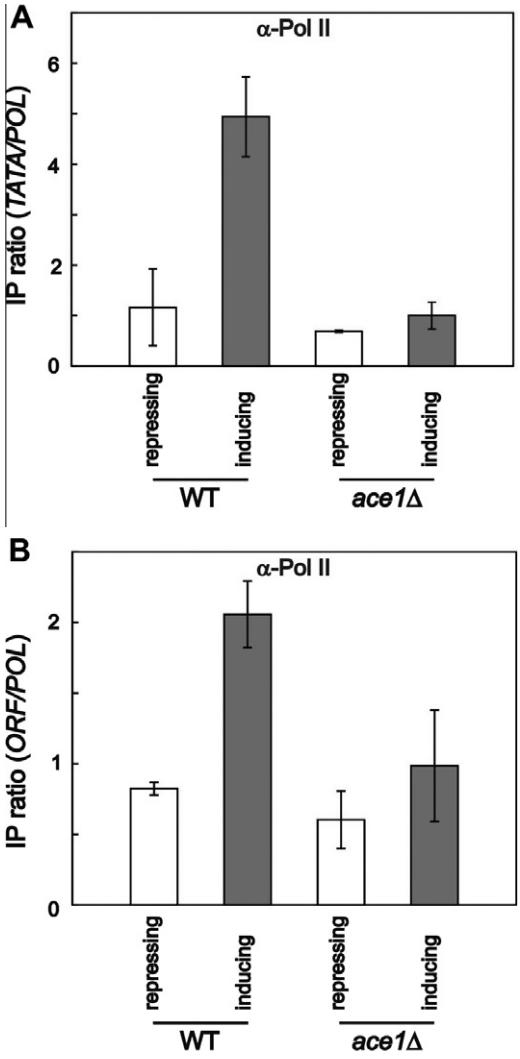

Fig. 2. Activator Ace1p is required to recruit RNA polymerase II during *CUP1* activation. Real-time PCR of DNA immunoprecipitated with antibodies against RNA polymerase II (α-Pol II) at the *CUP1 TATA*, *CUP1 ORF*, and part of the coding region of *POL1* gene (*POL*) for WT and *ace1Δ* cells. The IP ratios for the (A) *TATA*/*POL* and (B) *ORF*/*POL* are graphed as mean ± standard deviation. Annotation was as described in the legend of Fig. 1.

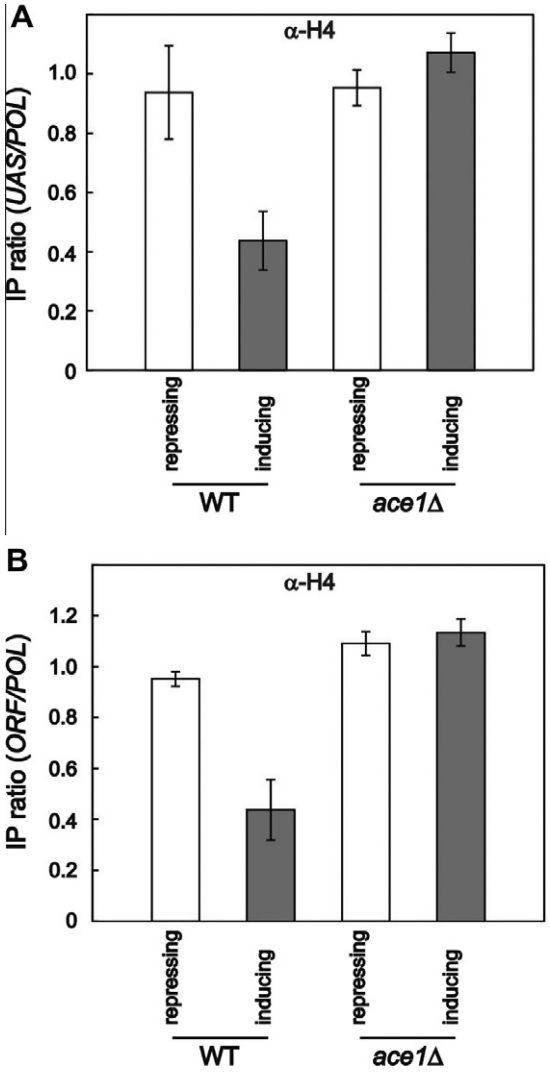

Fig. 3. The decrease of nucleosome density at the *CUP1* promoter during activation requires the presence of Ace1p. Real-time PCR of DNA immunoprecipitated with antibodies against histone H4 (α-H4) at the *CUP1 UAS*, *CUP1 ORF*, and *POL* for WT and *ace1Δ* cells. The IP ratios for the (A) *UAS*/*POL* and (B) *ORF*/*POL* are graphed as mean ± standard deviation. Annotation was as described in the legend of Fig. 1.

whether Ace1p is required to recruit chromatin remodeling activity. It has been shown that nucleosome density can accurately reflect chromatin remodeling activity by examining H3 or H4 occupancy [25]. As such, ChIP coupled with qPCR analysis was used to determine nucleosome density by examining the IP signals of histone H4 (H4-IP) at the *CUP1 UAS* region and *ORF* region for both WT and *ace1Δ* cells as described previously [25].

