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# Mcm1p binding sites in *ARG1* positively regulate Gcn4p binding and SWI/SNF recruitment

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## ABSTRACT

Transcription of the arginine biosynthetic gene *ARG1* is activated by Gcn4p, a transcription factor induced by starvation for any amino acid. Previously, we showed that Gcn4p binding stimulates the recruitment of Mcm1p and co-activator SWI/SNF to *ARG1* in cells via Gcn4p induction through amino acid starvation. Here, we report that Gcn4p binding is reduced by point mutations of the Mcm1p binding site and increased by overexpression of Mcm1p. This result suggests that Mcm1p plays a positive role in recruiting activator Gcn4p to *ARG1*, similar to the previously described cooperative interaction of Mcm1p with sequence-specific transcription factors at their promoters. In addition, the mutational analysis of Mcm1p binding sites showed that recruitment of the co-activator SWI/SNF correlated more closely with binding of Mcm1p than of Gcn4p at *ARG1*. Consistent with this, SWI/SNF co-immunoprecipitated with Mcm1p, but not with Gcn4p. These results support that Mcm1p increases the SWI/SNF recruitment at *ARG1*, a Gcn4p target promoter. The interaction between Mcm1p and SWI/SNF was abolished in a *snf2* deletion strain containing an intact SWI/SNF sub-complex, suggesting that Mcm1p targets the catalytic subunit, which has ATPase activity, during SWI/SNF recruitment. We propose that Mcm1p contributes to active transcription at the *ARG1* promoter by increasing the binding of the activator Gcn4p and by recruiting the co-activator complex SWI/SNF at *ARG1* under Gcn4p-induced conditions.

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## Introduction

Transcription of most amino acid biosynthesis genes, including four *ARG* (*ARG1*, *ARG3*, *ARG5,6*, and *ARG8*) genes, is induced by Gcn4p in cells starved for any amino acid due to increased expression of *GCN4* at the translational level [1,2]. Mcm1p is also an important regulator of the *ARG* gene promoters [1,3]. Mcm1p mediates the repression of the four *ARG* genes in response to exogenous arginine by forming a repressor complex, ArgR/Mcm1p, through interactions with Arg80p, Arg81p, and Arg82p [1,3]. Indeed, the ArgR/Mcm1p repressor and the transcriptional activator Gcn4p dually regulate the *ARG* gene promoters.

Mcm1p is an MCM1, AGAMOUS, DEFICIENS, and serum response factor (MADS) box transcription factor protein that regulates the response of yeast to environmental conditions [3]. Mcm1p also plays an important role in DNA synthesis by binding at the replication origin [4]. Mcm1p cooperates with diverse se-

quence-specific transcription factors such as  $\alpha$ 1p,  $\alpha$ 2p, Ste12p, Yox1p, Yhp1, and Fkh2p at their promoters, stimulating or impeding the function of transcription factors that regulate cell type specification or the cell cycle [3,5,6]. Previously, we showed that binding of Gcn4p transcription factor stimulates the recruitment of Mcm1p to *ARG1* [7]. Therefore, it is conversely possible that binding of Mcm1p transcription factor might increase Gcn4p recruitment.

The yeast SWI/SNF complex is an ATP-dependent chromatin remodeling complex that contains 11 different subunits. Deletion of Snf2p, the ATPase subunit of SWI/SNF, is not lethal but leads to altered transcription of a subset (1–2%) of genes in nutrient-rich medium [8,9]. Snf2p can also mediate repression of *SER3* without the cooperation of other SWI/SNF subunits [9,10]. Genetic studies suggest that many of these subunits are required for the chromatin remodeling function of the complex [9]. The SWI/SNF complex is recruited to *ARG1* in a Gcn4p-dependent manner [11]. Mcm1p recruits the SWI/SNF complex to the *STE6* promoter and contributes to the activation of the complex [12]. Therefore, Mcm1p binding might contribute to recruitment of the SWI/SNF complex to the *ARG1* promoter.

Here, we report that Gcn4p binding is increased by Mcm1p binding. In addition, we provide strong evidence that Mcm1p bind-

**Abbreviations:** ARC, arginine control; MADS, MCM1, AGAMOUS, DEFICIENS, and serum response factor; ChIP, chromatin immunoprecipitations; UAS, upstream activating sequence; SM, sulfometuron methyl; h.c., high-copy; s.c., single-copy; WT, wild-type; WCE, whole cell extract.

