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TITLE

Iron-Induced Dissociation of the Aft1p Transcriptional Regulator from Target Gene Promoters is an Initial Event in Iron-Dependent Gene Suppression

Running title: Iron-regulated DNA binding of Aft1

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ABSTRACT

Aft1p is an iron-responsive transcriptional activator that plays a central role in the regulation of iron metabolism in *Saccharomyces cerevisiae*. Aft1p is regulated by accelerated nuclear export in the presence of iron, mediated by Msn5p. However, the transcriptional activity of Aft1p is suppressed in iron-replete conditions in the *Δmsn5* strain, although Aft1p remains in the nucleus. Aft1p dissociates from its target promoters in iron-replete conditions due to an interaction between Aft1p and the monothiol glutaredoxins Grx3p or Grx4p (Grx3/4p). The binding of Grx3/4p to Aft1p is induced by iron repletion and requires binding of an iron-sulfur cluster to Grx3/4p. The mitochondrial transporter Atm1p that has been implicated in the export of iron-sulfur clusters and related molecules is required not only for iron binding to Grx3p but also for dissociation of Aft1p from its target promoters. These results suggest that iron binding to Grx3p (and presumably Grx4p) is prerequisite for the suppression of Aft1p. Since Atm1p plays crucial roles in the delivery of iron-sulfur clusters from the mitochondria to the cytoplasm and nucleus, these results support the previous observations that the mitochondrial iron-sulfur cluster assembly machinery is involved in cellular iron sensing.

INTRODUCTION

Iron is essential for various 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 machinery for sensing and regulating intracellular iron (10, 15, 34). The yeast *Saccharomyces cerevisiae* has served as a model organism to investigate iron metabolism in eukaryotic cells because iron homeostatic mechanisms are highly conserved between yeast, plants and animals (7). Iron homeostasis in *S. cerevisiae* is maintained primarily by the transcriptional activator Aft1p (40). Aft1p is activated only under iron-limiting conditions, and then induces the expression of more than 20 genes that comprise the iron regulon (36, 40, 42, 46, 47). The iron regulon includes genes encoding proteins involved in iron uptake and utilization, such as the iron permease Ftr1p and the multicopper ferroxidase Fet3p that form a high affinity iron transporting complex (1, 43). Iron-dependent modulation of Aft1p localization is involved in this regulation (48). Aft1p is imported into the nucleus by the nuclear import receptor Pse1p regardless of cellular iron status (45). However, Aft1p is exported from the nucleus by the nuclear export receptor Msn5p in iron-replete conditions (44). As a result, Aft1p accumulates in the nucleus only under iron-limited conditions. But, no evidence has convincingly shown that iron-dependent nuclear export by Msn5p is essential for down-regulating the transcriptional activity of Aft1p.

In this study, the mechanism underlying the iron-dependent suppression of Aft1p transcriptional activity was investigated. Aft1p activity was suppressed in a *Δmsn5* yeast strain, in iron-replete conditions, even though Aft1p remained in the nucleus. Thus, iron regulates Aft1p by two mechanisms: first, Aft1p is dissociated from target promoters in iron-replete conditions; second, Aft1p is recognized by Msn5p and exported to the cytoplasm (44). This study shows that the dissociation of Aft1p from target DNA is the critical step in the regulation of iron metabolism by Aft1p, and that dissociation requires the extra-mitochondrial monothiol glutaredoxins, Grx3p and Grx4p (Grx3/4p), and the mitochondrial ABC exporter Atm1p. Grx3/4p are thought to participate in intracellular transport of iron-sulfur clusters or their related molecules that are assembled in the mitochondria. These results suggest that, in iron-replete conditions, iron-sulfur clusters produced by the mitochondria are exported by Atm1p into the cytosol, where the iron-sulfur clusters are bound to Grx3/4p, and are subsequently recognized by Aft1p upon interacting with Grx3/4p.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in this study are listed in Table 1. Y26 (*Δaft1Δmsn5*) was derived by sporulation and tetrad dissection of crosses between Y23 (*Δaft1*, *MATa*) and Y25 (*Δmsn5*, *MATα*). Y30 (*Δgrx3Δgrx4*) was derived by sporulation and tetrad dissection of crosses between Y27 (*Δgrx3*, *MATa*) and Y29 (*Δgrx4*, *MATα*). Disruption of each gene was verified by PCR using specifically designed primers. Strains containing the *GRX3-HA* or *GRX4-HA* gene integrated at the chromosomal loci of *GRX3* or *GRX4*, respectively, were constructed using the classical pop-in/pop-out gene replacement method (39). Proper gene replacement was confirmed by PCR using specifically designed primers and DNA sequencing. The *GAL-ATM1* strains were maintained in YPGal or SGal medium supplemented with appropriate amino acids. Other cells were grown routinely in YPD or SD medium supplemented with appropriate amino acids. To produce iron-starved conditions, before each assay, cells were cultured for 20 h in iron-free medium, which consisted of yeast nitrogen base without iron, 2% glucose, 50 mM MES buffer (pH 6.1), and 500 μM ferrozine. To deplete Atm1p, the *GAL-ATM1* strains were cultivated in SD medium for 48 h prior to analysis.

Plasmids. Expression plasmids for HA-tagged Aft1p (Aft1-HA), tandem affinity purification (TAP)-tagged Aft1p (Aft1-TAP), or their mutants, driven by the *AFT1* promoter have been described previously (44). For yeast two-hybrid assays, pAD-AFT1 (44),

92 pBD-GRX3, pBD-GRX4, and pBD-NBP35 were used. The pBD-GRX3, pBD-GRX4, and
93 pBD-NBP35 constructs were created by inserting DNA fragments covering the complete
94 coding regions of *GRX3*, *GRX4* or *NBP35* into pGBKT7 (Invitrogen). Expression plasmids
95 for Grx3-HA, Grx4-HA, or their mutants, were created by inserting the open reading frames
96 (ORF) of *GRX3-HA*, *GRX4-HA*, or their mutants, into pRS415 containing the *ADH1*
97 promoter.
98 **Indirect immunofluorescence microscopy.** The subcellular localization of HA-tagged
99 proteins was examined by indirect immunofluorescence microscopy as described previously
100 (44). Briefly, cells expressing HA-tagged proteins were fixed in 4% formaldehyde. Cell walls
101 were digested with 300 units of zymolyase (Seikagaku Kogyo), followed by the addition of
102 2% SDS. Spheroplasts were fixed on poly-lysine-coated cover slips, permeabilized with
103 0.05% saponin, and then incubated with an anti-HA antibody (HA.11, Covance). Signals
104 were amplified and visualized using an Alexa Fluor 594 Signal-Amplification Kit
105 (Invitrogen). Nuclei were stained by incubation with 500 ng/ml 4',6'-diamino-2-phenylindole
106 (DAPI) for 5 min. Fluorescent and differential interference contrast (DIC) images were
107 captured using a FV-1000 confocal microscope (Olympus). Expression of the tagged proteins
108 was measured by immunoblotting and found to be similar in each of the strains under the
109 different iron conditions employed.
110 **Northern blotting.** Cells cultured to mid-log phase were harvested and total RNA was
111 isolated, as described (17). The RNA was separated on a 1% agarose gel containing

112 formaldehyde, transferred to a Biodyne B membrane (Pall Corporation) and hybridized with
113 ^{32}P-labeled probes for *FTR1* (nucleotides 1 to 649 of the *FTR1* ORF), *FET3* (nucleotides 1 to
114 582 of the *FET3* ORF), *FRE1* (nucleotides 1 to 2058 of the *FRE1* ORF), *SIT1*/*ARN3*
115 (nucleotides 1 to 1884 of the *SIT1*/*ARN3* ORF), and *ACT1* (nucleotides 37 to 1070 of the
116 *ACT1* ORF). The hybridized membranes were analyzed using a BAS-2000 Imager (GE
117 Healthcare).
118 **Chromatin immunoprecipitation.** Cells carrying the Aft1-TAP plasmid were incubated in
119 1% formaldehyde for 15 min followed by 125 mM glycine for 5 min at 30 °C. Cells were
120 collected and lysed in 50 mM HEPES (pH 7.5), 500 mM NaCl, 1 mM EDTA, 1% Triton
121 X-100, 0.1% deoxycholate using Multi-Beads Shocker (Yasui Kikai), and the homogenate
122 was then sonicated to shear chromatin. Aft1-TAP was precipitated from an aliquot of the
123 soluble fraction (500 μg total protein) using rabbit IgG conjugated Dynabeads M-270 Epoxy
124 (Invitrogen). Co-precipitated DNA fragments were incubated in 10 mM Tris-Cl (pH 8.0), 1
125 mM EDTA, 1% SDS at 65 °C for 12 h for reverse-crosslinking, and then analyzed by
126 semi-quantitative PCR to amplify -702 to -3 nt of the *FET3* promoter and -292 to -33 nt of the
127 *ACT1* promoter. PCR-amplified DNA was separated on 2% agarose gels and analyzed using
128 an LAS-3000 Imager (GE Healthcare).
129 **Yeast two-hybrid screening and assays.** Yeast two-hybrid screening for Aft1p-interacting
130 proteins has been described previously (23). Yeast two-hybrid assay was performed by
131 examining the growth of PJ69-4A strains expressing both AD- and BD-fused proteins in

media lacking adenine and histidine. Cells were spotted using 3-fold serial dilutions beginning at 600 cells. All cells showed similar growth on medium containing adenine and histidine.

Co-precipitation. Cells expressing TAP- and HA- fused proteins were lysed in 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 2 mM PMSF using Multi-Beads Shocker. The soluble fraction was incubated with rabbit IgG conjugated Dynabeads M-270 Epoxy in 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100 at 4 °C for 2 h. The beads were washed five times with the same buffer, and precipitates were separated using 9% SDS-PAGE, and subjected to immunoblotting using anti-HA or anti-TAP antibodies (Thermo Scientific). Expression levels of the fusion proteins were confirmed by immunoblotting an aliquot of each cell lysate using the anti-HA and anti-TAP antibodies.

Iron binding to Grx3p *in vivo*. Cells expressing Grx3-TAP were cultured in iron-depleted medium to mid-log phase growth. Cells (OD_{600} = 10) were radiolabeled with 370 KBq of ^{55}Fe for 30 min. Cells were washed with 50 mM citrate (pH 7.4), 1 mM EDTA, and lysed in 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 2.5 mM EDTA, 10% glycerol, and 2 mM PMSF using Multi-Beads Shocker. Grx3-TAP was precipitated from lysates (5 mg total protein) with rabbit IgG conjugated Dynabeads M-270 Epoxy, and co-precipitated ^{55}Fe was quantified by scintillation counting. The precipitated protein was assessed by immunoblotting using an anti-TAP antibody.

