Mechanism Underlying the Iron-dependent Nuclear Export of the Iron-responsive Transcription Factor Aft1p in *Saccharomyces cerevisiae*

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Aft1p is an iron-responsive transcriptional activator that plays a central role in maintaining iron homeostasis in *Saccharomyces cerevisiae*. Aft1p is regulated primarily by iron-induced shuttling of the protein between the nucleus and cytoplasm, but its nuclear import is not regulated by iron. Here, we have shown that the nuclear export of Aft1p is promoted in the presence of iron and that Msn5p is the nuclear export receptor (exportin) for Aft1p. Msn5p recognizes Aft1p in the iron-replete condition. Phosphorylation of S210 and S224 in Aft1p, which is not iron dependent, and the iron-induced intermolecular interaction of Aft1p are both essential for its recognition by Msn5p. Mutation of Cys291 of Aft1p to Phe, which causes Aft1p to be retained in the nucleus and results in constitutive activation of Aft1-target genes, disrupts the intermolecular interaction of Aft1p. Collectively, these results suggest that iron induces a conformational change in Aft1p, in which Aft1p Cys291 plays a critical role, and that, in turn, Aft1p is recognized by Msn5p and exported into the cytoplasm in an iron-dependent manner.

INTRODUCTION

The ability of iron to efficiently donate or accept an electron is essential for many biological processes, including oxygen transport, electron transfer, and many catalytic reactions. However, iron is potentially toxic because it accelerates the generation of reactive oxygen species. Therefore, cells must be equipped with the proper machinery for sensing and regulating the intracellular iron availability (Askwith *et al.*, 1996; De Freitas *et al.*, 2003). The yeast *Saccharomyces cerevisiae* has served as a model organism for the investigation of iron homeostasis (De Freitas *et al.*, 2003). The high-affinity iron uptake system of *S. cerevisiae* consists of two pathways. One pathway involves the iron permease Ftr1p and the multicopper ferroxidase Fet3p, which transports ferrous ion that has been reduced by the ferric reductases Fre1p and Fre2p (Dancis, 1998). The other pathway includes four siderophore transporters, each of which mediates the uptake of iron chelated to siderophores (Philpott *et al.*, 2002). One of these transporters, Arn3p/Sit1p, is a ferrioxamine B (FOB) transporter (Lesuisse *et al.*, 1998; Yun *et al.*, 2000). The tran-

scriptional activator Aft1p plays a central role in the homeostatic regulation of iron metabolism in *S. cerevisiae* by regulating the expression of genes whose products are involved in iron uptake and iron use, including Ftr1p, Fet3p, and Arn3p/Sit1p, in an iron-responsive manner (Yamaguchi-Iwai *et al.*, 1995; Yamaguchi-Iwai *et al.*, 1996; Philpott *et al.*, 2002; Puig *et al.*, 2005). We have shown that the iron-dependent alteration of Aft1p localization is important for this regulation: Aft1p accumulates in the nucleus and activates the transcription of its target genes in the iron-starved condition, whereas it is localized in the cytoplasm when there is excess iron (Yamaguchi-Iwai *et al.*, 2002). Importins and exportins mediate the nucleocytoplasmic localization of proteins by regulating nuclear import and export, respectively (Hood and Silver, 1999; Jans *et al.*, 2000; Komeili and O'Shea, 2000). We have previously shown that Pse1p is the importin that translocates Aft1p and that Pse1p-mediated nuclear import of Aft1p is not affected by the iron status of cells (Ueta *et al.*, 2003). This observation strongly suggests that the nuclear export of Aft1p is regulated by the availability of iron. The identification of the exportin for Aft1p will therefore be crucial for understanding the mechanism underlying the iron-regulated nucleocytoplasmic localization of Aft1p, and, therefore, iron homeostasis.

Four exportins have been identified in *S. cerevisiae* (Strom and Weis, 2001), each of which has a different mechanism of cargo recognition (Pemberton and Paschal, 2005). However, the precise mechanisms for target recognition have not been elucidated, with the exception that Xpo1p, also known as Crm1p, has been shown to recognize the well-defined short amino acid sequences referred to as the leucine-rich nuclear export signal (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). In this study,

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Abbreviations used: AD, Gal4 transactivation domain; BD, Gal4 DNA-binding domain; CIAP, calf intestine alkaline phosphatase; DAPI, 4',6'-diamino-2-phenylindole; FOB, ferrioxamine B; GFP, green fluorescence protein; HA, hemagglutinin; NES, nuclear export signal; NLS, nuclear localization signal; TAP, tandem affinity purification.

Table 1. Yeast strain genotypes

Strain	Genotype	Source
PSY580	<i>MATa ura3-52 leu2Δ1 trp1Δ63</i>	Winston <i>et al.</i> (1995)
PSY1105 (<i>xpo1-1</i>)	<i>MATα ura3-1 trp1-1 his3-11 ade2-1 can1-1 xpo1Δ::LEU2 <pKW457(xpo1-1 HIS3)></i>	Stade <i>et al.</i> (1997)
PSY1201 (<i>pse1-1</i>)	<i>MATa ura3-52 leu2Δ1 trp1Δ63 pse1-1</i>	Seedorf and Silver (1997)
PSY1569 (<i>Δmsn5</i>)	<i>MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 msn5Δ::HIS3</i>	P. A. Silver
PJ69-4A	<i>MATa ura3-52 leu2-3,112 trp1-901 his3-200 gal4Δ gal80Δ GAL2-ADE2</i> <i>LYS2::GAL1-HIS3 met2::GAL7-lacZ</i>	James <i>et al.</i> (1996)
Y22 (PJ69-4AΔ <i>fet3</i>)	PJ69-4A strain background, <i>fet3Δ::KanMX</i>	This study
BY4741	<i>MATa uraΔ0 leu2Δ his3Δ0 met15Δ0</i>	Brachmann <i>et al.</i> (1998)
Y23 (<i>Δaft1</i>)	BY4741 strain background, <i>aft1Δ::KanMX</i>	This study
Y24 (<i>Δaft1/ARN3</i>)	BY4741 strain background, <i>aft1Δ::KanMX <pADH-ARN3 (ADH1-ARN3 LEU2)></i>	This study

we show that the nuclear export of Aft1p is crucial for its iron-regulated changes in localization, and we identify Msn5p as the exportin responsible for the iron-dependent nuclear export of Aft1p. Analysis of the iron-dependent recognition of Aft1p by Msn5p indicates that the phosphorylation and intermolecular interaction of Aft1p are important for the iron-dependent nucleocytoplasmic trafficking of Aft1p. Moreover, Cys291 of Aft1p, whose mutation to Phe in the Aft1-1^{up} variant has been shown to render Aft1p constitutively active in iron-replete cells, is critical for the intermolecular interaction of the protein.