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ing at *ARG1* contributes to the recruitment of SWI/SNF. Our results indicate that Mcm1p and Gcn4p cooperatively play a role in recruiting SWI/SNF to *ARG1*.

## Materials and methods

**Yeast strains and plasmids.** The strains used in this study are listed in Table 1 in the Supporting Information. The wild-type (WT) parent strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and deletion derivatives, generated by the *Saccharomyces* Genome Deletion Project, were purchased from Research Genetics. The strain SS5 (*MATα ura3Δ arg1-PH*) was a gift from Marjolaine Craebel [13]. The construction of novel strains to generate myc epitope tags, mutations of the *ARG1* promoter, or *gcn4Δ* strains is described in the Supporting Information.

Plasmids pHQ1293, pHQ1239, pED40, and pSK1 were described previously [7,11,14]. The empty vectors were the *URA3 CEN4* plasmid YCp50, the *LEU2* plasmid pRS315, and pRS425. The details of the construction of novel plasmids are provided in the Supporting Information.

**Chromatin immunoprecipitations.** Cells were grown to OD<sub>600</sub> of ~0.8 in an appropriate synthetic complete (SC) media (SC, SC-Ura, SC-Leu, SC-Ura-Leu). Cells were starved for isoleucine/valine at 30 °C, treated for 2 h with SM (0.6 μg/ml), and cross-linked with formaldehyde. Chromatin immunoprecipitation (ChIP) assays were conducted using anti-myc antibodies as previously described [7,11,14]. PCR analysis was conducted as described in the Supporting Information.

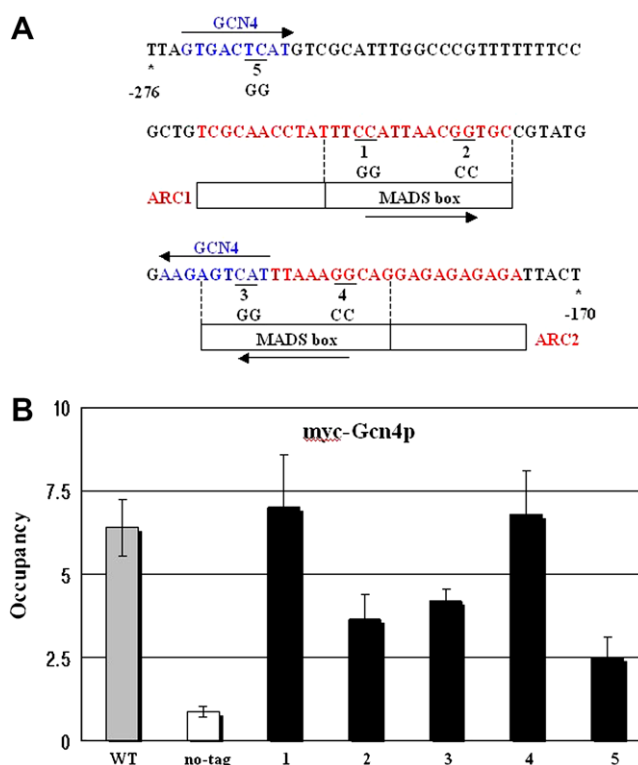
**Co-immunoprecipitation assays.** Cells were grown as described for ChIP analysis. Co-immunoprecipitation analysis was performed as described previously [7,11]. Briefly, whole cell extracts (WCEs) were prepared as described for GST-pull down assays [15]. The WCEs were incubated for 1 h at 4 °C with 1 μg anti-myc antibody, 100 μg BSA dissolved in PBS, and buffer MTB. The immune complexes were washed three times with 1 ml MTB, dissolved in SDS-PAGE loading buffer, and subjected to Western blot analysis.