RESULTS

Iron mediates growth suppression in constitutively active Aft1p cells but not in *Δmsn5* cells. Aft1-1^{up}, which is a C291F mutant, is constitutively nuclear and activates the expression of target genes regardless of the iron status of the cells (46-48). The growth of the *AFT1-1^{up}* mutant strain is inhibited in iron-replete conditions, possibly because of iron-toxicity (46). We confirmed that the growth of the *AFT1-1^{up}* mutant strain was inhibited in the presence of as little as 50 μM iron in the medium (Fig. 1A). The nuclear export receptor Msn5p is required for the iron-mediated nuclear export of Aft1p, and, consequently, Aft1p resides in the nucleus regardless of the iron concentration in the medium in the *Δmsn5* strain (44) (Fig. 1C). However, in contrast to the *AFT1-1^{up}* strain, the growth of the *Δmsn5* strain was not retarded in iron-replete conditions and appeared similar to the wild-type strain (Fig. 1A).

Several amino acid residues of Aft1p, including Leu99, Leu102, Ser210, Ser224, Cys291, Cys293, Thr421, Thr423, Thr431 and Thr435, play important roles in the iron-responsive nuclear export of Aft1p (44, 48). Aft1p mutants, in which some of those amino acid residues were substituted for alanine, were generated (Aft1pLeu99/102Ala [Aft1p(LA)], Aft1pSer210/224Ala [Aft1p(SA)], Aft1pCys291/293Ala [Aft1p(CA)] and Aft1pThr421/423/431/435Ala [Aft1p(TA)]). The mutants were introduced in *Δaft1* cells to generate cells expressing only the Aft1p mutant. The Aft1p mutants were localized in the

171 nucleus even in iron-replete conditions (Fig. 1C) and the effect of iron on the growth of cells
172 expressing these Aft1p-mutants was examined. Although cells expressing Aft1p(LA) or
173 Aft1p(CA) grew poorly in high iron medium, as observed in the *AFT1-1*^{up} strain, the growth
174 of cells expressing Aft1p(SA) or Aft1p(TA) was not retarded in iron-rich medium (Fig. 1B).
175 These results suggest that nuclear retention of Aft1p is not sufficient to induce iron-mediated
176 growth suppression.
177
178 **Aft1p dissociates from its target promoters in response to iron repletion.** The growth of
179 the *AFT1-1*^{up} strain and of cells expressing Aft1p(LA) or Aft1p(CA) was retarded in high
180 iron medium, whereas iron exerted virtually no effect on the growth of the *Δmsn5* strain, or
181 cells expressing Aft1p(SA) or Aft1p(TA), despite the fact that Aft1p localizes in the nucleus
182 in iron-replete conditions (Fig 1). Although Ser210, Ser224, Thr421, Thr423, Thr431 and
183 Thr435, which are mutated in Aft1p(SA) or Aft1p(TA), are involved in interactions with
184 Msn5p, Leu99, Leu102, Cys291 and Cys293 do not appear to be involved in binding Msn5p
185 (44). To dissect the differential roles of these two groups of amino acid residues, we tested
186 the iron-regulated expression of Aft1p target genes (*FET3, FTR1, FRE1, and SIT1/ARN3)* by
187 Northern blotting (Fig. 2A). The mRNA levels for these genes were high in iron-depleted
188 conditions in all strains, suggesting that mutation of these amino acid residues did not affect
189 the transcriptional activation activity of Aft1p. Expression of the iron regulon decreased in
190 iron-replete conditions in the *Δmsn5* strain, as well as in Aft1p(SA)- and

191 Aft1p(TA)-expressing cells, similar to cells expressing wild-type Aft1p. However, in
192 Aft1p(LA)- or Aft1p(CA)-expressing cells, the iron regulon was not substantially
193 down-regulated under iron-replete conditions. Previously, target DNA binding by Aft1p was
194 suggested to occur only in iron-limited conditions by *in vivo* footprinting (47). Indeed,
195 chromatin immunoprecipitation revealed that Aft1p bound to the Aft1p-regulated *FET3*
196 promoter only under iron-limited conditions in the *Δmsn5* strain, as well as in cells
197 expressing Aft1p(SA) or Aft1p(TA) and in wild-type cells (Fig. 2B). By contrast, the
198 Aft1p(LA) and Aft1p(CA) mutants bound to the *FET3* promoter even in iron-replete
199 conditions (Fig. 2B). The *Δaft1* strain transformed with a centromere-based plasmid
200 expressing Aft1-TAP under the *Aft1* promoter expressed and regulated Aft1p-target genes in
201 a manner identical to cells expressing the wild-type Aft1p (Fig. 2C), indicating that
202 Aft1-TAP is functional. These results are consistent with the previous observations that
203 substitution of Leu99 with Ala and Cys291 with Phe generated a constitutively active Aft1p
204 transcriptional activator (32, 46, 48). Thus, iron-replete conditions appear to induce not only
205 Aft1p-nuclear export but also the dissociation of Aft1p from target promoters, and Cys291,
206 Cyc293, Leu99 and Leu102 appear to be involved in the dissociation of Aft1p from the iron
207 regulon promoters.
208
209 **Involvement of Leu99, Leu102, Cys291, and Cys293 in Aft1p interactions with Grx3p**
210 **and Grx4p.** To elucidate the mechanism underlying dissociation of Aft1p from the iron

211 regulon promoters in iron-replete cells, yeast two-hybrid screening was used to identify
212 proteins that are involved in regulation of Aft1p (23). Using Aft1p lacking the transactivation
213 domain [Aft1(1-413)] as prey, we identified six candidate genes, three of which encode
214 iron-sulfur proteins: Nbp35p and two monothiol glutaredoxin homologs, Grx3p and Grx4p
215 (Grx3/4p). We tested if the interaction between Aft1p and these candidates for
216 Aft1p-interacting protein requires Leu99, Leu102, Cys291, and Cys293 of Aft1p, and found
217 that Grx3/4p did not bind to Aft1p(LA) or Aft1p(CA) in a yeast two-hybrid assay, although
218 Aft1p(SA) and Aft1p(TA) did interact with Grx3/4p (Fig. 3A). Immunoblot analyses of
219 cellular extracts using both anti-HA (for AD-fused proteins) and anti-myc (for BD-fused
220 proteins) showed that the fusion proteins were expressed in co-transformed cells although
221 expression of BD-Grx4p was higher than that of BD-Grx3p (Fig 3B). The interaction
222 between Aft1p and Grx3/4p was also examined by co-immunoprecipitation analyses.
223 Consistent with the results obtained by the yeast two-hybrid assays, neither Aft1p(LA) nor
224 Aft1p(CA) co-precipitated with Grx3/4p, whereas Aft1p, Aft1p(SA) and Aft1p(TA) did
225 co-precipitate (Fig. 3C and D). These results indicate that Aft1p interacts with Grx3/4p, and
226 that the Aft1p residues Leu99, Leu102, Cys291, and Cys293 are involved in this interaction,
227 raising the possibility that Grx3/4p are involved in the dissociation of Aft1p from target
228 promoters in response to iron repletion.
229
230 **Grx3/4p are required for the iron-dependent dissociation of Aft1p from target**

231 **promoters.** *GRX3* and/or *GRX4* deletion strains were constructed to test whether the binding
232 of Aft1p to the *FET3* promoter is regulated by iron. In the strains that lacked either Grx3p or
233 Grx4p, Aft1p dissociated from the *FET3* promoter in response to iron repletion as observed
234 in the wild-type strain (Fig.4A). When both *GRX3* and *GRX4* were deleted, Aft1p occupied
235 the *FET3* promoter even in iron-replete conditions (Fig. 4A). The Aft1p-regulated iron
236 regulon was highly expressed even in iron-replete conditions in *Δgrx3Δgrx4* cells, but not
237 *Δgrx3* or *Δgrx4* cells (Fig. 4B), similar to previous results (32). These results indicate that
238 Grx3/4p are required for iron-suppression of transcription of the iron regulon by inducing
239 iron-dependent dissociation of Aft1p from target promoters.
240
241 **The interaction between Aft1p and Grx3/4p is augmented in iron-replete conditions.**
242 The above results suggested that the interaction between Aft1p and Grx3/4p plays a crucial
243 role in dissociation of Aft1p from target promoters in iron-rich conditions. Hence, we
244 examined whether the interaction between Aft1p and Grx3/4p is induced upon iron repletion,
245 in order to suppress the transcriptional activation activity of Aft1p. Cells containing both
246 *AFT1-TAP* and *GRX3-HA* integrated at the chromosomal loci of *AFT1* and *GRX3*,
247 respectively, were constructed. Grx3-HA is functional because the expression of the iron
248 regulon was normally regulated by iron in *Δgrx3Δgrx4* cells expressing Grx3-HA (Fig. 4B).
249 Cells expressing Aft1-TAP and Grx3-HA were first cultured in iron-free medium (SD
250 lacking ferric chloride, and supplemented with 500 μM ferrozine) for 20 h to ensure iron

starvation. Cells were then cultured for 15 min in the presence of 200 μM ferrous sulfate. Cell lysates were prepared before, or 15 min after, the addition of 200 μM ferrous sulfate, and co-precipitation experiments were performed. Grx3p-HA was effectively co-precipitated with Aft1p-TAP in lysates from cells after iron repletion, whereas minimal amounts of Grx3p-HA co-precipitated with Aft1p-TAP in lysates from iron-starved cells (Fig. 5A). Similarly, Grx4-HA was effectively co-precipitated with Aft1-TAP only after iron repletion (Fig. 5B). By contrast, previous reports suggest that Grx3/4p interact with Aft1p regardless of the iron status of cells (19, 32). Kumanovics *et al* indicated that Aft1-TAP is effectively co-precipitated with Grx3p both under high- and low-iron conditions (19). In their analysis, cells were cultivated in SD medium containing either 250 μM iron sulfate or 40 μM BPS, an impermeable iron (II) chelator, for 6 h, to produce high- or low-iron conditions, respectively (19). The expression of iron regulon genes including *FET3* and *FTR1* was induced in the iron-depleted condition. The levels of *FTR1* and *FET3* mRNAs were higher in cells cultivated for 20 h in SD medium lacking iron than in cells cultivated for 6 h in SD medium containing BPS (Fig. 5C). This result suggests that treatment of BPS for 6 h does not completely deprive the cells of iron and a substantial amount of iron bound to Grx3/4p, accounting for the interaction between Aft1p and Grx3/4p in cells treated with BPS for 6 h, as observed in the previous report (18). However, in the BPS treated cells, the Grx3/4p may not be fully saturated with iron and some free Aft1p may be available to induce the expression of the iron regulon. Moreover, indirect immunofluorescence analyses revealed

that Grx3/4p reside both in the nucleus and the cytoplasm, and changes in cellular iron status did not overtly affect their subcellular localization (Fig. 5D), as reported previously (19, 24). Taken together, these results strongly suggest that iron induces the interaction between Aft1p and Grx3/4p.