MATERIALS AND METHODS

Yeast Strains and Media

The yeast strains used in this study are listed in Table 1. Cells were grown as described previously (Ueta *et al.*, 2003) in YPD or SD medium supplemented with amino acids. The media used for iron repletion or iron starvation have been described previously (Ueta *et al.*, 2003). FeSO₄ (200 μM) was added to the medium to create excess iron conditions for a two-hybrid analysis using PJ69-4AΔ*fet3* cells (Y22). To use FOB as an iron source, 0–200 μM each of FeCl₃ and deferroxamine were added to SD-Fe medium containing 100 μM bathophenanthroline disulfonate (BPS).

Plasmids

Plasmids for expressing Aft1p-hemagglutinin (HA) and Aft1pC291F-HA from its own promoter were generated by subcloning those DNA fragments into pRS415. Open reading frames (ORFs) of Aft1 and Aft1C291F, fused C-terminally with the tandem affinity purification (TAP)-tag (Open Biosystems, Huntsville, AL; Rigaut *et al.*, 1999), were subcloned into pRS416 containing the Aft1 promoter. Plasmids for the yeast two-hybrid assay were constructed as follows. To construct pAD-AFT1 and its derivatives, NcoI-XhoI fragments encoding the entire AFT1 coding region, or fragments thereof, were inserted into the NcoI-XhoI site of pACT2 (Clontech, Mountain View, CA). pBD-MSN5 was created by inserting an EcoRI-XhoI fragment containing the open reading frame of MSN5 into the EcoRI-XhoI site of pGBKT7 (Clontech). To construct the expression plasmids for Gal4 DNA-binding domain (BD)-fused fragments of Aft1p, NcoI-XhoI fragments encoding Aft1p regions were inserted into the NcoI-SalI site of pGBKT7. The expression plasmid for simian virus 40 (SV40) nuclear localization signal (NLS)-protein kinase inhibitor (PKI) nuclear export signal (NES)-green fluorescent protein (GFP) (pPS1372) (Taura *et al.*, 1998) was a gift from Dr. P. A. Silver (Harvard Medical School). Expression plasmids for GFP-fused Aft1p fragments from the MET25-driven promoter were constructed by inserting DNA fragments encoding various regions of Aft1p between the ClaI and EcoRI sites of pMET25-SV40 NLS-GFP (Ueta *et al.*, 2003). The pET-GSP1^{G21V} and pET-GSP1^{T26N} vectors used to express mutant histidine-tagged Gsp1 proteins have been described previously (Ueta *et al.*, 2003). The ORF of Msn5 was amplified from BY4741 genomic DNA by polymerase chain reaction (PCR). The Msn5 ORF was C-terminally myc-tagged and subcloned into pFastBac HT-A (Invitrogen, Carlsbad, CA) to generate the pFastBac-His₆-Msn5-myc vector.

Yeast Two-Hybrid Assays

Yeast two-hybrid assays were performed by examining the growth of PJ69-4A or PJ69-4AΔ*fet3* (Y22) strains expressing both Gal4 transactivation domain (AD)- and BD-fused proteins in medium lacking adenine and histidine. In some experiments, 3-aminotriazole (3-AT; 10 mM final concentration) was added to the medium. Cells were spotted using 10-fold serial dilutions

beginning at OD₆₀₀ = 0.6. Cells showed similar growth on medium containing adenine and histidine. Adequate expression of fusion proteins in cells was confirmed by immunoblotting using anti-HA (12CA5) (Roche Diagnostics, Mannheim, Germany) and anti-Myc (9E10) (Covance, Princeton, NJ) antibodies.

Immunoblotting

Lysates of yeast cells, prepared by the bead lysing method, were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes. After blocking in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% skim milk, the membrane was incubated with the appropriate primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and visualization with the SuperSignal chemiluminescent detection system (Pierce Chemical, Rockford, IL). Signals obtained by immunoblotting were quantified by the LAS1000 luminescent image analyzer (Fuji Film, Tokyo, Japan).

Detection of Aft1p Phosphorylation

To detect Aft1p phosphorylation, a cell lysate was prepared using the bead lysing method from cells expressing Aft1p-HA or its derivatives in buffer (50 mM HEPES-KOH, pH 7.5, 50 mM NaCl, and 1 mM EDTA) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A) and 10 mM β-glycerophosphate. The lysate was cleared by centrifugation, and the buffer was changed to calf intestine alkaline phosphatase (CIAP) buffer (10 mM Tris-Cl, pH 8.0, 50 mM NaCl, and 10 mM MgCl₂) using protein desalting spin columns (Pierce Chemical). CIAP-treated samples were incubated with 1 U of CIAP (Takara, Kyoto, Japan) at 37°C for 1 h before the addition of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, and 1% β-mercaptoethanol) and boiling. Proteins samples were separated by 4% SDS-PAGE and subjected to immunoblotting with an anti-HA antibody.

Indirect Immunofluorescence and GFP Microscopy

Subcellular localization of proteins was examined by indirect immunofluorescence microscopy or by GFP microscopy, essentially as described previously (Ueta *et al.*, 2003). Briefly, for indirect immunofluorescence microscopy, cells expressing HA-tagged wild-type or mutant Aft1p were fixed in 4% formaldehyde. Cell walls were digested with 300 U of Zymolyase (Seikagaku Kogyo, Tokyo, Japan), followed by the addition of 2% SDS. Spheroplasts were fixed on poly-lysine-coated coverslips, permeabilized with 0.05% saponin, and then incubated with an anti-HA antibody. Signals were amplified and visualized using an Alexa Fluor 594 signal amplification kit (Invitrogen). Nuclei were stained by incubation with 500 ng/ml 4',6'-diamino-2-phenylindole (DAPI) for 5 min. For GFP microscopy, cells were cultured in medium lacking methionine to induce the expression of the GFP-fused proteins. Cells were fixed in 70% ethanol for 5 min and suspended in mounting medium containing 50 ng/ml DAPI. GFP-fused proteins and nuclei were visualized using an IX81 fluorescence microscope or an FV-1000 confocal microscope (Olympus, Tokyo, Japan). Expression of the tagged proteins was measured by immunoblotting and found to be similar in each of the strains under the different iron conditions used.