## Results and discussion

### *Gcn4p* binding is reduced by point mutations in the MADS box

The *ARG1* promoter contains two arginine control elements (ARC1 and ARC2) and two Gcn4p binding sites, which are designated by blue and red letters in Fig. 1A, respectively. Genetic and biochemical analyses of the *ARG1* promoter have shown that two ARC elements are required for efficient repression of *ARG1* expression by the ArgR/Mcm1p complex in medium containing arginine. Two Gcn4p binding sites contribute to efficient activation of *ARG1* by Gcn4p [1,3,13,16]. The consensus sequence for Gcn4p contains nine nucleotides, ATGACTCAT. Both the 5'-most Gcn4p binding site and the second Gcn4p binding site (present in reverse orientation) contain eight consensus nucleotides. The half sequences of the ARC elements contain the MADS box, which is the binding site for Mcm1p [13,16]. The consensus sequence for the MADS box, called the P box, contains 16 nucleotides, TTTCCCTATTAGGTAA [17]. The first MADS box in ARC1 includes a greater number of consensus nucleotides (nine) than the second MADS box in ARC2 (six nucleotides). The two MADS boxes are also in opposite orientations, as designated by the arrows in Fig. 1A. Six base pairs in the second Gcn4p site overlap the second MADS box.

Mcm1p binding has been shown to enhance recruitment of other transcription factors [17]. In order to determine whether Mcm1p also plays a role in Gcn4p binding at the *ARG1* promoter, we introduced point mutations into the predicted MADS box binding sites in the ARC elements. To eliminate Mcm1p binding at each



**Fig. 1.** Gcn4p binding is reduced by point mutations in the MADS box. (A) Nucleotide sequence of the *ARG1* promoter region. The sequence is numbered (–276 to –170, indicated by asterisks) relative to the main transcription start site. The Gcn4p binding sites (GTGACTCAT and AAGAGTCAT) are overlined and designated with blue. The site-directed mutagenized sequences are underlined with black and indicated below by numbers 1–5. There are two arginine control elements (ARC1 and ARC2), which are binding sites for the ArgR/Mcm1p repressor complex, indicated by red letters and boxes. The putative Mcm1p binding sites are boxed within the ARC elements and designated as MCM1, AGAMOUS, DEFICIENS, and serum response factor (MADS) boxes. The two Gcn4p binding sites and MADS boxes are in opposite orientations and designated with arrows. (B) The *gcn4Δ* SS5 strains containing mutations in the *ARG1* alleles are indicated below the histogram; WT (SY722), mutant 1 (SY772), mutant 2 (SY723), mutant 3 (SY583), mutant 4 (SY724), and mutant 5 (SY773). The strains were transformed with a single-copy *GCN4-myc* plasmid (pSK1). For the non-tagging (no-tag) condition, *gcn4Δ* (SY722) strains were transformed with an empty vector. ChIP analysis of the transformants was conducted to measure binding of myc-Gcn4p to *ARG1*, as described in Materials and methods and Supporting Information. The percentage of *ARG1* UAS that immunoprecipitated with myc antibodies was measured for each strain and normalized to the nonspecific immunoprecipitation of *POL1* ORF sequences (Occupancy). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

ARC element, we replaced the conserved CC residues in each predicted half-site with GG residues in each of the two ARC elements (Fig. 1A). In the crystal structure, these residues in the MADS box are important for Mcm1p binding with Matα2 transcription factor to *STE6* promoter [17]. They make base-specific contacts with Lys-38 of Mcm1p, and substitution of these CC residues with GG residues strongly impairs the transcriptional repression mediated by Mcm1p/Matα2 binding to the *STE6* promoter [17].

We also made GG mutations in one of the half-sites in the Gcn4p binding sites to determine the effect of mutating Gcn4p binding sites on myc-Gcn4p binding (Fig. 1A). Note that the GG mutation in the second Gcn4p binding site also mutated two residues in the MADS box in ARC2. We designated the mutants as numbers 1–5 (Fig. 1A). The mutations were introduced into the chromosomal copy of *ARG1*. The *gcn4* deletion was generated in each mutant and transformed with a *GCN4-myc* plasmid encoding a functional myc-tagged form of Gcn4p [11,14]. After inducing myc-Gcn4p protein by incubating cells with sulfometuron methyl