Grx3/4p require an iron-sulfur cluster to interact with Aft1p. Several lines of evidence suggest that ligation of a [2Fe-2S] cluster plays a crucial role in dimer formation by monothiol glutaredoxins *in vitro* and *in vivo* (26, 35). Because Grx3/4p-Aft1p binding appeared to be regulated by the iron status of cells, a [2Fe-2S] cluster and/or [2Fe-2S] cluster dependent dimerization of Grx3/4p may be involved in the Grx3/4p-Aft1p interactions. To address the possibility, we first tested the iron-dependent dimerization of Grx3/4p. We expressed Grx3-TAP, which was shown to be functional (Fig 4B), and Grx3-HA in *Δgrx3Δgrx4* cells. Cells were cultured in iron-free medium for 20 h, and co-precipitation experiments were performed using cell lysates that were prepared before, or 15 min after, the addition of 200 μM ferrous sulfate. As shown in Fig 6A, Grx3-HA is co-precipitated effectively with Grx3-TAP in the lysate of iron-replete cells, but not in the lysate of iron-starved cells. To verify iron-dependent dimerization of monothiol glutaredoxins in physiological settings, we constructed cells with both *GRX3-TAP* and *GRX4-HA* integrated at the chromosomal loci of *GRX3* and *GRX4*, respectively. Co-precipitation analyses revealed that Grx3-TAP and Grx4-HA form stable complexes only in iron-rich conditions (Fig. 6B).

These results indicate that Grx3/4p dimerize in an iron-dependent manner, raising the possibility that Grx3/4p bind to Aft1p in the dimer form.

Next, Grx3p mutants that cannot bind iron were constructed. Since Cys31 and glutathione binding residues such as Lys23 and Thr71 of a cyanobacterium Grx3p, which is homologous to Grx3/4p, is involved in [2Fe-2S] cluster ligation (35), the corresponding residues of *S. cerevisiae* Grx3p were mutated: Cys211 to Ser, Lys203 to Gln, and Thr251 to Val (Grx3pC211S, Grx3pK203Q and Grx3pT251V). To analyze iron binding by these Grx3p mutants *in vivo*, *Δgrx3Δgrx4* cells expressing TAP-tagged wild-type or mutant Grx3p were cultured in the presence of ^{55}Fe. Subsequently, TAP-tagged Grx3p proteins were immunoprecipitated and the amount of co-precipitated ^{55}Fe was quantified by scintillation counting. Wild-type Grx3p bound ^{55}Fe, but the Grx3pC211S, Grx3pK203Q and Grx3pT251V mutants failed to bind ^{55}Fe (Fig 6C), indicating that these residues are important for iron-sulfur ligation by *S. cerevisiae* Grx3p. Whether these Grx3p mutants can bind to Aft1p was examined by co-immunoprecipitation analyses. The wild-type Grx3p, but not the C211S, K203Q, or T251V mutants, co-precipitated with Aft1p (Fig. 6D), suggesting that [2Fe-2S] cluster ligation by Grx3p is prerequisite for Aft1p-Grx3p interactions. The Grx3p mutants were expressed in *Δgrx3Δgrx4* cells and promoter occupation by Aft1p and expression of Aft1p target genes was evaluated. As shown in Fig. 6E, Aft1p occupied the *FET3* promoter even in iron-replete conditions in cells expressing the Grx3p mutants that failed to ligate the iron-sulfur cluster, but Aft1p dissociated from the *FET3* promoter in

311 iron-replete cells expressing wild-type Grx3p. Northern blotting revealed that expression of
312 the iron regulon was not reduced in response to iron in *Δgrx3Δgrx4* cells expressing the
313 C211S, K203Q, or T251V mutants of Grx3p, but was reduced in cells expressing wild-type
314 Grx3p (Fig. 6F). Collectively, these results indicate that ligation of a [2Fe-2S] cluster by
315 Grx3p is necessary for dissociation of Aft1p from its target promoters.
316
317 **Atm1p is required for iron-sulfur loading onto Grx3/4p and Aft1p regulation by iron.**
318 Defects in the iron-sulfur cluster assembly (ISC) machinery in mitochondria lead to
319 reductions in iron binding by Grx3/4p *in vivo* (26). Thus, whether iron-sulfur clusters
320 transported from the mitochondria are involved in iron-sulfur cluster loading by Grx3/4p in
321 the cytosol or nucleus was examined. The mitochondrial ABC-transporter Atm1p has been
322 suggested to be an exporter of iron-sulfur clusters or related molecules (16, 22). Whether
323 Atm1p is required for iron-sulfur cluster loading onto Grx3/4p and, therefore, the
324 iron-dependent dissociation of Aft1p from target promoters was examined. A strain in which
325 *ATM1* gene expression was driven by the glucose-repressible *GAL1-10* promoter
326 (*GAL-ATM1*) was constructed, as described previously (2). To repress the *GAL1-10* promoter
327 and deplete Atm1p, the *GAL-ATM1* strain was grown for 48 h on synthetic medium
328 containing D-glucose as a sole carbon source (2). As shown in Fig. 7A, the expression of
329 *ATM1* in the *GAL-ATM1* strain was undetectable by Northern blotting after culture in the
330 glucose medium. Wild-type control cells were also cultured on the glucose containing

331 medium for these experiments. In the *GAL-ATM1* strain, expression of the iron regulon did
332 not decrease in iron-rich conditions, as reported previously (41), indicating that expression of
333 Atm1p was adequately suppressed in the *GAL-ATM1* strain (Fig. 7B). Expression of iron
334 regulon transcripts decreased in the wild-type, but not in the *GAL-ATM1* strain, in the
335 presence of iron. Chromatin immunoprecipitation analyses revealed that Aft1p bound to the
336 *FET3* promoter regardless of iron status in the Atm1p-depleted (*GAL-ATM1*) cells (Fig. 7C).
337 The interaction between Aft1p and Grx3p in Atm1p-depleted cells was assessed by
338 co-immunoprecipitation. In the wild-type strain, Grx3p co-precipitated with Aft1p in
339 iron-replete conditions, but Grx3p failed to interact with Aft1p in iron-replete
340 Atm1p-depleted cells (Fig. 7D). Since ligation of the iron-sulfur cluster by Grx3p appears to
341 be a prerequisite for interactions between Grx3p and Aft1p, ^{55}Fe-binding to Grx3p in
342 iron-replete cells was assessed, as described in Fig 6C. The amount of ^{55}Fe in Grx3p
343 immunoprecipitates was significantly lower in Atm1p-depleted cells than in wild-type cells
344 (Fig. 7E). Collectively, these results indicate that Atm1p is involved in iron-sulfur cluster
345 loading by Grx3p, the net result of which is dissociation of Aft1p from target promoters in
346 iron-replete cells.

DISCUSSION

The activity of Aft1p, a central transcriptional regulator of iron metabolism in *S. cerevisiae*, is regulated by iron-dependent nuclear export of Aft1p mediated by the nuclear export receptor Msn5p (44). This study demonstrates another layer of iron regulation of Aft1p: Aft1p dissociates from target promoters in response to iron repletion.

Several transcriptional regulators are exported by Msn5p. Crz1p, a calcineurin-responsive transcriptional activator is regulated, in part, by Msn5p-mediated nuclear export. Calcineurin-mediated dephosphorylation of Crz1p suppresses Msn5p-mediated nuclear export. In *Δmsn5* cells, some Crz1p is localized in the nucleus and activates the Crz1p-responsive CDRE promoter (4). Thus, Msn5p-mediated nuclear export is required to suppress Crz1p activity. However, Msn5p-mediated nuclear export of some transcriptional regulators is not required for their suppression. For example, Pho4p is a transcription factor that regulates a variety of genes in response to phosphate availability, and Msn5p-mediated nuclear export of Pho4p is regulated by Pho4p phosphorylation in phosphate-rich conditions (31). Pho4p has multiple phosphorylation sites and phosphorylation of some of these induces the Pho4p interaction with Msn5p (14). However, phosphorylation of Pho4p at a different site disrupts the interaction with its dimerization partner, Pho2p, and thereby suppresses Pho4p transcriptional activator activity (18). Nuclear export of Mig1p, a transcriptional repressor that represses a number of genes in the presence of glucose (28, 29), is also

367 mediated by Msn5p (8). However, phosphorylation of Mig1p, induced by glucose
368 deprivation, not only facilitates Msn5p-mediated nuclear export of Mig1p, but also induces
369 dissociation from the co-repressor complex (8, 33). Therefore, Msn5p-mediated nuclear
370 export is not required for suppression of Pho4p or Mig1p activity. Here, Msn5p-mediated
371 nuclear export was shown to be dispensable for iron-mediated suppression of Aft1p
372 transcriptional activity (Fig. 2). Msn5p-mediated nuclear export of Crz1p, Pho4p and Mig1p
373 is regulated by phosphorylation/dephosphorylation (4, 8, 14). Although phosphorylation of
374 Aft1p is necessary for recognition by Msn5p, Aft1p phosphorylation is not regulated by
375 cellular iron status (44).
376 The present study has demonstrated that iron-mediated dissociation of Aft1p from target
377 DNA sequences occurs prior to Msn5p-mediated nuclear export. Since earlier work showed
378 that dimerization or multimerization of Aft1p was required for Aft1p-Msn5p binding (44),
379 Aft1p must form multimers after dissociation from the target sequences, and is then
380 recognized by Msn5p. Therefore, it would be of interest to determine whether
381 phosphorylation of Crz1p, Pho4p or Mig1p induces dimerization of the transcriptional
382 regulators as an essential step in Msn5p recognition.
383 Grx3/4p play crucial roles in iron-mediated suppression of the transcriptional activity of
384 Aft1p (32, 37). However, the precise roles played by Grx3/4p in Aft1 regulation had not been
385 determined previously. Here, we showed that Grx3/4p induced Aft1p dissociation from the
386 target promoters through an enhanced interaction with Aft1p in iron-replete conditions.

Aft1p mutants that were unable to interact with Grx3/4p did not dissociate from the Aft1p-regulated gene promoters even in iron-rich conditions (Fig. 1-3). Grx3/4p failed to dimerize in severe iron-deprived conditions (Fig 6). Several lines of evidence suggest that iron bound to Grx3/4p in cells is in the form of a [2Fe-2S] cluster. When overproduced in *Escherichia coli,* the GRX domain of Grx3/4p binds a [2Fe-2S] cluster utilizing the cysteine located within the conserved CGFS motif (Cys211 for Grx3p and Cys171 for Grx4p) and a glutathione cofactor (35). The conserved Cys within the CGFS motifs and the amino acids that are involved in glutathione binding are thus implicated in [2Fe-2S] binding. Mutations of these amino acids in Grx3/4p reduced iron incorporation into Grx3/4p to near background levels *in vivo* and suppressed the binding of Grx3p to Aft1p (Fig. 6) (26). Moreover, glutathione depletion of cells impairs iron incorporation into Grx3/4p (26). Therefore, iron binding to Grx3/4p appears to be prerequisite for dimerization and Aft1p interactions with the monothiol glutaredoxins. Whether dimerization of Grx3/4p is sufficient for binding to Aft1p or iron itself, bound to Grx3/4p, is required for the interaction with Aft1p will be of interest to determine in future studies.