Purification of Recombinant Proteins

His-GSP1^{G21V} and His-GSP1^{T26N} were expressed in *Escherichia coli*, purified, and loaded with GTP or GDP as described previously (Ueta *et al.*, 2003). Recombinant baculovirus expressing His₆-Msn5-myc was generated using the Bac-to-Bac Baculovirus Expression system (Invitrogen). Hi Five cells were cultured in Grace's insect medium plus 10% fetal bovine serum at 27°C and infected with the recombinant virus. Sixty hours after infection, cells were lysed with 50 mM Tris-Cl, pH 7.5, and 2 mM PMSF and homogenized with a

Dounce homogenizer. His₆-Msn5-myc was purified from the soluble fraction using nickel-nitrilotriacetic acid agarose (QIAGEN, Valencia, CA). The His₆-tag was cleaved using the AcTEV protease (Invitrogen).

Detection of Intermolecular Interaction of Aft1p

Cells expressing Aft1p-HA and Aft1p-TAP or Aft1pC291F-HA and Aft1pC291F-TAP were lysed in 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 2 mM PMSF using Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). The soluble fraction was incubated with IgG-Sepharose (GE Healthcare) in 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 2% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS at 4°C for 2 h. The beads were washed five times with the same buffer, and precipitates were separated by 6% SDS-PAGE, and subjected to immunoblotting using an anti-HA antibody (16B12) (Covance).

Detection of Aft1p-Msn5p Interaction In Vitro

Lysates (2 mg) of cells expressing Aft1p-HA or Aft1pC291F-HA, prepared as above, were incubated with 1.5 μ g of purified Msn5p-myc, in the presence of 1.5 μ g of either GTP-loaded His₆-Gsp1p^{G21V} or GDP-loaded His₆-Gsp1p^{T26N} in 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, and 10% glycerol at 4°C for 2 h, followed by immunoprecipitation of Msn5p-myc by using an anti-myc antibody and protein A-Sepharose (GE Healthcare). The beads were washed four times with the same buffer, and precipitates were separated by 6% SDS-PAGE and subjected to immunoblotting using an anti-HA antibody.

RESULTS

The Nuclear Export of Aft1p Is Promoted in the Presence of Iron

We have previously shown that Pse1p-mediated nuclear import of Aft1p does not affect the subcellular localization of Aft1p in response to iron administration (Ueta *et al.*, 2003), which raises the possibility that nuclear export of the protein occurs in an iron-dependent manner. To examine whether the nuclear export of Aft1p is regulated by iron, we used *pse1-1* mutant cells carrying a temperature-sensitive *PSE1* gene (Stade *et al.*, 1997). We first grew *pse1-1* cells expressing Aft1p-HA at the permissive temperature (25°C) in the absence of iron to accumulate Aft1p in the nucleus, followed by cultivation for 4 h at the restrictive temperature (37°C) in the continued absence of iron to inhibit additional nuclear import of Aft1p (Figure 1A). Cycloheximide was added to the culture at the restrictive temperature for the last 30 min to inhibit further synthesis of Aft1p. Aft1p accumulated in the nuclei of cells cultured in iron-deficient medium at the restrictive temperature (Figure 1B1). The culture was then divided into two aliquots, and cells were cultured in the presence or absence of 200 μ M iron in the continuous presence of cycloheximide at the restrictive temperature, and the effect of iron on the subcellular localization of Aft1p was monitored by immunofluorescence microscopy. Aft1p was exported to the cytoplasm within 30 min of the addition of iron (Figure 1B2), whereas the protein remained in the nucleus in the absence of iron (Figure 1B3). Anti-HA immunoblotting revealed that the amount of Aft1p was not affected by iron treatment, although it decreased by 30 min in both iron-rich and -depleted cells (Figure 1C). These results suggest that Aft1p is exported from nucleus in an iron-dependent manner.

Msn5p Is Required for the Nuclear Export of Aft1p

To elucidate the mechanism of this iron-dependent nuclear export of Aft1p, we used yeast cells carrying defects in exportin genes to identify the exportin that transports Aft1p. We first examined regulation of the nucleocytoplasmic localization of Aft1p in *xpo1-1* cells, which carry a temperature-sensitive mutation in the *XPO1* gene, whose gene product is a major exportin (Stade *et al.*, 1997). Wild-type cells and *xpo1-1* cells expressing Aft1p-HA were cultured to the logarithmic growth phase in iron-replete or iron-depleted conditions at the permissive temperature (25°C) and further

Figure 1. Nuclear export of Aft1p is promoted by iron. (A) Schematic diagram of the monitoring of Aft1p nuclear export. Shading represents the localization of Aft1p in the nucleus. (B) The nuclear export of Aft1p is promoted by iron. *pse1-1* (PSY1201) cells expressing Aft1p-HA were grown to the logarithmic growth phase under conditions of iron starvation at 25°C, followed by cultivation at 37°C for 4 h to inactivate Pse1p. Cycloheximide was added to the cultures to inhibit further synthesis of Aft1p (1). Cells were further cultured at 37°C in the presence (2) or absence (3) of iron. At each of the indicated time points, cells were fixed, and the subcellular localization of Aft1p-HA was examined by indirect immunofluorescence microscopy. (C) The amount of Aft1 protein was not affected by the iron status of the cells. Lysates from yeast cells as treated described in B were separated by SDS-PAGE, and Aft1-HA was probed with an anti-HA antibody.

cultured for 3 h at the restrictive temperature (37°C). As a control, we examined GFP-fused PKI NES, which is exported by Xpo1p (Wen *et al.*, 1995). The cells were fixed, and the subcellular localization of Aft1p was examined by immunofluorescence microscopy. Aft1p was localized in the nucleus in iron-depleted wild-type cells as well as in *xpo1-1* cells (Figure 2A, 1–4). In iron-replete *xpo1-1* cells, Aft1p localized to the cytoplasm. In contrast, nuclear export of the control GFP-fused PKI NES was inhibited (Figure 2B, compare 1 and 2). These results indicate that Xpo1p is not the exportin for Aft1p. We next probed the subcellular localization of Aft1p in Δ *msn5* cells to assess the involvement of Msn5p in nuclear export of Aft1p. In Δ *msn5* cells, Aft1p accumulated in the nucleus regardless of iron availability (Figure 2A, 5 and 6), although nuclear export of GFP-fused PKI NES was not perturbed (Figure 2B3). These results indicate that Msn5p is required for the nuclear export of Aft1p.