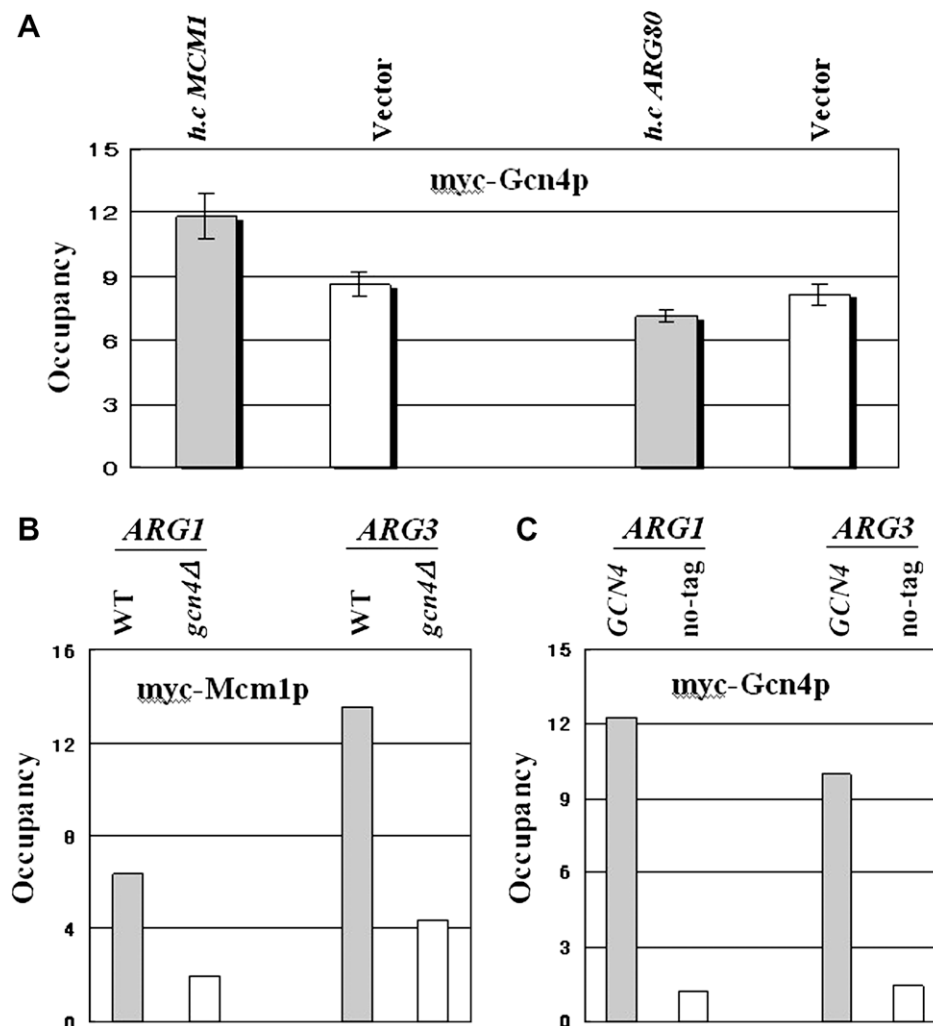
(SM), which produces starvation for isoleucine and valine, we performed ChIP analysis to measure myc-Gcn4p occupancy of the *ARG1* promoter.

As shown in Fig. 1B, mutants 2, 3, and 5 exhibited reduced myc-Gcn4p binding. Mutant 5 produced the most severe binding defect, whereas mutants 2 and 3 reduced the binding of myc-Gcn4p to similar levels (Fig. 1B). Even though mutation 3 altered the Gcn4p binding site and the ARC2 element, we assume that the reduction in myc-Gcn4p binding in mutant 3 resulted from the defective Gcn4p binding site, not from defective Mcm1p binding in the ARC element. Interestingly, mutation 2 also reduced myc-Gcn4p binding, suggesting that Mcm1p binding was involved in the increased myc-Gcn4p binding at *ARG1*. However, myc-Gcn4p binding was not affected by mutants 1 and 4, suggesting that the conserved 5'-distal CC nucleotides sequences in the MADS box were more critical for Gcn4p binding than the 5'-proximal CC nucleotides. This mutational analysis revealed that the MADS box in the ARC1 element is important for Gcn4p binding. This implies that binding of Mcm1p to ARC1 stimulates Gcn4p binding to the *ARG1* promoter and that Mcm1p binding to ARC1 plays a positive role in *ARG1* transcription through increased Gcn4p binding.