For H4-IP, the relative IP values of *UAS*/*POL* were 0.94 ± 0.16 and 0.44 ± 0.10 for repressing and inducing WT cells, respectively (Fig. 3A). For the *ORF* region, the relative IP values of *ORF*/*POL* were 0.95 ± 0.03 and 0.44 ± 0.12 for repressing and inducing WT cells, respectively (Fig. 3B). The decrease of nucleosome density during induction suggests the presence of both chromatin remodeling activity and nucleosome movement. The reconfiguration of chromatin structure is occurring not only at the *CUP1* promoter but also at the *ORF* region under inducing conditions.

For the *ace1Δ* cells, the relative IP values of *UAS*/*POL* were 0.95 ± 0.06 and 1.07 ± 0.07 for repressing and inducing cells, respectively (Fig. 3A). For the *ORF* region, the relative IP values of *ORF*/*POL* were 1.09 ± 0.05 and 1.13 ± 0.05 for repressing and inducing conditions, respectively (Fig. 3B). These results indicate that nucleosome density remains constant in *ace1Δ* cells *UAS* and *ORF* regions, suggesting that no chromatin remodeling activity in the absence of Ace1p under inducing conditions. As such, chromatin remodeling activity is recruited by Ace1p.

4. Discussion

Tight regulation of copper homeostatic machinery can ensure that sufficient copper is present in the cell to perform essential biochemical processes yet prevent the accumulation to toxic levels. In *S. cerevisiae*, Mac1p regulates copper uptake genes, and Ace1p regulates the transcriptional activation of detoxification genes. These two copper ion sensors detect copper and respond by appropriately regulating the expression of copper homeostasis genes in order to maintain the delicate balance between essential and toxic levels. In this report, we examined how Ace1p regulates *CUP1* expression at the molecular level. We have demonstrated that *CUP1* exhibits Ace1p-dependent expression. We also showed that *CUP1* expression is accurately reflected in the observed occupancy of Pol II. Pol II was not observed at *CUP1* for *ace1Δ* cells under

inducing conditions, suggesting a crucial role for Ace1p in recruiting transcription machinery. Furthermore, chromatin remodeling activity was absent at the *CUP1* promoter under both repressing and inducing conditions in the *ace1Δ* strain. This suggests that the Ace1p activator is required in recruiting chromatin remodeling activity. Chromatin remodeling activity is brought in by the transcriptional coactivators – chromatin remodelers. Thus, the chromatin remodelers that are responsible for *CUP1* expression are clearly activator-dependent remodelers.

For transcriptional activation, it normally requires the presence of chromatin remodeling activity and transcription machinery. Here, we provide the evidence that Ace1p directly participates in the *CUP1* activation under inducing conditions. Ace1p is required to recruit both chromatin remodeling activity and transcription machinery to the promoter (Fig. 2A and 3A). Furthermore, we found that remodeling activity present not only at the promoter region but also at the *ORF* region (Fig. 3B). This result indicates a gene-wide chromatin remodeling under inducing condition and agrees with previous findings [19]. Previously, it was shown that nucleosome repositioning was observed over the entire *CUP1* gene under inducing conditions through the analysis of a 2.4-kb episome. Therefore, the induction of *CUP1* results in the global chromatin remodeling.

Such global nucleosome repositioning is not unexpected. This is because the process of transcription would require the continuous action of transcription machinery on the entire gene. Indeed, we did also observe the presence of Pol II on the entire *CUP1* gene under inducing conditions (Fig. 2B). For Pol II to go through the entire gene, it is necessary that remodeling activity must present in the entire gene as well. As such, our observations provide the clear evidence for such transcription event.

In the Ace1p occupancy experiment, we observed the presence of Ace1p at the *CUP1* promoter under repressing conditions for WT cells. Upon induction, more Ace1p was recruited to the promoter (Fig. 1C). Despite the presence of Ace1p at the *CUP1* promoter under repressing conditions, we did not observe elevated levels of Pol II at the *TATA* region or *ORF* region for WT cells (Fig. 2), suggesting the absence of transcriptional activity. Indeed, *CUP1* mRNA levels remained constant in the WT cells under repressing conditions (Fig. 1B). Upon induction, more Ace1p was recruited to the promoter and more Pol II was recruited to both *CUP1 TATA* and *ORF* regions (Fig. 2). As a result, the *CUP1* mRNA levels increased dramatically (Fig. 1B). These results suggest that Ace1p is present at the *CUP1* promoter under both repressing and inducing conditions. However, Ace1p may not be functional under repressing conditions. Under inducing conditions, more Ace1p is recruited to the *CUP1* promoter and it becomes functional. Subsequently, chromatin remodeling activity is recruited to the promoter, resulting in the chromatin remodeling and Pol II recruitment. As a result, transcription is initiated.

The constitutive binding of the activator to the promoter has been observed in other genes [22,26,27]. In *INO1*, the transcriptional activator and chromatin remodelers are present at the promoter under repressing conditions. The activator is not functional as the repressor binds to the activator. Under inducing conditions, the repressor dissociates from the activator and the activator becomes functional. Subsequently, the functional activator stimulates chromatin remodelers in the absence of the repressor, and thus the activation process is initiated [24]. Here, we also observed constitutive binding of Ace1p to the *CUP1* promoter. Although the chromatin remodelers have not been identified for *CUP1* expression, it is possible that *CUP1* expression also follows such an activation pathway. Further analysis is necessary to uncover the specific transcriptional coactivators that are responsible for *CUP1* expression, which will provide key insight into the mechanism of the activation process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.047.

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