Msn5p Recognizes Aft1p in an Iron-dependent Manner

The recognition of cargo proteins by exportins is critical for nuclear export of the proteins (Nakielnny and Dreyfuss, 1997; Ossareh-Nazari *et al.*, 2001). Because Aft1p is ex-

B : SV40 NLS-PKI NES-GFP

Figure 2. Aft1p accumulates in the nucleus of the Δ *msn5* strain independently of iron status. (A) Wild-type (PSY580) (1 and 2), *xpo1-1* (PSY1105) (3 and 4), or Δ *msn5* (PSY1569) cells (5 and 6) were grown to the logarithmic growth phase in iron-starved (-Fe; 1, 3, and 5) or iron-replete (+Fe; 2, 4, and 6) conditions. After fixation, the subcellular localization of Aft1p-HA was examined by indirect immunofluorescence microscopy. Images taken with transmitted light and DAPI staining of nuclei are provided for comparison. (B) The subcellular localization of the SV40 NLS-PKI NES-GFP fusion protein was observed in wild-type (1), *xpo1-1* (2), or Δ *msn5* (3) cells. *xpo1-1* cells were cultured at 37°C for 3 h before fixation (A and B).

ported to the cytoplasm only in the iron-replete condition, we examined whether the interaction between Aft1p and Msn5p is regulated by the iron status of cells using a yeast two-hybrid analysis. Aft1p was fused to the Gal4 trans-activation domain (AD-Aft1p) and Msn5p was fused to the DNA-binding domain (BD-Msn5p). PJ69-4A cells, which possess *GAL1-HIS3* and *GAL2-ADE2* reporter

genes, grew on SD plates lacking adenine and histidine when both AD-Aft1p and BD-Msn5p were expressed, whereas cells expressing either the AD-Aft1p or BD-Msn5p fusion protein alone did not grow (Figure 3A), indicating that Aft1p and Msn5p interact with each other. Next, we used a yeast two-hybrid assay to determine whether the interaction between Aft1p and Msn5p was

Figure 3. Aft1p and Aft2p interact with Msn5p in an iron-dependent manner. PJ69-4A (A and D) or PJ69-4A Δ *fet3* (Y22; B, C, E, and F) cells were transformed with plasmids expressing AD-fused Aft1p (A–C), AD-fused Aft2p (D–F), or the corresponding empty vector, as well as a plasmid expressing BD-fused Msn5p or the corresponding empty vector. Cells were grown at 30°C for 4 d on medium containing adenine and histidine (+ADE +HIS), or lacking adenine and histidine but containing 10 mM 3-AT (-ADE -HIS). In C and F, 200 μ M FeSO₄ was added to the medium to create an excess iron condition. Cells were spotted using 10-fold serial dilutions beginning at an OD₆₀₀ = 0.6. AD-fused SV40 Large T antigen (LargeT) and BD-fused p53 were used as a positive control.

Figure 4. The Msn5p-interacting domain within Aft1p. (A) Schematic presentation of the fragments of Aft1p tested for interaction with Msn5p. (B) Aft1p(147-270) and Aft1p(304-498) interact with Msn5p. Using PJ69-4A cells expressing the AD-fused Aft1p fragment indicated on the left and BD-Msn5p, the interaction between the fragments of Aft1p and Msn5p was tested as in Figure 3. (C) Subcellular localization of GFP fused Aft1p fragments. Y24 cells expressing GFP-Aft1p(1-690) (1 and 2), GFP-Aft1p(147-270) (3 and 4), GFP-Aft1p(304-498) (5 and 6), or GFP-Aft1p(147-498) (7 and 8) were cultured in the absence (-Fe; 1, 3, 5, and 7) or presence (+Fe; 2, 4, 6, and 8) of FOB, and the localization of GFP-fusion proteins was observed.

affected by the iron status of the cells. We first generated yeast two-hybrid reporter cells in which we could manipulate the iron status. Because regular SD medium is iron replete (Yamaguchi-Iwai *et al.*, 1995, 1996; Philpott *et al.*, 2002; Puig *et al.*, 2005), to achieve the iron-starved condition we disrupted the *FET3* gene, whose gene product is required for high-affinity iron uptake (Askwith *et al.*, 1994; Stearman *et al.*, 1996) in PJ69-4A cells, forming PJ69-4AΔ*fet3*. PJ69-4AΔ*fet3* cells expressing both AD-Aft1p and BD-Msn5p fusion proteins were not able to grow on media lacking adenine and histidine, whereas the binding between SV40 large T and p53 was not affected (Figure 3B). However, the interaction between Aft1p and Msn5p in PJ69-4AΔ*fet3* was restored by the addition of excess iron (200 μM) to the plates (Figure 3C). These results indicate that Msn5p recognizes Aft1p only in iron-replete conditions. A paralogue of Aft1p, Aft2p, activates its target genes in response to iron starvation, similar to Aft1p (Blaiseau *et al.*, 2001; Rutherford *et al.*, 2001). We therefore examined whether Msn5p also recognizes Aft2p only in iron-rich conditions. Msn5p interacted with Aft2p in PJ69-4A cells and PJ69-4AΔ*fet3* cells in the presence, but not in the absence, of excess iron (Figure 3, D–F), suggesting that the activity of Aft2p is regulated by iron via the same mechanism as that of Aft1p. Collectively, these results indicate that Msn5p recognizes both Aft1p and Aft2p in the iron-replete condition.