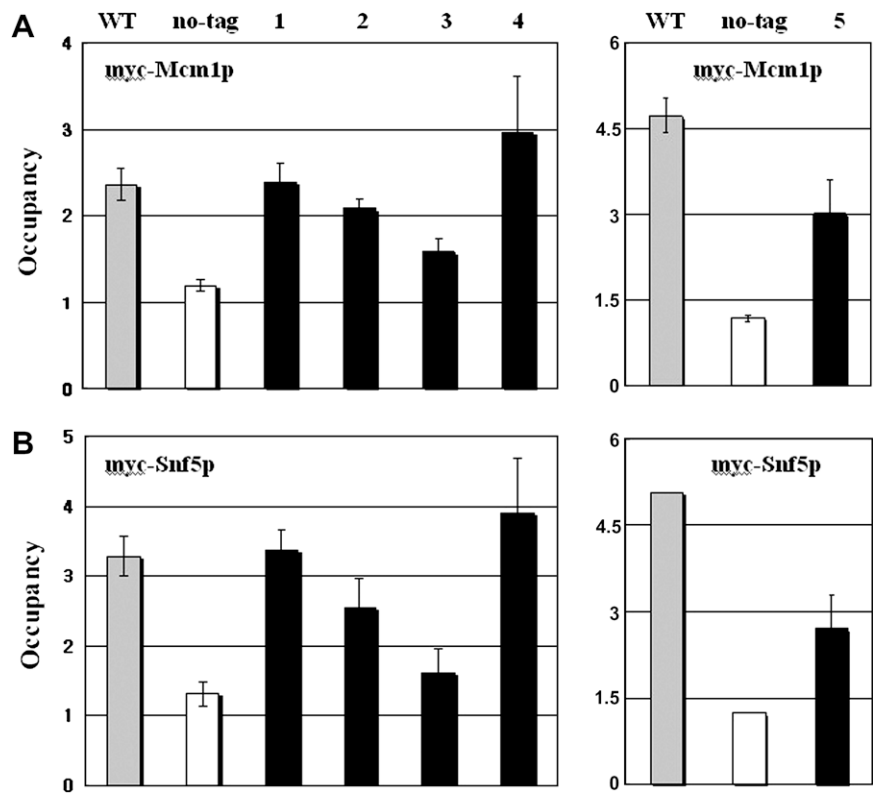
#### Levels of Gcn4p binding positively correlate with Mcm1p binding levels at Gcn4p target promoters

We further assessed the positive relationship between Mcm1p binding and recruitment of Gcn4p by testing whether increased Mcm1p levels led to enhanced Gcn4p binding. After transformation with a high-copy *MCM1* plasmid, we measured the binding of myc-Gcn4p (Fig. 2A). The binding of myc-Gcn4p increased with Mcm1p overexpression compared to that with empty vector transfection (ANOVA,  $P = 0.015$ ). Arg80p is a component of the ArgR/Mcm1p complex and MADS box protein, which is recruited by Gcn4p [7]. But, overexpression of Arg80p protein did not result in increased binding of myc-Gcn4p (Fig. 2A), suggesting that Mcm1p could increase recruitment of Gcn4p at the ARC element regardless of the ArgR/Mcm1p complex.