Iron-sulfur clusters are generated in mitochondria (27) and defects in proteins in the mitochondrial ISC machinery result in constitutive expression of Aft1p-regulated genes (41). A previous report indicated that the ISC machinery is required for iron incorporation into Grx3/4p *in vivo* (26). The mitochondrial ABC exporter Atm1p has been implicated in the export of iron-sulfur clusters or related molecules from mitochondria into the cytosol

because iron loading of several extra-mitochondrial iron-sulfur proteins, such as Leu1p and Nbp35p, is decreased in Atm1p-depleted strains (16, 22). In this study, Atm1p depletion significantly decreased both binding of Grx3p to iron, and binding of Grx3p to Aft1p in iron-replete conditions, and resulted in misregulation of Aft1p (Fig. 8). Although heme and intermediates in the heme biosynthetic pathway may also be Atm1p-substrates (2, 20), heme is not required for Aft1p inhibition by iron (6). The possibility that the iron bound to Grx3/4p that is required for the interaction between Grx3/4p and Aft1p is not in the form of an iron-sulfur cluster cannot be excluded. However, our results strongly indicate that iron-sulfur clusters or molecules that invoke iron-sulfur cluster formation, which are generated in mitochondria and exported from mitochondria by Atm1p, are involved in iron-sulfur cluster loading of Grx3p and probably Grx4p, although the mechanism for cluster loading of Grx3/4p in the nucleus reminds to be elucidated. Since Grx3/4p binding to Aft1p is strongly augmented in iron-replete cells and iron binding of Grx3p is prerequisite for the interaction with Aft1p (Fig 6), iron-sulfur clusters or related molecules generated in the mitochondria and transported by Atm1p appear to play crucial roles in Grx3/4p binding to Aft1p in iron-replete cells. The net result of these interactions is dissociation of Aft1p from target promoters (Fig 8). This result is consistent with the previous observation that the mitochondrial ISC machinery is involved in cellular iron sensing (41).

Grx3/4p participate in iron loading of several iron-containing proteins (26, 49). Grx3/4p may be involved in iron loading of Rnr2p, a cofactor of ribonucleotide reductase containing a

427 di-iron center, together with Dre2p (49). Grx3/4p are also involved in the insertion of
428 iron-sulfur clusters into cytosolic iron-sulfur containing proteins such as Leu1p or Rli1p (26).
429 Dre2p and Nbp35p, components of the cytoplasmic iron-sulfur assembly machinery, are also
430 required for the insertion of iron-sulfur clusters (9, 30, 50). Although Nbp35p was identified
431 as a candidate Aft1p-interacting protein by yeast two-hybrid screening (Fig. 3A), no
432 significant interaction between Aft1p and Nbp35p was detected by co-immunoprecipitation
433 and Aft1p was normally regulated by iron in Nbp35p-depleted cells (data not shown).
434 Moreover, suppression of Dre2p expression had no influence on iron-mediated suppression
435 of expression of Aft1p target genes (data not shown). Thus, neither Nbp35p, consistent with
436 earlier reports (41), nor Dre2p are required for suppression of Aft1p activity in iron-replete
437 cells, while Grx3/4p are indispensable.
438 Fra1p and Fra2p are also involved in iron-mediated suppression of Aft1p activity (19). Both
439 Fra1p and Fra2p bind to Grx3/4p (11, 21). Therefore, both proteins may function as a
440 bridging molecule between Aft1p and Grx3p. However, no interactions between Aft1p and
441 Fra1p or Fra2p have been detected and both proteins are localized primarily in the cytosol
442 (19), suggesting that either Fra1p or Fra2p is not involved in the Grx3/4p-mediated
443 dissociation of Aft1p from target promoters. Grx5p, a mitochondrial monothiol glutaredoxin,
444 is involved in storage and delivery of [2Fe-2S] clusters in the organelle (25, 38). Since forced
445 expression of Grx3/4p in mitochondria partially rescues Grx5p function and growth defects
446 of *Δgrx5* mutants (24), one role of Grx3/4p may be to deliver iron-sulfur clusters. The

activities of other metal-regulated transcriptional activators, Zap1p and Mac1p, are inhibited by the direct binding of zinc and copper (3, 13, 40), respectively. Direct metal binding may be the case with Aft1p as well. Aft1p forms homodimers or multimers in iron-rich cells and Cys291 of Aft1p is involved in this interaction (44). In this study, Grx3p and Grx4p were shown to form homo- or hetero-dimers in an iron-dependent manner (Fig. 6). Considering that *in vivo* and *in vitro* analyses suggest that Grx3/4p dimerize through bridging a [2Fe-2S] cluster (26, 35), it is tempting to speculate that Grx3/4p dimers deliver iron, possibly an iron-sulfur cluster, to Aft1p at Cys291 and Cys293 to form iron-sulfur cluster-bridging Aft1p dimers. Alternatively, Aft1p may indirectly sense cellular iron status through increased interactions with a [2Fe-2S] cluster-containing Grx3/4p dimer. Resolution of these complex interactions will require further biochemical and structural studies.

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459

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462

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620 **FIGURE LEGENDS**

621

622 **FIG 1.** The *Δmsn5* strain is not sensitive to excess iron. (A) The indicated strains were
623 spotted on SD medium supplemented with the indicated concentrations of $FeSO_4$ using
624 3-fold serial dilutions beginning at 200 cells per spot. Cell growth was observed after
625 incubation at 30 °C for 3 days. (B) The growth of cells expressing the indicated Aft1p
626 mutants was tested as in (A). (C) Aft1p accumulates in the nucleus of the *Δmsn5* strain
627 independent of cellular iron status. *Δaft1* or *Δmsn5Δaft1* cells carrying an expression plasmid
628 for wild-type Aft1-HA or the indicated mutants were cultured in iron-depleted (-Fe) or
629 iron-replete (+Fe) medium to mid-log phase growth. After fixation, the subcellular
630 localization of Aft1-HA was examined by indirect immunofluorescence microscopy using an
631 anti-HA antibody. DAPI staining of nuclei and differential interference contrast (DIC)
632 images are provided for comparison.

633

634 **FIG 2.** Aft1p dissociates from a target promoter in response to iron repletion. (A) Expression
635 of the iron regulon is suppressed in iron-replete *Δmsn5* cells. *Δaft1* or *Δmsn5Δaft1*cells
636 carrying an expression plasmid for wild-type Aft1-HA or the indicated mutants were cultured
637 in iron-free medium to mid-log phase growth. Cells were cultured for an additional 30 min in
638 the absence (- iron) or presence (+ iron) of 200 μM $FeSO_4$, and total RNA was extracted. The
639 mRNA levels of the indicated genes were then analyzed by Northern blotting. (B) Aft1p

binds to the *FET3* promoter only under iron-deprived conditions in *Δmsn5* cells. *Δaft1* or *Δmsn5Δaft1* cells carrying an expression plasmid for wild-type Aft1-TAP or the indicated mutants were cultured as in (A) and Aft1p binding to the *FET3* and *ACT1* promoters was detected by chromatin immunoprecipitation. (C) Aft1-TAP is functional. BY4741 (*AFT1*) cells, *Δaft1* cells, and *Δaft1* cells carrying an expression plasmid for Aft1-TAP, Aft1-HA, or Aft1-1^{up}-HA were cultured in iron-free medium to mid-log phase growth. Total RNA was isolated after the addition of iron at the indicated times, and mRNA levels of the indicated genes were analyzed by Northern blotting.

FIG 3. Leu99, Leu102, Cys291, and Cys293 are required for the Aft1p-Grx3/4p interaction. (A) The PJ69-4A strain was transformed with an expression plasmid for AD-fused Aft1p, mutant Aft1p, or the corresponding empty vector, as well as an expression plasmid for the BD-fused indicated proteins or the corresponding empty vector. Cells were grown at 30 °C for 4 days on SD medium lacking (-ADE -HIS) or containing (+ADE +HIS) adenine and histidine. Cells were spotted using 3-fold serial dilutions beginning at 600 cells per spot. (B) Lysates from the cells in (A) were analyzed by immunoblotting with anti-HA (for the detection of AD-fused proteins) or with anti-myc (for the detection of BD-fused proteins). *: non-specific bands. (C and D) *Δgrx3Δgrx4* cells carrying expression plasmids for Aft1-TAP or the indicated mutants, and Grx3-HA (C) or Grx4-HA (D) were cultured in iron-depleted medium to mid-log phase growth. Cells were cultured for an additional 15 min in the

660 presence of 200 μM $FeSO_4$. TAP-precipitates and lysates were probed with the indicated
661 antibodies.
662
663 **FIG 4.** Grx3/4p are required for the dissociation of Aft1p from DNA in response to iron. (A)
664 Grx3/4p are required for the iron-dependent dissociation of Aft1p from the *FET3* promoter.
665 BY4741 (WT), *Δgrx3*, *Δgrx4* or *Δgrx3Δgrx4* strains carrying expression plasmids for
666 Aft1-TAP were cultured in iron-free medium to mid-log phase growth. Cells were cultured
667 for an additional 30 min in the absence (-) or presence (+) of 200 μM $FeSO_4$, and Aft1p
668 binding to the *FET3* and *ACT1* promoters was detected by chromatin immunoprecipitation.
669 (B) Iron regulon expression is not suppressed even under iron-replete conditions in the
670 *Δgrx3Δgrx4* strain. The BY4741 (WT), *Δgrx3*, *Δgrx4*, *Δgrx3Δgrx4* strains, or *Δgrx3Δgrx4*
671 cells carrying expression plasmids for Grx3-TAP or Grx3-HA were cultured as in (A) and
672 expression of the indicated genes was analyzed by Northern blotting.
673
674 **FIG 5.** The Grx3/4p interaction with Aft1p is augmented in the presence of iron. (A and B)
675 Binding of Grx3/4p to Aft1p was enhanced in iron-replete conditions. (A) Cells expressing
676 both Aft1-TAP and Grx3-HA (+ Aft1-TAP), or Grx3-HA alone (- Aft1-TAP) from their
677 natural chromosomal loci were cultured in iron-free medium to mid-log phase growth (20 h).
678 Cells were cultured for an additional 15 min in the absence (- iron) or presence (+ iron) of
679 200 μM $FeSO_4$, and TAP-immunoprecipitates and cell lysates were probed with the indicated