Two Regions of Aft1p Are Involved in Interaction with Msn5p

To identify the regions in Aft1p that are required for its interaction with Msn5p, we constructed AD-fusion proteins of several Aft1p fragments (Figure 4A). The interaction between these Aft1p fragments and Msn5p was tested in a yeast two-hybrid assay. Aft1p fragments containing amino acids 147-270 [Aft1p(147-270)] and 304-498 [Aft1p(304-498)] interacted with Msn5p in PJ69-4A cells (Figure 4B), although the interaction of these fragments to Msn5p seemed to be weaker than that of full-length Aft1p. We next examined whether Aft1p(147-270) or Aft1p(304-498) alone is sufficient to mediate the nuclear export of Aft1p by analyzing the subcellular localization of GFP fused to these fragments [GFP-Aft1p(147-270) and GFP-Aft1p(304-498)]. Because the expression of GFP-fused Aft1p fragments may affect the expression of Aft1p target genes and therefore the cellular iron concentration, we constructed yeast cells expressing an FOB transporter, Arn3p/Sit1p, under the control of the ADH1 promoter (Y24). The resultant cells can take up iron independently of the activity of Aft1p when FOB is present in the medium (data not shown). GFP-fused full-length Aft1p localized in the nucleus in the absence of iron (Figure 4C1), but it was distributed in the cytoplasm in iron-replete cells (Figure 4C2), confirming that the localization of Aft1p was properly regulated in Arn3p/Sit1p-expressing cells. In contrast, GFP-fused Aft1p(147-270) and Aft1p(304-498) ac-

accumulated in the nucleus even in iron-rich conditions (Figure 4C, 4 and 6). GFP-fused Aft1p(147-498), which contains both the Aft1p(147-270) and Aft1p(304-498) fragments, was localized primarily in the cytoplasm in iron-rich conditions, although some of the protein remained in the nucleus (Figure 4C8). These results suggest that these two regions are necessary for the interaction between Aft1p and Msn5p but that either region alone may not be sufficient to support the nuclear export of Aft1p.

Involvement of Two Serines in Aft1p(147-270) and Four Threonines in Aft1p(304-498) in the Nuclear Export of Aft1p

The cargo proteins regions recognized by Msn5p do not share any sequence homology. However, it has been suggested that phosphorylation of serine/threonine residue(s) within these regions is important for recognition by Msn5p (Kaffman *et al.*, 1998; DeVit and Johnston, 1999; Boustany and Cyert, 2002). As we showed above, both Aft1p and Aft2p are recognized by Msn5p in the presence of sufficient iron, and Aft1p is a phosphoprotein (Casas *et al.*, 1997). We therefore searched for serine/threonine residues in the two Msn5p-interacting regions that are conserved in Aft1p and Aft2p as well as in predicted Aft1p orthologues in other fungal species in the *Saccharomyces* Genome Database. Four serine residues (S170, S210, S224, and S240) in Aft1p(147-270) are conserved between Aft1p and Aft2p (Figure 5A), and S210 and S224 are also highly conserved in other Aft1p orthologues. We replaced these two serine residues with alanines or phospho-mimetic aspartates to create the Aft1p(SA) and Aft1p(SD) mutants, respectively. We also identified a highly conserved sequence in Aft1p(304-498), amino acids 421-435, and mutated four threonine residues within this region (T421, T423, T431, and T435) to alanine to create Aft1p(TA) (Figure 5B). We then assessed the effect of these mutations on the localization of Aft1p in iron-replete cells. Most Aft1p(SA) was localized to the nucleus even in the presence of excess iron. However, individual serine mutations of Aft1p [Aft1p(S170A) and Aft1p(S240A)] did not affect the iron-dependent localization of the protein, indicating that phosphorylation of S210 and/or S224 may be important for the nuclear export of Aft1p (Figure 5C, 3–8). Aft1p(TA) also localized primarily to the nucleus in the presence of excess iron (Figure 5C, 11 and 12), although the distribution was less distinct than with Aft1p(SA). We next generated a combined mutant, Aft1p(STA), in which the two serine (S210 and S224) and four threonine (T421, T423, T431, and T435) residues were replaced with alanine. Aft1p(STA) discretely accumulated in the nucleus even in the presence of excess iron (Figure 5C, 13 and 14). Aft1p with either S210 or S224 mutated to alanine (Aft1pS210A or Aft1pS224A), but not both, was exported to the cytoplasm in iron-replete cells (data not shown). However, the phospho-mimetic mutant of S210 and S224 [Aft1p(SD)] was not exported to the cytoplasm in the iron-depleted condition, suggesting that phosphorylation of S210 and S224 is not enough to create a signal for iron-dependent nuclear export of Aft1p (Figure 5C, 9 and 10). Although we have not examined the effect of single mutations of the four threonines on Aft1p localization, S220 and S224 in Aft1p(147-270) and at least one of the four threonines in Aft1p(304-498) are likely to be involved in creating motifs recognized by Msn5p in the presence of iron.

Iron-Dependent Intermolecular Interaction of Aft1p

To analyze the precise roles of these serine/threonine residues in Msn5p-mediated nuclear export of Aft1p, we first examined whether the residues are involved in the interac-

tion between Msn5p and Aft1p. We generated Aft1p(147-270, SA), Aft1p(147-270, SD) and Aft1p(304-498, TA) mutants in which S210 and S224 were substituted with alanine (SA) or aspartic acid (SD), and T421, T423, T431, and T435 were substituted with alanine, respectively, and probed the interaction of these mutants with Msn5p in the iron-replete condition by using a yeast two-hybrid analysis. Aft1p(147-270, SA) failed to interact with Msn5p, although Aft1p(147-270, SD) and Aft1p(147-270) each interacted with the exportin (Figure 6A). In contrast, the alanine substitutions for T421, T423, T431, and T435 had little effect on the Aft1p(304-498)–Msn5p interaction (Figure 6B). These results suggest that phosphorylation of S210 and S224 is crucial for the Aft1p–Msn5p interaction, whereas phosphorylation of the four threonine residues in Aft1p(304-498) is dispensable.

We next tested whether these serine residues are indeed phosphorylated in Aft1p. Anti-HA immunoblotting of an extract from cells expressing Aft1p-HA revealed the presence of a slower-migrating species of Aft1p (Figure 6C, lane 1), which disappeared after treatment with phosphatase (Figure 6C, CIAP, lane 2). The administration of iron had no effect on the amount of the slower migrating species of Aft1p (Figure 6C, lanes 3 and 4). The slower migrating species of Aft1p(SA) was barely visible (Figure 6C, compare lanes 5 and 6), although we have observed that both Aft1pS210A and Aft1pS224A were phosphorylated (data not shown). These results indicate that Aft1p is phosphorylated at S210 and S224 in the nucleus and that Aft1p phosphorylation is not affected by the iron status of the cells.