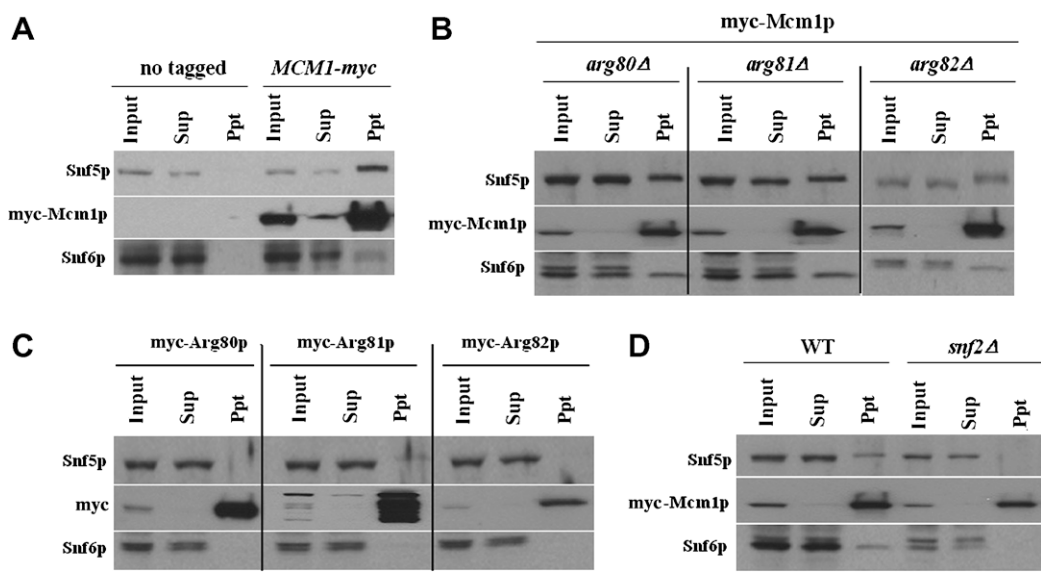
We also tested whether Gcn4p binding positively correlated with Mcm1p binding at two different Gcn4p target promoters, *ARG1* and *ARG3*. These two *ARG* genes are dependent on Gcn4p for their transcription and are known to bind ArgR/Mcm1p [1,7,11,14]. As shown in Fig. 2B and C, *ARG1* and *ARG3* both exhibit high Mcm1p and myc-Gcn4p binding. Consistent with our previ-



**Fig. 2.** Mcm1p binding levels positively correlate with Gcn4p binding levels at Gcn4p target promoters. (A) The transformants of *gcn4Δ* (249) strains containing myc-tagged *GCN4* plasmid (pSK1) were re-transformed with an empty vector, a high-copy plasmid pED40 harboring *MCM1*, or high-copy plasmid pSY365 harboring *ARG80*. ChIP analysis of the transformants was conducted to measure binding of myc-Gcn4p to *ARG1* UAS, as described in Fig. 1B. (B) The target genes (*ARG1* and *ARG3*) of Gcn4p are indicated above the histogram. High-copy plasmid pHQ1239 harboring the *GCN4*-HA allele was introduced into WT *MCM1*-myc (SY337) strains. An empty vector was introduced into the *gcn4Δ* *MCM1*-myc (SY339) strains. ChIP analysis of the transformants was conducted to measure binding of myc-Mcm1p to *ARG1* UAS or to *ARG3*. Signals for *ARG1* UAS or *ARG3* in the immunoprecipitate (IP) were normalized to the corresponding *POL1* signal and plotted in the histogram (Occupancy). (C) The *gcn4Δ* (249) strains were transformed with an empty vector (no-tag) or myc-tagged *GCN4* plasmid (pSK1). ChIP analysis of the transformants was conducted to measure binding of myc-Gcn4p to *ARG1* UAS or *ARG3*, as described in Fig. 2B.



**Fig. 3.** Recruitment of SWI/SNF more closely depends on Mcm1p binding than Gcn4p binding. (A and B) The strains contained a non-tagged allele (no-tag; SY539) or 13 myc-tagged alleles (*MCM1-myc* and *SNF5-myc*), designated above each histogram (Table 1 in the Supporting Information). The *MCM1-myc* strains containing mutations in the *ARG1* alleles are indicated at the top of each graph; WT (SY476), mutant 1 (SY650), mutant 2 (SY494), mutant 3 (SY499), mutant 4 (SY504), and mutant 5 (SY760). The *SNF5-myc* strains containing mutations in the *ARG1* alleles are indicated at the top of each graph; WT (SY480), mutant 1 (SY663), mutant 2 (SY498), mutant 3 (SY503), mutant 4 (SY508), and mutant 5 (SY759). High-copy *GCN4* plasmid pHQ1239 was introduced into the strains. ChIP analysis of the transformants was conducted to measure binding of myc-Mcm1p or myc-Snf5p to *ARG1* UAS, as described in Fig. 1B.