680 antibodies. (B) Cells expressing both Aft1-TAP and Grx4-HA (+ Aft1-TAP), or Grx4-HA
681 alone (- Aft1-TAP) from their natural chromosomal loci were cultured as in (A) and the
682 interaction between Aft1-TAP and Grx4-HA was analyzed by immunoprecipitation. (C)
683 Induction of the expression of the iron regulon is incomplete after a 6 h treatment with 40 μM
684 BPS. BY4741 cells were cultured under the following conditions: SD medium containing 40
685 μM BPS for 6 h (BPS), SD medium containing 250 μM $FeSO_4$ for 6 h (Fe), SD medium
686 lacking iron for 20 h (Fe; -), or SD medium lacking iron for 20 h with an additional 15 min
687 cultivation in the presence of 200 μM $FeSO_4$ (Fe; +). Total RNA was extracted and
688 expression of the indicated genes was analyzed by Northern blotting. The values indicated
689 below each panel are relative mRNA levels as a percentage of the amount in the cells cultured
690 in SD medium lacking iron for 20 h. (D) Grx3/4p reside both in the nucleus and in the
691 cytoplasm. *Δgrx3Δgrx4* cells carrying expression plasmids for Grx3/4-HA were cultured in
692 iron-free medium to mid-log phase growth. Cells were cultured for an additional 30 min in
693 the absence (-Fe) or presence (+Fe) of 200 μM $FeSO_4$. After fixation, the subcellular
694 localization of Grx3/4-HA was examined by indirect immunofluorescence microscopy using
695 an anti-HA antibody. Differential interference contrast (DIC) images are provided for
696 comparison.
697
698 **FIG 6.** Grx3/4p require an iron-sulfur cluster to interact with Aft1p. (A and B) Grx3-TAP and
699 Grx3-HA interact in iron-replete conditions. (A) *Δgrx3Δgrx4* cells carrying expression

700 plasmids for Grx3-TAP and Grx3-HA were cultured in iron-free medium to mid-log phase
701 growth (20 h). Cells were cultured for an additional 15 min in the absence (-) or presence (+)
702 of 200 μM $FeSO_4$, and TAP-immunoprecipitates and cell lysates were probed with the
703 indicated antibodies. (B) Cells expressing both Grx3-TAP and Grx4-HA (+ Aft1-TAP), or
704 Grx4-HA alone (- Aft1-TAP) from their original chromosomal loci were cultured as in (A)
705 and interactions between Grx3-TAP and Grx4-HA were analyzed by immunoprecipitation.
706 (C) Lys203, Thr251 and Cys211 of Grx3p are important for iron binding by Grx3p *in vivo*.
707 *Δgrx3Δgrx4* cells carrying an expression plasmid for Grx3 (-), Grx3-TAP (WT) or the
708 indicated mutants were cultured in iron-free medium to mid-log phase growth. Cells were
709 radiolabeled with 370 KBq of ^{55}Fe for 2 h. TAP-tagged proteins were precipitated, and bound
710 ^{55}Fe was quantified by scintillation counting. Data represent mean values from three
711 independent experiments. Error bars indicate ± SD. The amount of precipitated proteins was
712 assessed by immunoblotting using an anti-TAP antibody. (D) Lys203, Thr251 and Cys211 of
713 Grx3p are important for the Aft1p-Grx3p interaction. *Δgrx3Δgrx4* cells carrying expression
714 plasmids for Aft1-TAP and Grx3-HA or the indicated mutants were cultured in iron-depleted
715 medium to mid-log phase growth. Cells were cultured for an additional 15 min in the
716 presence of 200 μM $FeSO_4$, and TAP-immunoprecipitates and cell lysates were probed with
717 the indicated antibodies. (E) Lys203, Thr251 and Cys211 of Grx3p are important for the
718 iron-dependent dissociation of Aft1p from the FET3 promoter. *Δgrx3Δgrx4* cells carrying
719 expression plasmids for Aft1-TAP and Grx3p-HA or the indicated mutants were cultured in

720 iron-free medium to mid-log phase growth. Cells were cultured for an additional 30 min in
721 the absence (-) or presence (+) of 200 μM $FeSO_4$, and Aft1p binding to the *FET3* and *ACT1*
722 promoters was probed by chromatin immunoprecipitation. (F) Lys203, Thr251 and Cys211
723 of Grx3p are important for the regulation of *FTR1* expression by iron. *Δgrx3Δgrx4* cells
724 carrying an expression plasmid for Grx3p-HA or the indicated mutants were cultured in
725 iron-depleted medium to mid-log phase growth. Cells were cultured for an additional 30 min
726 in the absence (-) or presence (+) of 200 μM $FeSO_4$, and the expression of *FTR1*, *FET3*,
727 *FRE1*, *SIT1* and *ACT1* was analyzed by Northern blotting.
728
729 **FIG7.** Atm1p is required for Aft1p inactivation in response to iron. (A) The *ATM1* mRNA is
730 depleted in *GAL-ATM1* strains cultured in glucose containing medium. The wild-type or
731 Atm1p-depleted *GAL-ATM1* strains were cultured in iron-free medium containing galactose
732 (Gal) or glucose (Glc) as a sole carbon source to mid-log phase growth. Cells were cultured
733 for an additional 30 min in the absence (-) or presence (+) of 200 μM $FeSO_4$, and the *ATM1*
734 and *ACT1* mRNA levels were analyzed by Northern blotting. (B) Iron regulon expression
735 remains high in iron-replete conditions in Atm1p-depleted cells. The wild-type or
736 Atm1p-depleted *GAL-ATM1* strains were cultured in iron-free medium to mid-log phase
737 growth. Cells were cultured for an additional 30 min in the absence (-) or presence (+) of 200
738 μM $FeSO_4$, and the expression of *FTR1*, *FET3*, *FRE1*, *SIT1* and *ACT1* was analyzed by
739 Northern blotting. (C) Atm1p is required for the iron-dependent dissociation of Aft1p from

740 the *FET3* promoter. The wild-type or Atm1p-depleted *GAL-ATM1* strains expressing
741 Aft1-TAP were cultured in iron-free medium to mid-log phase growth. Cells were cultured
742 for an additional 30 min in the absence (-) or presence (+) of 200 μM $FeSO_4$, and Aft1p
743 binding to the *FET3* and *ACT1* promoters was analyzed by chromatin immunoprecipitation.
744 (D) Atm1p is required for the Aft1p-Grx3p interaction. The wild-type or Atm1p-depleted
745 *GAL-ATM1* strains expressing Aft1-TAP and Grx3-HA were cultured in iron-free medium to
746 mid-log phase growth. Cells were cultured for an additional 15 min in the absence (-) or
747 presence (+) of 200 μM $FeSO_4$, and the interaction between Aft1-TAP and Grx3-HA was
748 analyzed by co-immunoprecipitation. (E) Atm1p is important for iron binding by Grx3p *in*
749 *vivo*. The wild-type or Atm1p-depleted *GAL-ATM1* strains expressing non-tagged Grx3p (-)
750 or Grx3-TAP (+) were cultured in iron-free medium to mid-log phase growth. Cells were
751 radiolabeled with 370 kBq of ^{55}Fe for 30 min. TAP-tagged proteins were immunoprecipitated,
752 and bound ^{55}Fe was quantified by scintillation counting. Data represent mean values from
753 three independent experiments. Error bars indicate ± SD. The amount of precipitated protein
754 was assessed by immunoblotting using an anti-TAP antibody.
755
756 **FIG 8.** Proposed model for iron sensing by Aft1p. During iron starvation, iron-sulfur
757 assembly in the mitochondria and dimeric Grx3/4p with bound iron-sulfur clusters are
758 minimal. Under these conditions, Grx3/4p binding to Aft1p is attenuated, and Aft1p binds to
759 target promoters to increase the expression of the iron regulon. In response to iron

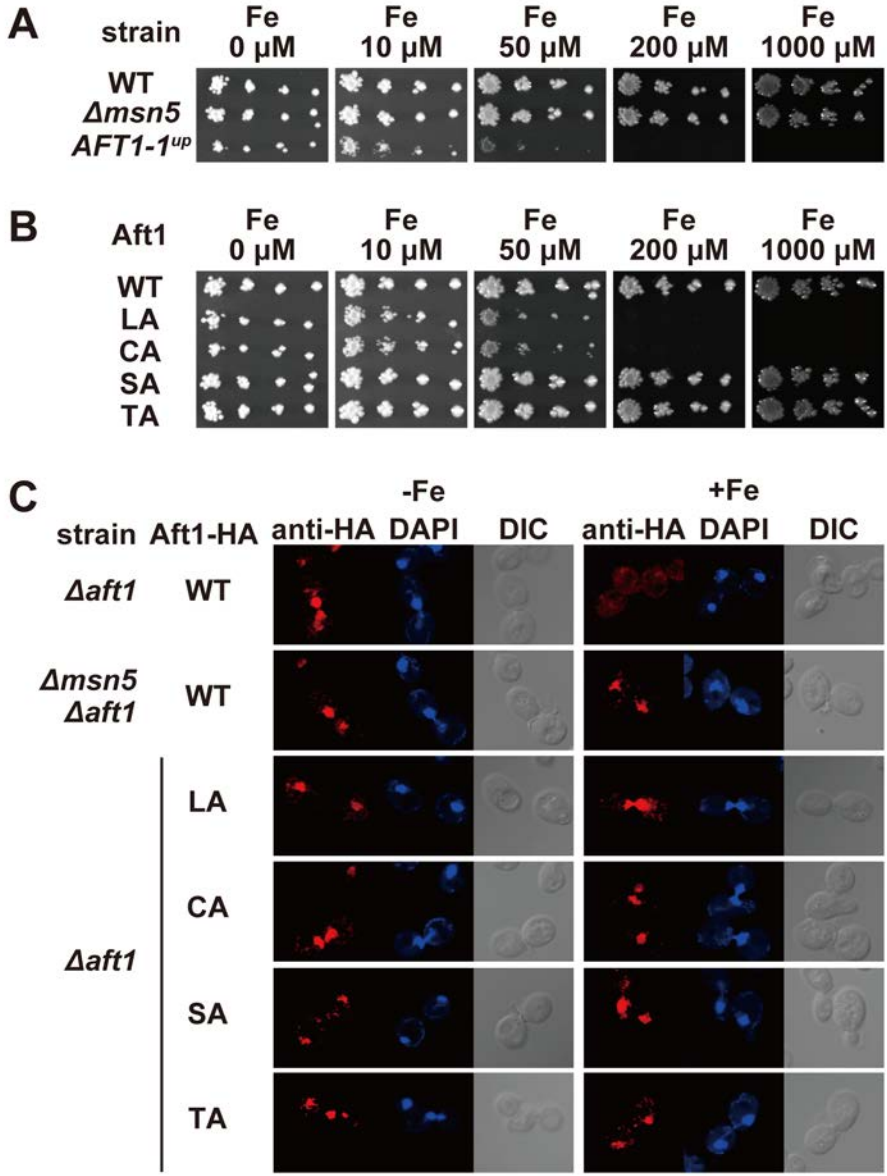
availability (i), iron-sulfur cluster assembly in the mitochondria increases (ii), and the iron-sulfur clusters, or signals that invoke iron-sulfur cluster formation, are delivered to the monothiol glutaredoxins Grx3/4p, which resides both in the nucleus and cytoplasm, via the mitochondrial ABC exporter Atm1p (iii). Grx3/4p with bound iron-sulfur clusters bind to Aft1p (iv), which induces dissociation of Aft1p from its target promoters (v), leaving Aft1p available for nuclear export by Msn5p (vi). The expression of the iron regulon is thereby down-regulated.