The results presented in Figure 4C suggested that two regions of Aft1p, Aft1p(147-270) and Aft1p(304-498), are necessary for the nuclear export of Aft1p. We analyzed interactions between the N-terminal or C-terminal regions of Aft1p by a yeast two-hybrid assay by using PJ69-4A cells (Figure 6D). We did not observe any interaction between Aft1p(1-270) and Aft1p(304-690), leading us to suspect that the sequences intervening between the two Msn5p-interacting regions, amino acids 271-303 [Aft1p(271-303)], may be involved in the interaction between the two domains. Although the addition of Aft1p(271-303) to one fragment had little effect on the interaction between the two fragments, PJ69-4A cells expressing BD-Aft1(1-303) and AD-Aft1p(271-690) were able to grow on –His and –Ade plates. The interaction between the two Aft1p fragments was confirmed to be iron-sensitive, because the interaction between the two domains was not observed in PJ69-4AΔ*fet3* cells (Figure 6E). We next examined the involvement of S210 and S224, as well as T421, T423, T431, and T435, in the interaction between Aft1p(1-303) and Aft1p(271-690) (Figure 6F). Although Aft1p(1-303, SA) interacted with Aft1p(271-690), Aft1p(271-690, TA) failed to interact with Aft1p(1-303). These results indicate that at least some of the threonine residues, which are dispensable for the interaction with Msn5p (Figure 6B), do participate in the iron-mediated interaction between the N-terminal and C-terminal regions of Aft1p. Thus, phosphorylation of S210 and S224 may be involved in the interaction with Msn5p (Figure 6, A and C), but not in the interaction between the two fragments of Aft1p.

We examined iron induces intermolecular interaction of Aft1p in cells using coimmunoprecipitation assays (Figure 6F). Δ*msn5* cells expressing Aft1p-TAP and Aft1p-HA were cultured in the presence or absence of iron and Aft1p-TAP was immunoprecipitated with IgG beads and the amount of Aft1p-HA that coprecipitated with Aft1p-TAP was assessed. Aft1p-HA was specifically coprecipitated from lysates of cells cultured in the presence of iron, indicating that two

Figure 5. Two serine and four threonine residues are important for Aft1p-nuclear export. (A and B) Amino acid sequence of the Msn5p-interacting regions of Aft1p aligned with the relevant regions of predicted Aft1p orthologues in other fungal species and with that of Aft2p. The sequences outside amino acids 202-270 (A) and amino acids 387-451 (B) are not presented because there is little conservation in these sequences. Identical amino acids are boxed and similar residues are shaded. The serine and threonine residues that were mutated for the experiments shown in C and Figure 6 are indicated by asterisks. (C) Two serine and four threonine residues are involved in the cytoplasmic localization of Aft1p. Y24 cells expressing Aft1p-HA (1 and 2), Aft1p(SA)-HA (3 and 4), Aft1p(S170A)-HA (5 and 6), Aft1p(S240A)-HA (7 and 8), Aft1p(SD)-HA (9 and 10), Aft1p(TA)-HA (11 and 12), or Aft1p(STA)-HA (13 and 14) were cultured to the logarithmic growth phase in the presence (+Fe; 1, 3, 5, 7, 9, 11, and 13) or absence (−Fe; 2, 4, 6, 8, 10, 12, and 14) of FOB. Cells were fixed, and the subcellular localization of Aft1p-HA and indicated mutants was examined by indirect immunofluorescence microscopy.

Aft1p proteins interact each other in an iron-dependent manner.

Intermolecular Interaction of Aft1p Is Required for Msn5p Recognition and the Mutation Found in Aft1-1^{np} Disrupts the Iron-mediated Intermolecular Interaction and Msn5p Recognition of Aft1p

The results presented above suggest that the iron-dependent intermolecular interaction of Aft1p is involved in its iron-dependent nuclear export. Because Aft1p(271-303) is necessary for the iron-dependent interaction of the N-terminal and C-terminal fragments of Aft1p (Figure 6, D and E), an amino acid(s) in Aft1p (271-303) may be critical for the

iron-dependent intermolecular interaction of Aft1p. We have shown that the Aft1-1^{np} mutant (Aft1pC291F) constitutively activates Aft1 target genes, because it localizes exclusively in the nucleus (Yamaguchi-Iwai *et al.*, 2002). Here, we assessed whether Cys291 is necessary for the interaction of the N- and C-terminal regions of Aft1p by using a yeast two-hybrid analysis (Figure 7A). Introduction of the C291F mutation in either Aft1p(1-303) or Aft1p(271-690) weakened the interaction between the two fragments, and the interaction between the two fragments was completely disrupted when the Cys291 in both fragments was mutated to phenylalanine. We next examined whether the C291F mutation of Aft1p affects the iron-dependent intermolecular interaction