**Figure 4.** Mcm1p interacts with the SWI/SNF complex and requires Snf2p subunit for its interaction. (A) Non-tagged allele (BY4741) or myc-tagged *MCM1* (SY337) were cultured. WCEs were immunoprecipitated with myc antibodies, as described in Materials and Methods. Ten percent of the input samples (Input), 100% of the immunoprecipitates (Ppt), and 10% of the supernatant (Sup) fractions were subjected to Western blot analysis using myc antibodies or rabbit polyclonal antibodies against Snf5p and Snf6p. (B) *GCN4* strains containing *arg80Δ MCM1-myc* (SY366), *arg81Δ MCM1-myc* (SY372), and *arg82Δ MCM1-myc* (SY367) were grown and subjected to co-immunoprecipitation with myc antibodies as described in (A). They were then subjected to Western blot analysis using antibodies against myc, Snf5p, and Snf6p. (C) *GCN4* strains containing *Arg80-myc* (SY373), *Arg81-myc* (SY375), and *Arg82-myc* (SY377) were grown and subjected to co-immunoprecipitation with myc antibodies as described in (A). They were then subjected to Western blot analysis using myc antibodies to detect myc-Arg80p, myc-Arg81p, or myc-Arg81p. In addition, rabbit polyclonal antibodies against Snf5p and Snf6p were used for the analysis. (D) *MCM1-myc* (SY337) and *snf2Δ MCM1-myc* (SY358) strains were grown and subjected to co-immunoprecipitation with myc antibodies as described in (A). They were then subjected to Western blot analysis using antibodies against myc, Snf5p, and Snf6p.



ous results [7], myc-Mcm1p occupancy is greatly reduced in *gcn4Δ* cells (Fig. 2B). These results fit with the idea that Gcn4p binding is enhanced by Mcm1p binding at ARG gene promoters, which would imply that Gcn4p and Mcm1p mutually enhance their binding to these promoters. Considering that this is similar to the previously described interaction of Mcm1p with other transcriptional partners such as  $\alpha$ 1p, Ste12p, and Fkh2p in target promoters [3,5,6], we assumed that Gcn4p is newly identified as a transcription partner for promoters including ARC elements.

#### *SWI/SNF recruitment depends on Mcm1p binding more than Gcn4p binding*

Mcm1p contributes to recruitment of the co-activator SWI/SNF complex at *STE6* [17]. Since SWI/SNF complex is recruited to *ARG1* in a Gcn4p-dependent manner [11], we tested the relationship between Mcm1p and recruitment of the SWI/SNF complex at *ARG1* under Gcn4p-induced conditions by comparing Mcm1p binding and recruitment of Snf5p in mutants 1–5 (Fig. 1A). We were able to measure detectable and distinguishable levels of their recruitment with a high-copy *GCN4* plasmid, which increased Gcn4p protein levels and recruitment of SWI/SNF 4- to 5-fold more than single-copy *GCN4* plasmid at *ARG1* [11]. Mutants 3 and 5 exhibited greatly reduced binding of both Mcm1p and Snf5p (Fig. 3A and B), suggesting that their recruitment was primarily affected by the mutations in the Gcn4p binding sites. Mutant 2 showed a lesser decrease in recruitment of both Mcm1p and Snf5p, while mutation 4 modestly increased the recruitment of both Mcm1p and Snf5p. We observed a similar pattern between Mcm1p and Snf5p by mutational analysis, suggesting that the SWI/SNF recruitment largely depends on Mcm1p binding at *ARG1*.

Since SWI/SNF recruitment also depends on Gcn4p at *ARG1* [11], we compared Snf5p recruitment (Fig. 3B) with Gcn4p binding (Fig. 1B). Based on the recruitment of Gcn4p, Mcm1p, and Snf5p in mutants 2, 3, and 4, we concluded that SWI/SNF recruitment more closely correlated with Mcm1p binding than with Gcn4p binding, which indicates that Mcm1p binding contributes more to SWI/SNF recruitment than Gcn4p binding. It was puzzling that we did not observe greatly reduced recruitment of both Mcm1p and Snf5p in mutant 2, since Gcn4p binding was significantly reduced in this mutant. Presumably, the residual Gcn4p binding in mutant 2 was sufficient to support nearly WT occupancies of Mcm1p and SWI/SNF. Taken together with the previous finding that SWI/SNF recruitment depends on Gcn4p [11], SWI/SNF recruitment was apparently enhanced by both Mcm1p and Gcn4p at *ARG1*. As myc-Gcn4p binding was unaffected by deletions of SWI/SNF subunits *SWI3* or *SNF5* at *ARG1* [11], we found that myc-Mcm1p occupancy was also unaffected by their deletions (Supplementary Fig. 1A). This result indicates that binding of both Gcn4p and Mcm1p occurs before SWI/SNF recruitment.