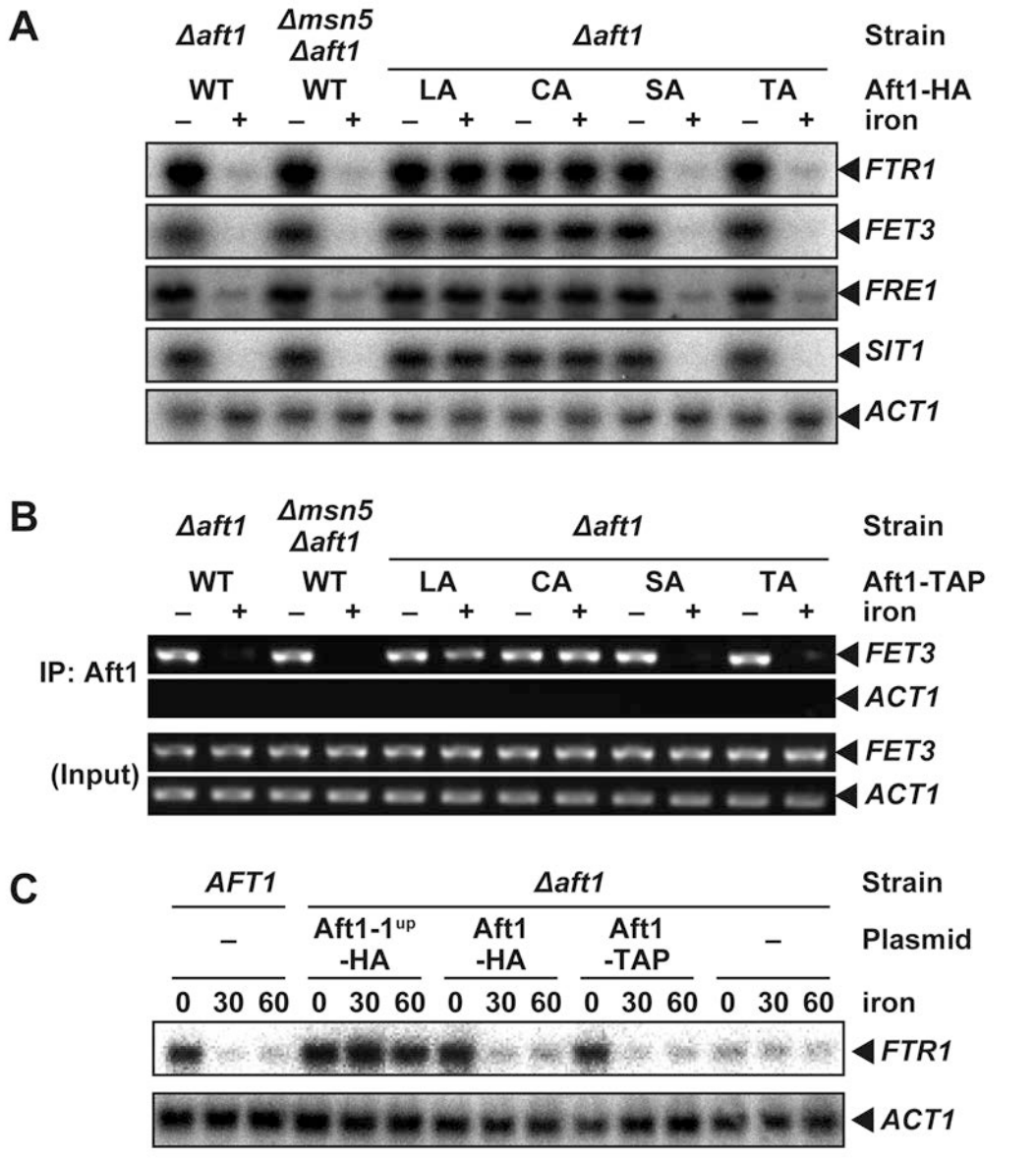
767

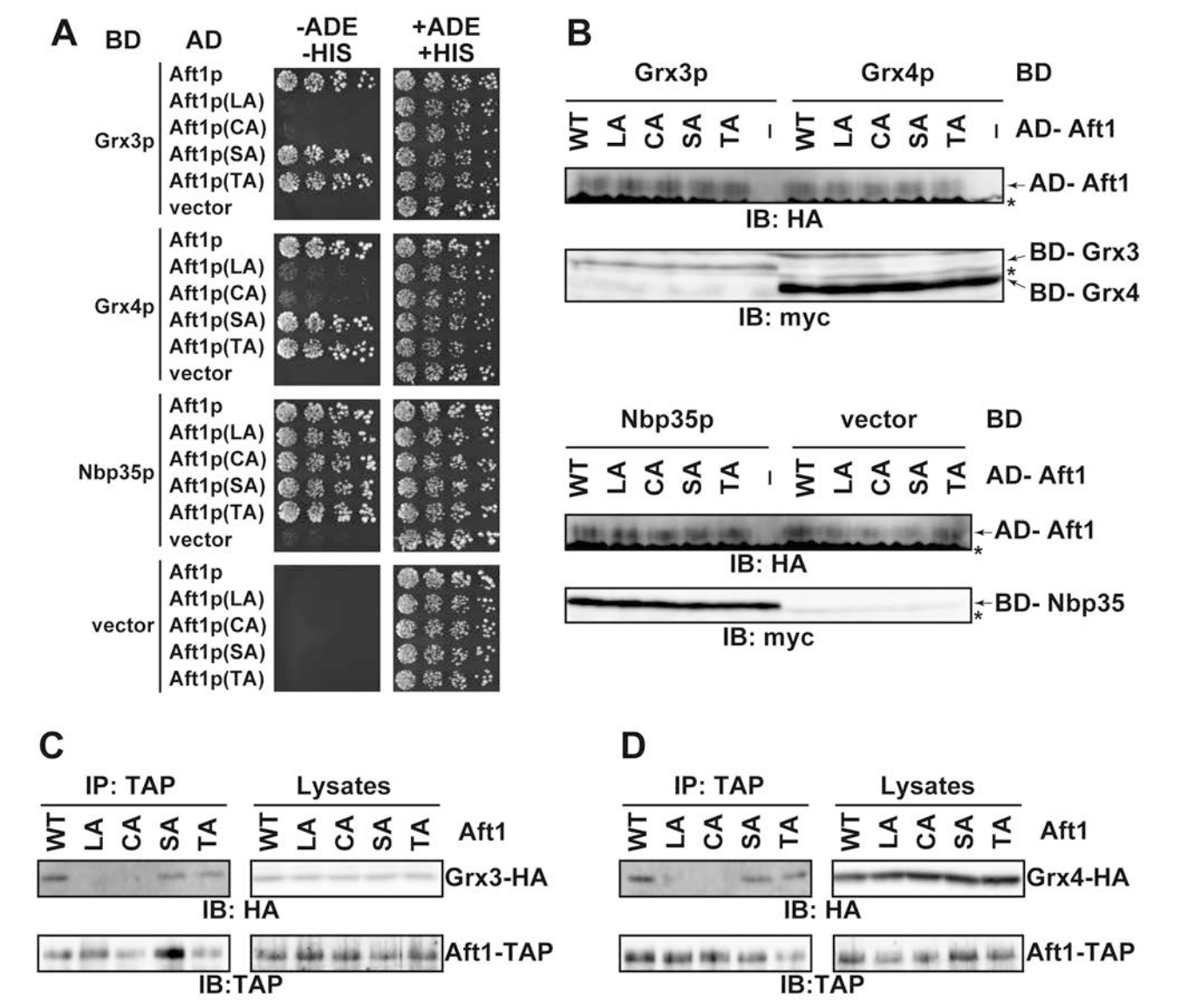
768 **TABLE 1.** Yeast Strain Genotypes.

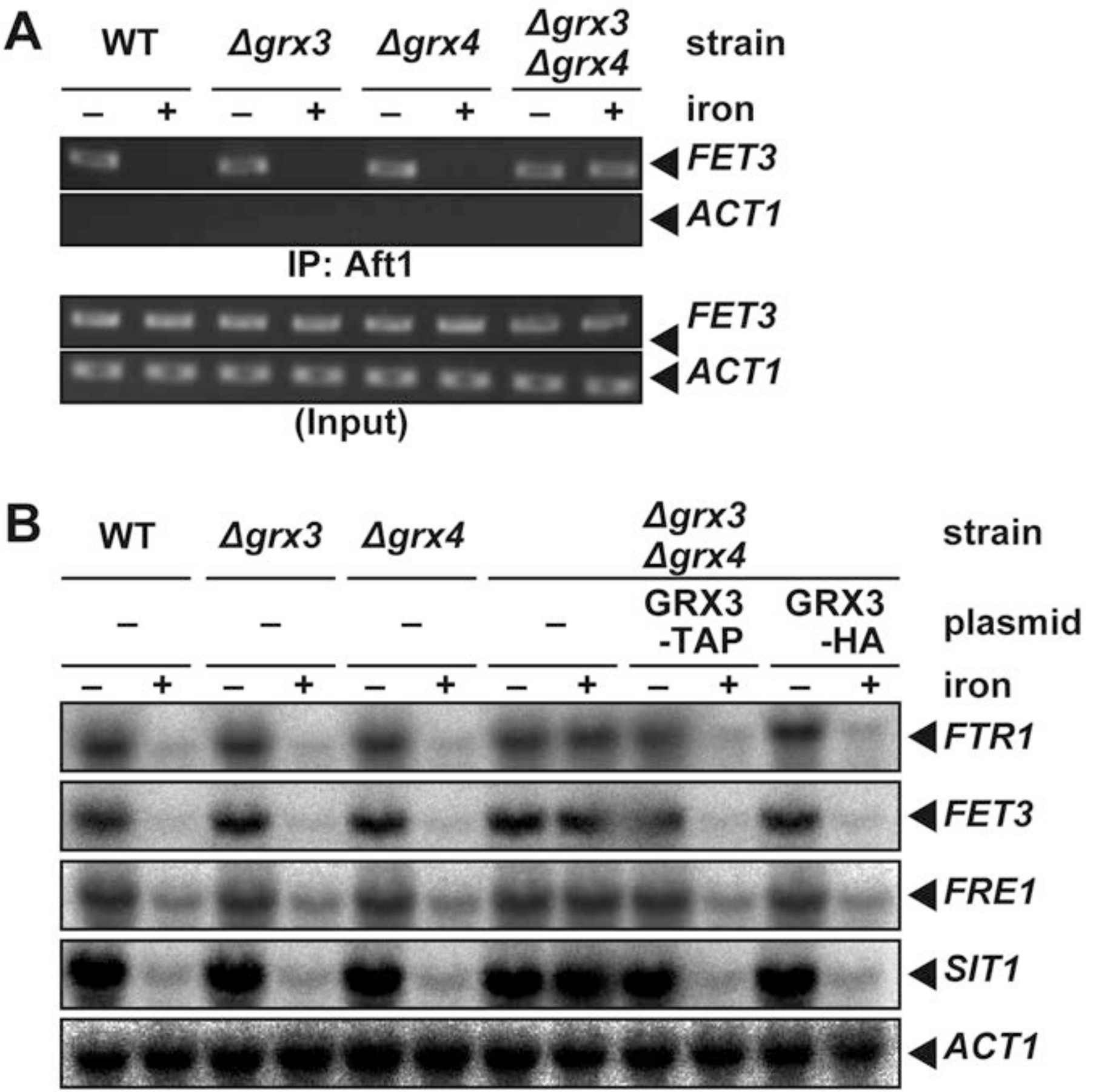
Strain	Genotype	Source
PJ69-4A	*MATa ura3-52 leu2-3,112 trp1-901 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*	(12)
BY4741	*MATa ura3Δ0 leu2Δ his3Δ1 met15Δ0*	(5)
BY4742	*MATα ura3Δ0 leu2Δ his3Δ1 lys2Δ0*	(5)
Y23 (*Δaft1*)	BY4741 background, *aft1Δ::KanMX*	(44)
Y24 (*Δmsn5*)	BY4741 background, *msn5Δ::KanMX*	This study
Y25	BY4742 background, *msn5Δ::KanMX*	This study
Y26 (*Δaft1Δmsn5*)	BY4741 background, *aft1Δ::KanMX msn5Δ::KanMX*	This study
Y27 (*Δgrx3*)	BY4741 background, *grx3Δ::KanMX*	This study
Y28 (*Δgrx4*)	BY4741 background, *grx4Δ::KanMX*	This study
Y29	BY4742 background, *grx4Δ::KanMX*	This study
Y30 (*Δgrx3Δgrx4*)	BY4741 background, *grx3Δ::KanMX grx4Δ::KanMX*	This study
Y31	BY4741 background, GRX3-HA	This study
Y32	BY4741 background, AFT1-TAP::HIS3MX6, GRX3-HA	This study
Y33	BY4741 background, GRX4-HA	This study
Y34	BY4741 background, GRX3-TAP::HIS3MX6, GRX4-HA	This study
Y35 (*GAL-ATM1*)	BY4741 background, *pATM1::pGAL1-10-KanMX*	This study