Figure 6. Requirements for iron-dependent intermolecular interaction of Aft1p. (A) S210 and S224 are important for the interaction between Aft1p(147-270) and Msn5p. Interaction of Aft1p(147-270, SA) and Aft1p(147-270, SD) with Msn5p was examined with a yeast two-hybrid assay, as in Figure 3. (B) T421, T423, T431, and T435 are not important for the interaction between Aft1p(304-498) and Msn5p. Interaction of Aft1p(304-498, TA) with Msn5p was examined with a yeast two-hybrid assay, as in Figure 3. (C) S210 and S224 are involved in the phosphorylation of Aft1p. Proteins extracted from wild-type (BY4741) cells expressing Aft1p-HA (1-5 and 8), Aft1p(SA)-HA (6 and 9), or Aft1p(TA)-HA (7 and 10) were treated (+; 2 and 8-10) or not treated (-; 1 and 3-7) with CIAP and separated by SDS-PAGE. Aft1p-HA and its derivatives were detected by immunoblotting by using an anti-HA antibody. (D and E) Aft1p(1-303) and Aft1p(271-690) interact with each other in an iron-dependent manner. PJ69-4A (D) or PJ69-4A Δ fet3 (E) cells were transformed with expression vectors for AD-fused Aft1p(271-690) or Aft1p(304-690) and BD-fused Aft1p(1-270) or Aft1p(1-303). Interactions between these Aft1p fragments were tested, as in Figure 3. (F) T421, T423, T431, and T435 are essential for the interaction between Aft1p(271-690) and Aft1p(1-303). The interactions between these Aft1p fragments were tested, as in Figure 3, using PJ69-4A cells expressing AD-fused Aft1p(271-690) or Aft1p(271-690, TA) and BD-fused Aft1p(1-303) or Aft1p(1-303, SA). (G) Aft1p interacts each other in iron-rich conditions. Δ msn5 cells expressing Aft1p-TAP and Aft1p-HA were cultured in the presence (1 and 3) or absence (2 and 4) of iron, and Aft1p-TAP was immunoprecipitated with IgG beads. Precipitates (lanes 1 and 2) and 1/20 of the precipitated lysates (3 and 4) were resolved by SDS-PAGE and analyzed by immunoblotting with an anti-HA antibody and an anti-TAP antibody (Open Biosystems).

of the protein (Figure 7B). Aft1pC291F-HA failed to coprecipitate with Aft1pC291F-TAP, whereas Aft1p-HA was able to interact with Aft1p-TAP in the presence of iron. These results confirm that two Aft1p proteins interact each other in the presence of iron and that Cys291 is critical for that intermolecular interaction. Finally, we examined whether the iron-dependent intermolecular interaction of Aft1p is critical for recognition by Msn5p (Figure 7, C and D). Because the interaction between cargo and exportin depends on the GTP-bound form of Ran (Mattaj and Englmeier, 1998), mutants of the yeast homologue of Ran, Gsp1, which lack the GTPase activity (G21V) or specifically bind GDP (T26N), were prepared (Ueta *et al.*, 2003). Lysates of yeast cells expressing Aft1p-HA were incubated with Msn5p-myc in the presence of either GTP-loaded His₆-Gsp1^{G21V} or GDP-bound His₆-Gsp1^{T26N}, and Msn5p-myc was immunoprecipitated from the mixture. Anti-HA immunoblotting clearly showed that Aft1p-HA interacted with Msn5p in the presence of Ran-GTP (Figure 7C). Moreover, Aft1pC291F cannot interact with Msn5p in the presence of Ran-GTP (Figure 7D). Because the C291F mutation of Aft1p abolishes its ability to

interact each other, the intermolecular interaction of Aft1p seems to be critical for the recognition of Aft1p by Msn5p. Aft1-1^{up} may therefore localize in the nucleus because the C291F mutation disrupts the iron-dependent interaction of Aft1p, which in turn blocks its recognition by Msn5p.

DISCUSSION

Aft1p, an iron-sensing transcriptional activator, is a central regulator of iron metabolism in *S. cerevisiae* (Dancis, 1998; Philpott *et al.*, 2002; De Freitas *et al.*, 2003). Change in its nucleocytoplasmic localization in response to iron concentration is a key event in the regulation of iron-dependent transcriptional activation of genes involved in iron metabolism (Yamaguchi-Iwai *et al.*, 2002). Because Pse1p-mediated nuclear import of Aft1p is not regulated by cellular iron status (Ueta *et al.*, 2003), it was of great interest to identify the mechanism underlying nuclear export of the protein. In the present study, we have shown that nuclear export of Aft1p is regulated by the iron status of the cells and that Msn5p is an exportin for Aft1p. The interaction between

Figure 7. Intermolecular interaction of Aft1p is required for Msn5p recognition and the mutation found in Aft1-1^{up} disrupts the iron-mediated intermolecular interaction and Msn5p recognition of Aft1p. (A) Cys291 is important for the interaction between Aft1p(271-690) and Aft1p(1-303). PJ69-4A cells were transformed with expression vectors for AD-fused Aft1p(271-690) wild-type (WT) or C291F, and BD-fused Aft1p(1-303) WT or C291F, and the interactions between these Aft1p fragments were tested, as described in Figure 3. (B) Aft1pC291F failed to interact each other in iron-rich conditions. $\Delta msn5$ cells expressing Aft1p-TAP and Aft1p-HA (lanes 1 and 3) or Aft1pC291F-TAP and Aft1pC291F-HA (lanes 2 and 4) were cultured in the presence of iron. Aft1p-TAP or Aft1pC291F-TAP was immunoprecipitated with IgG beads, and the precipitates were analyzed, as described in Figure 6G. (C) Aft1p was recognized by Msn5p in the presence of Ran-GTP, but not Ran-GDP. Lysates of yeast cells expressing Aft1p-HA were incubated with Msn5p-myc in the presence of either GTP-loaded His₆-Gsp1^{G21V} (lanes 2 and 4) or GDP-bound His₆-Gsp1^{T26N} (lanes 1 and 3). Anti-myc immunoprecipitates from the mixture (lanes 1 and 2) and 1/100 of the precipitated mixtures (lanes 3 and 4) were separated in SDS-PAGE and probed with an anti-HA antibody and an anti-myc antibody. (D) Aft1p, but not Aft1pC291F, was recognized by Msn5p in the presence of Ran-GTP. Lysates of yeast cells expressing Aft1p-HA (lanes 1 and 3) or Aft1pC291F-HA (lanes 2 and 4) were incubated with Msn5p-myc in the presence of GTP-loaded His₆-Gsp1^{G21V}. Anti-myc immunoprecipitates from the mixture (lanes 1 and 2) and 1/100 of the precipitated mixtures (lanes 3 and 4) were separated in SDS-PAGE and probed as described in C.

Aft1p and Msn5p did not occur under conditions of iron starvation, and Aft1p nuclear export was promoted by iron. We therefore propose that the iron-dependent interaction between Aft1p and Msn5p is critical for the iron-regulated nucleocytoplasmic shuttling of Aft1p.