#### *The interaction between Mcm1p and SWI/SNF requires the Snf2p subunit*

It is possible that protein–protein interactions contribute to the positive relationship between Mcm1p binding and the recruitment of SWI/SNF. However, the interaction between SWI/SNF and Mcm1p has not been identified. To determine whether Mcm1p interacts with the SWI/SNF complex, we performed co-immunoprecipitation experiments. Precipitation of Snf5p and Snf6p subunits was not detected in a non-tagged control strain, while the SWI/SNF subunits co-immunoprecipitated with myc-Mcm1p (Fig. 4A). To the best of our knowledge, this is the first report demonstrating an interaction between Mcm1p and SWI/SNF complex. This suggests that Mcm1p directly interacts with the SWI/SNF complex in solution. However, using a similar approach, we did

not detect an interaction between Gcn4p and the SWI/SNF complex in cells containing either high- or single-copy *GCN4-myc* plasmid (Supplementary Fig. 1B and 1C). Considering that recombinant GST-Gcn4p interacted with SWI/SNF complex in yeast extracts in previous GST-pull down assays [15], our results suggest that the interaction between Gcn4p and SWI/SNF is weaker than the interaction between SWI/SNF and Mcm1p and support our ChIP data that Mcm1p contributes more to the recruitment of SWI/SNF complex at *ARG1* than does Gcn4p.

Mcm1p forms the ArgR/Mcm1p complex with Arg80p, Arg81p, and Arg82p, which are also recruited to *ARG1* [7]. We tested whether the SWI/SNF complex could still interact with Mcm1p when the other subunits of ArgR/Mcm1p complex were absent. Mcm1p precipitated with both Snf5p and Snf6p after deletion of *ARG80*, *ARG81*, or *ARG82* (Fig. 4B). We also tested whether SWI/SNF interacted with myc-tagged forms of Arg80p, Arg81p, and Arg82p, but found no interaction (Fig. 4C). These results indicate that the interaction between Mcm1p and the SWI/SNF complex did not involve the ArgR/Mcm1p complex. Taken together, our findings suggest that Mcm1p alone may contribute to the increased SWI/SNF binding to ARC elements at *ARG1*, regardless of the ArgR/Mcm1p complex. Gcn4p binding was not increased by Arg80p overexpression (Fig. 2A), which also supports our conclusion.

Since Snf2p is the catalytic subunit in the SWI/SNF complex, we tested whether Mcm1p interacted with the rest of the SWI/SNF complex in a *SNF2* deletion strain. We found that the SWI/SNF complex and Mcm1p no longer interacted when the Snf2p subunit was deleted (Fig. 4D). It was important to determine that Snf2p subunit-dependence in the interaction was not from complex-disruption in the *SNF2* deletion strain. We confirmed that the *SNF2* deletion did not disrupt the rest of the complex, since the *SNF2* deletion did not disrupt the interactions among Snf5p, Snf6p, Swi1p, and Swp73p (Supplementary Fig. 1D and 1E). We conclude that the Snf2p subunit is important for the interaction between Mcm1p and the SWI/SNF complex. Our results reflect that SWI/SNF recruitment by Mcm1p might also depend on Snf2p subunit at *STE6* promoter [12]. Previously, we showed that SWI/SNF recruitment at *ARG1* was not reduced by the *snf2* mutation [11], which was ostensibly at odds with the idea that Mcm1p contributes to SWI/SNF recruitment at *ARG1*. However, Gcn4p-myc binding was increased almost 2.5-fold in the *snf2* strain [14]. This may indicate that enhanced, direct recruitment of SWI/SNF by Gcn4p can compensate for the impaired interaction of Mcm1p with SWI/SNF complex in the *snf2* strain.

Our results suggest that Mcm1p binding to the MADS boxes contributes to binding of the activator Gcn4p and recruitment of the co-activator complex SWI/SNF at *ARG1* under Gcn4p-induced conditions. The strong interaction between Mcm1p and the SWI/SNF complex could explain the proposed contribution of Mcm1p to SWI/SNF recruitment at *ARG1*.

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#### **Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.02.045.

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