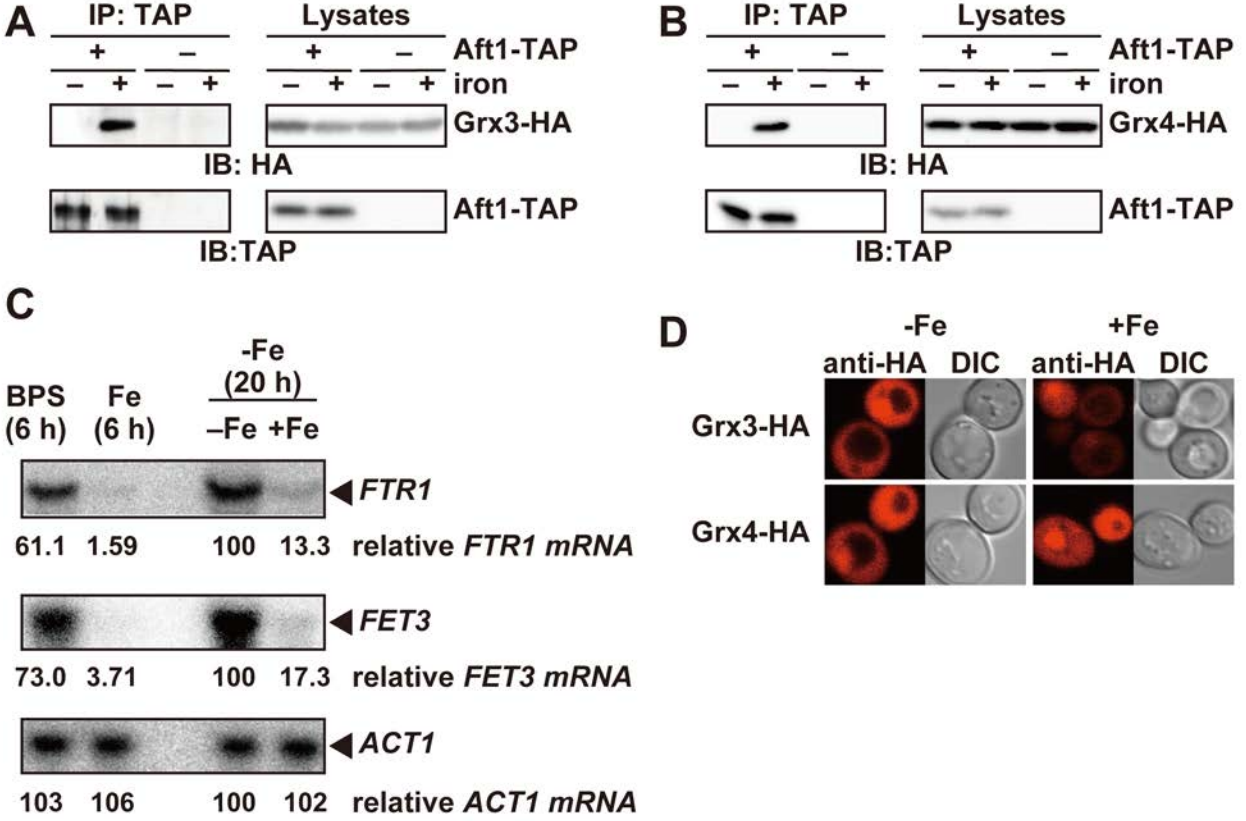
769

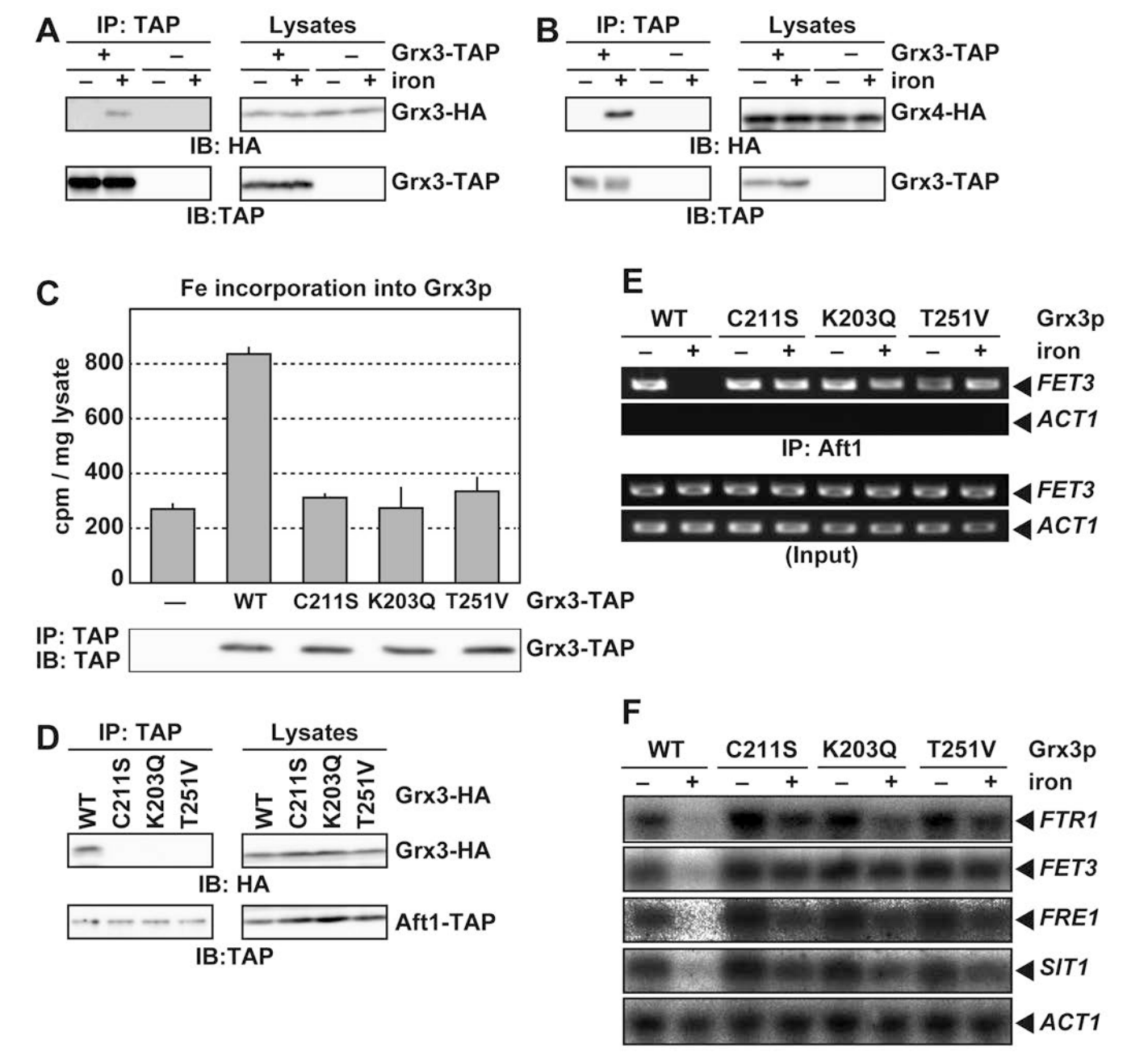

A
strain
Fe 0 μM
Fe 10 μM
Fe 50 μM
Fe 200 μM
Fe 1000 μM
WT
Δmsn5
AFT1-1up
B
Aft1
Fe 0 μM
Fe 10 μM
Fe 50 μM
Fe 200 μM
Fe 1000 μM
WT
LA
CA
SA
TA
C
-Fe
+Fe
strain
Aft1-HA
anti-HA
DAPI
DIC
anti-HA
DAPI
DIC
Δaft1
WT
Δmsn5 Δaft1
WT
Δaft1
LA
CA
SA
TA