Msn5p was identified as a factor involved in the pheromone response and in calcium signaling (Akada *et al.*, 1996;

Matheos *et al.*, 1997) and has since been identified as a nuclear export receptor for many proteins, including transcription factors (Hopper, 1999). The Msn5p-interacting regions within these cargo proteins are large stretches of amino acid sequences (~100 amino acids or more) that do not share any homology (Blondel *et al.*, 1999; DeVit and Johnston, 1999; Boustany and Cyert, 2002; Jaquenoud *et al.*, 2002). Here, we have identified two regions of Aft1p, amino acids 147-270 and 304-498, that are necessary for interacting with Msn5p as well as for nuclear export of Aft1p. Phosphorylation of S210 and S224 within the former region is involved in the Msn5p-mediated nuclear export of Aft1p (Figures 5C and 6, A-C). Serine/threonine phosphorylation within the Msn5p-interacting region is similarly required for Msn5p recognition of many proteins (Kaffman *et al.*, 1998; DeVit and Johnston, 1999; Jaquenoud *et al.*, 2002). Mig1p, a transcriptional repressor responsible for the repression of many genes in *S. cerevisiae* in the presence of glucose, is regulated by Msn5p-mediated nuclear export in low glucose conditions (DeVit and Johnston, 1999). Phosphorylation of serine residues within the Msn5p-interacting region of Mig1p by the Snf1p kinase is critical for the glucose-induced nuclear export of Mig1p (DeVit and Johnston, 1999). Snf1p is also involved in the activation of Aft1p in the diauxic shift (the transition between fermentative and oxidative metabolism) (Haurie *et al.*, 2003), but neither S210 nor S224 of Aft1p is an Snf1 phosphorylation site (Dale *et al.*, 1995). Moreover, the activity of Aft1p is regulated by iron in $\Delta snf1$ cells (Haurie *et al.*, 2003). In fact, we observed that iron had no effect on Aft1p phosphorylation (Figure 6C) and that the phospho-mimetic mutations of S210 and S224 did not promote nuclear export of Aft1p in the iron-depleted condition (Figure 5C). Thus, we suspect that S210 and S224 of Aft1p are constitutively phosphorylated in the nucleus, but these phosphoserines are not sufficient for Msn5p-dependent export of Aft1p. We first hypothesized that the phosphorylation of at least one of the threonines, T421, T423, T431, and T435, in the second Msn5p interaction region would also be required for Msn5p recognition. This turned out not to be the case, but our further analysis suggested that at least one of these four threonines is involved in the iron-dependent interaction between the two regions of Aft1p (Figure 6, B and C). We also observed that two Aft1p proteins interact each other in iron-rich cells, which seems to be a key event in the regulation of Aft1p nuclear export (Figure 6G).

Other metal-sensing transcriptional activators are regulated by metal-dependent intramolecular interactions. Mac1p, a copper-responsive transcriptional activator in *S. cerevisiae*, is inactivated in the presence of copper through the intramolecular interaction of Cys-rich motifs within the transactivation domain (Jensen and Winge, 1998). Zap1p, a zinc-responsive transcriptional activator in *S. cerevisiae*, is inactivated in the presence of zinc, and its inactivation is similarly mediated by a zinc-dependent intramolecular interaction (Bird *et al.*, 2003; Wang *et al.*, 2006). Recently, Cuf1p, a copper-sensing transcription factor from *Schizosaccharomyces pombe*, has also been reported to undergo a copper-induced intramolecular interaction leading to the cytoplasmic retention of Cuf1p (Beaudoin and Labbe, 2006). However, in the case of Aft1p, an intermolecular, but not an intramolecular interaction of Aft1p is critical for its nuclear export and subsequent down-regulation of the expression of genes in the iron regulon. From the results presented here, we propose the following mechanism for the iron-dependent nuclear export of Aft1p. In iron-replete conditions, iron induces the intermolecular interaction of Aft1p. Msn5p recognizes a motif created by the intermolecular interaction of Aft1p and the phosphor-

ylated S210 and S224, and then it exports Aft1p to the cytoplasm. Cys291 is critical for intermolecular interaction of the protein, and its absence in the Aft1-1^{up} mutant (Aft1pC291F) may in part explain why Aft1-1^{up} localizes exclusively in the nucleus and constitutively activates Aft1-target genes.

If intermolecular interaction of Aft1p in iron-replete cells is necessary for the regulation of Aft1p by iron (Figures 6 and 7), what signal induces this intermolecular interaction? It has been hypothesized that Aft1p itself senses iron, which triggers the nucleocytoplasmic shuttling of the protein (Yamaguchi-Iwai *et al.*, 1995; Winge *et al.*, 1998). A recent study demonstrated that the mitochondrial but not the cytoplasmic Fe-S cluster metabolism is important for Aft1p activation, and the binding of the Fe-S cluster directly to Aft1p was questioned (Rutherford *et al.*, 2005). Additionally, an unidentified molecule that is transported from mitochondria to the cytoplasm by the Atm1p ABC transporter has been suggested to regulate Aft1p (Rutherford *et al.*, 2005). Another report indicates that direct binding of glutaredoxin-3 (Grx3p) and -4 (Grx4p) to Aft1p functions in the iron regulation of Aft1p (Ojeda *et al.*, 2006). Glutaredoxin-5 is involved in the Fe-S cluster biogenesis in mitochondria, where it is localized (Rodriguez-Manzanique *et al.*, 2002), and mitochondrial expression of Grx3p or Grx4p compensates for the defect in Δ grx5 cells (Molina *et al.*, 2004). Indeed, in Δ grx3 Δ grx4 cells, Fe incorporation into cytoplasmic Fe-S proteins, as well as iron-dependent nuclear export of Aft1p, are affected (Ojeda *et al.*, 2006; Pujol-Carrion *et al.*, 2006). These observations again raise the possibility that Aft1p may sense iron through its binding to an Fe-S cluster. The ²⁹¹CXC²⁹³ motif of Aft1p is essential for its regulation by iron (Yamaguchi-Iwai *et al.*, 1995), and both Grx3p and Grx4p fail to bind to the Aft1 C291,293F mutant (Ojeda *et al.*, 2006). We showed here that the C291F mutation disrupts the iron-dependent intermolecular interaction of Aft1p (Figure 7B). Thus, it is tempting to speculate that the binding of an Fe-S cluster to the ²⁹¹CXC²⁹³ motif of Aft1p mediates the intermolecular interaction and creates the motif recognized by Msn5p. More work will be needed to clarify the detailed mechanism underlying the iron-induced conformational change that creates the motif recognized by Msn5p.

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