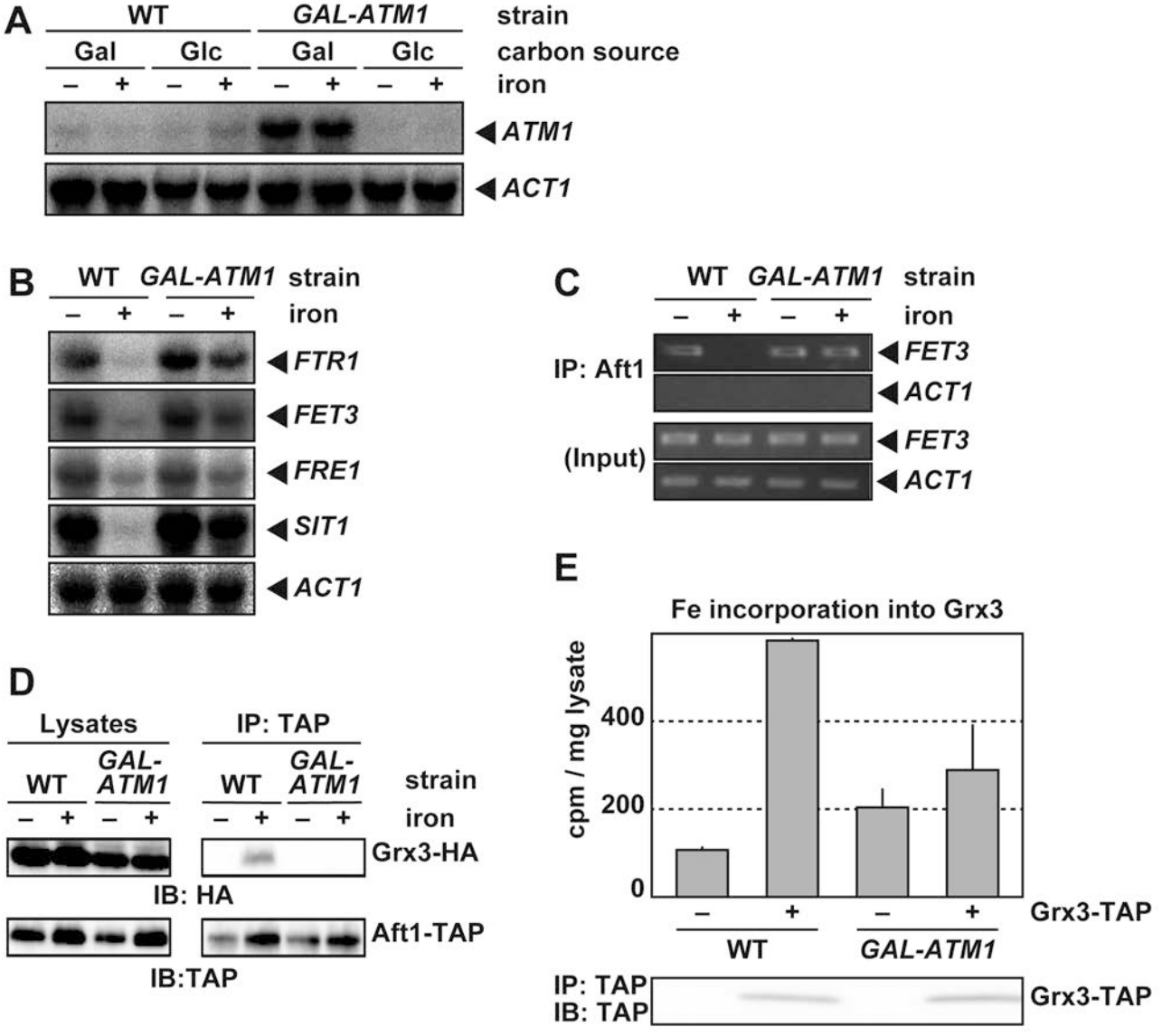

A
Δaft1
Δmsn5 Δaft1
Δaft1
Strain
WT
WT
LA
CA
SA
TA
Aft1-HA
– + – + – + – + – + – +
iron
FTR1
FET3
FRE1
SIT1
ACT1
B
Δaft1
Δmsn5 Δaft1
Δaft1
Strain
WT
WT
LA
CA
SA
TA
Aft1-TAP
– + – + – + – + – + – +
iron
IP: Aft1
FET3
ACT1
(Input)
FET3
ACT1
C
AFT1
Δaft1
Strain
–
Aft1-1up -HA
Aft1 -HA
Aft1 -TAP
–
Plasmid
0 30 60 0 30 60 0 30 60 0 30 60 0 30 60
iron
FTR1
ACT1

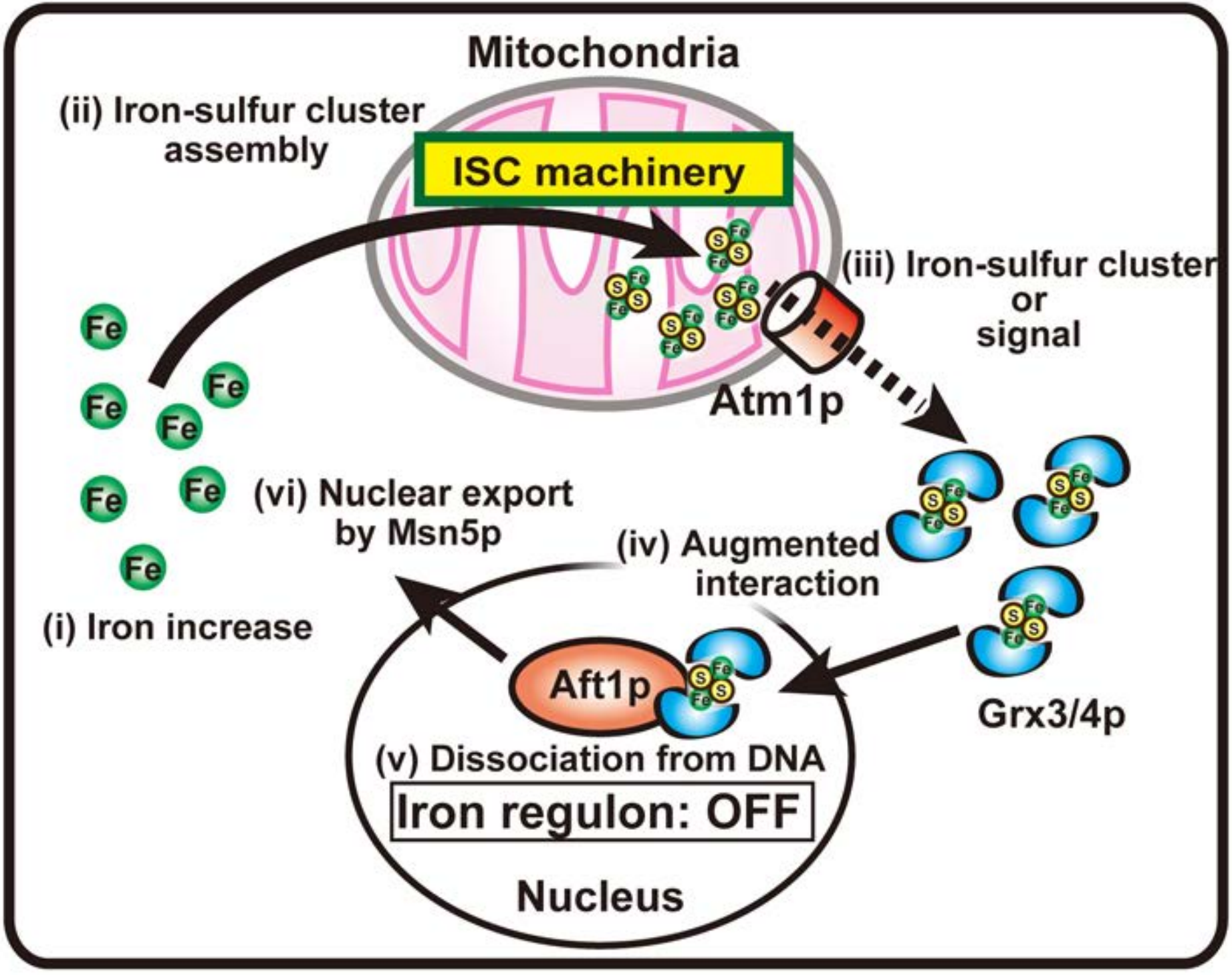

A
BD
AD
-ADE
-HIS
+ADE
+HIS
Grx3p
Grx4p
Nbp35p
vector
Aft1p
Aft1p(LA)
Aft1p(CA)
Aft1p(SA)
Aft1p(TA)
vector
B
Grx3p
Grx4p
BD
WT
LA
CA
SA
TA
–
AD- Aft1
IB: HA
BD- Grx3
BD- Grx4
IB: myc
Nbp35p
vector
BD- Nbp35
C
IP: TAP
Lysates
Aft1
Grx3-HA
Aft1-TAP
IB:TAP
D
Grx4-HA


A
WT
Δgrx3
Δgrx4
Δgrx3 Δgrx4
strain
– + – + – + – +
iron
FET3
ACT1
IP: Aft1
FET3
ACT1
(Input)
B
WT
Δgrx3
Δgrx4
Δgrx3 Δgrx4
strain
–
–
–
–
GRX3 -TAP
GRX3 -HA
plasmid
– + – + – + – + – + – +
iron
FTR1
FET3
FRE1
SIT1
ACT1


A
IP: TAP
Lysates
Aft1-TAP
iron
Grx3-HA
IB: HA
Aft1-TAP
IB:TAP
B
IP: TAP
Lysates
Aft1-TAP
iron
Grx4-HA
IB: HA
Aft1-TAP
IB:TAP
C
BPS (6 h)
Fe (6 h)
-Fe (20 h)
–Fe +Fe
FTR1
61.1 1.59 100 13.3 relative FTR1 mRNA
FET3
73.0 3.71 100 17.3 relative FET3 mRNA
ACT1
103 106 100 102 relative ACT1 mRNA
D
-Fe
+Fe
anti-HA DIC
anti-HA DIC
Grx3-HA
Grx4-HA


A
IP: TAP
Lysates
Grx3-TAP
iron
Grx3-HA
IB: HA
Grx3-TAP
IB:TAP
B
IP: TAP
Lysates
Grx3-TAP
iron
Grx4-HA
IB: HA
Grx3-TAP
IB:TAP
C
Fe incorporation into Grx3p
cpm / mg lysate
800
600
400
200
0
—
WT
C211S
K203Q
T251V
Grx3-TAP
IP: TAP
IB: TAP
Grx3-TAP
D
IP: TAP
Lysates
WT
C211S
K203Q
T251V
Grx3-HA
Grx3-HA
IB: HA
Aft1-TAP
IB:TAP
E
WT
C211S
K203Q
T251V
Grx3p
iron
FET3
ACT1
IP: Aft1
FET3
ACT1
(Input)
F
WT
C211S
K203Q
T251V
Grx3p
iron
FTR1
FET3
FRE1
SIT1
ACT1


A
WT
GAL-ATM1
strain
Gal
Glc
Gal
Glc
carbon source
– + – + – + – +
iron
ATM1
ACT1
B
WT
GAL-ATM1
strain
– + – +
iron
FTR1
FET3
FRE1
SIT1
ACT1
C
WT
GAL-ATM1
strain
– + – +
iron
IP: Aft1
FET3
ACT1
(Input)
FET3
ACT1
D
Lysates
IP: TAP
WT
GAL-ATM1
WT
GAL-ATM1
strain
– + – + – + – +
iron
Grx3-HA
IB: HA
Aft1-TAP
IB:TAP
E
Fe incorporation into Grx3
cpm / mg lysate
400
200
0
– + – +
Grx3-TAP
WT
GAL-ATM1
IP: TAP
IB: TAP
Grx3-TAP


Mitochondria
(ii) Iron-sulfur cluster assembly
ISC machinery
(iii) Iron-sulfur cluster or signal
Atm1p
Fe
(vi) Nuclear export by Msn5p
(iv) Augmented interaction
(i) Iron increase
Aft1p
Grx3/4p
(v) Dissociation from DNA
Iron regulon: OFF
Nucleus