A novel function of Aft1 in regulating ferrioxamine B uptake: Aft1 modulates Arn3 ubiquitination in *Saccharomyces cerevisiae*

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Aft1 is a transcriptional activator in *Saccharomyces cerevisiae* that responds to iron availability and regulates the expression of genes in the iron regulon, such as *FET3, FTR1* and the *ARN* family. Using a two-hybrid screen, we found that Aft1 physically interacts with the FOB (ferrioxamine B) transporter Arn3. This interaction modulates the ability of Arn3 to take up FOB. The interaction between Arn3 and Aft1 was confirmed by β-galactosidase, co-immunoprecipitation and SPR (surface plasmon resonance) assays. Truncated Aft1 had a stronger interaction with Arn3 and caused a higher FOB-uptake activity than full-length Aft1. Interestingly, only full-length Aft1 induced the correct localization of Arn3 in response to FOB. Furthermore, we found Aft1 affected Arn3 ubiquitination. These results suggest that Aft1 interacts with Arn3 and may regulate the ubiquitination of Arn3 in the cytosolic compartment.

Key words: Aft1, Arn3, iron, *Saccharomyces cerevisiae*, siderophore, ubiquitination.

INTRODUCTION

Virtually all organisms require iron as it is an essential in diverse biological processes, including respiration and oxidation-reduction pathways [1]. At physiological pH, most iron is present in an insoluble ferric form (Fe³⁺) and is not accessible for cellular use [2]. Moreover, excess iron in the cell promotes the formation of ROS (reactive oxygen species) from the oxygen produced by the respiratory chain. These ROS can damage proteins, lipids and DNA [3]. For this reason, most organisms have developed diverse regulatory mechanisms to absorb and metabolize iron [4].

The budding yeast *Saccharomyces cerevisiae* has two different pathways to take up iron. One of these pathways is the reductive iron uptake pathway. Before entry into the cell, insoluble ferric iron is reduced to soluble ferrous iron (Fe²⁺) by the metallo-reductase membrane enzymes Fre1 and Fre2 [5,6]. Reduced iron is taken up by the Fet3–Ftr1 complex, which is a high-affinity iron transporter [7]. Membrane-bound Fet3, a ferroxidase, oxidizes the ferrous iron to the ferric form by consuming oxygen, and then the Ftr1 membrane iron permease transports iron into the cells [8–10].

The second pathway of iron uptake relies on a siderophore–iron complex. Siderophores are low-molecular-mass natural iron chelators that are synthesized and secreted by both bacteria and fungi, but not by the budding and fission yeasts. Siderophores bind ferric iron specifically and with high affinity [11]. Siderophore-mediated iron uptake in the yeast *S. cerevisiae* occurs by two different pathways, the Fet3/Ftr1-dependent pathway [12–14] and the Arn-dependent pathway [14]. In the former pathway, siderophore–bound ferric iron is reduced and released from the siderophore by the cell-surface ferric reductases Fre1, Fre2, Fre3, and Fre4. Subsequently, the reduced iron is taken up by the Fet3–Ftr1 complex, as in the case of the reductive iron uptake pathway [5,6,8,10,15]. The latter mechanism involves the uptake of the siderophore–iron complex by proteins of the Arn family, which consists of the transporters Arn1, Arn2 (also known as Taf1), Arn3 (also known as Sit1) and Enb1 (also known as Arn4). Each protein transports different classes of siderophores. Arn1 exhibits specificity for FCs (ferrichromes), Arn2 exhibits specificity for the fusarinines and Arn3 exhibits specificity for both ferrioxamines, including FOB (ferrioxamine B) and FC [14,16–20]. Each of these transporters has 14 transmembrane domains and can transport intact siderophore–iron substrates with high specificity [21].

A pathway for FC uptake by Arn1 has been identified. Low concentrations of substrate induce Arn1 to localize to the plasma membrane, yet little uptake of the substrate occurs. At high concentrations of substrate (1 μM and higher), Arn1 cycles between the plasma membrane and the endosome [22]. The intracellular trafficking of the protein is controlled by the binding of the substrate to the high-affinity binding site of the transporter [23]. Recently, it was reported that Sit1 also displayed identical membrane sorting and endocytic recycling [24]. Arn1 and Sit1 recycling, and their ubiquitination, is regulated by Rsp5. Ubiquitination in turn regulates the uptake of cellular siderophores [25,26].

Aft1, which consists of 690 amino acids, is a transcriptional activator of genes required for iron transport and metabolism and maintains the iron homoostasis of cells [27,28]. Under conditions of iron depletion, Aft1 is resident in the nucleus and induces the expression of several genes involved in iron transport and utilization. Aft1 senses the intracellular iron levels and translocates to the cytoplasm when cytosolic iron is abundant [29]. Pse1 and Msn5 mediate the import and export of Aft1 respectively [29,30]. The mitochondrial ISC (iron/sulfur cluster) assembly is also required for the iron-dependent nuclear export of Aft1 [31]. In addition to its function as a transcriptional factor, systematic synthetic lethal analysis in yeast suggests that Aft1 also plays a role in chromosomal segregation [32]. A recent study shows that Aft1, and the Aft1-regulated genes involved

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Abbreviations used: ER, endoplasmic reticulum; FC, ferrichrome; FOB, ferrioxamine B; GAL4AD, Gal4 activation domain; GAL4BD, Gal4-binding domain; GST, glutathione transferase; HA, haemagglutinin; MVB, multivesicular body; ROS, reactive oxygen species; RU, response units; SD, synthetic defined; SPR, surface plasmon resonance, YNB, yeast nitrogen base.
in metal metabolism, are induced during the acid-shock response and during acid adaptation with lactic acid. Furthermore, Aft1 localizes to the nucleus under acidic conditions, in a similar manner to that which occurs under conditions of iron depletion [33]. These reports indicate that Aft1 may have multiple functions beyond iron homeostasis.

In the present study, we identified proteins that interact with Arn3 using a yeast two-hybrid analysis. We found that Aft1, a transcriptional activator of the iron regulon, physically interacts with Arn3 and affects intracellular trafficking and the subsequent ubiquitination of Arn3. These results imply a novel role for Aft1 in regulating the proper localization of Arn3 to the cytoplasm in order to prevent cell cytotoxicity caused by excess iron.

EXPERIMENTAL PROCEDURES

Yeast strains and culture media

The yeast strains used in this study were \( \textit{S. cerevisiae} \) AH1109 (MATa, trp1–901, leu2–3, 112, ura3–52, his3–200, GAL4AD, GAL80D, LYS2::GALUAS-GALITATA-his3, GALUAS-GALITATA-ade2, URA3::MEL1UAS-MELITATA-lacZ), wild-type BY4741 (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0), and Δarn1,2,3 deletion mutant strain. Yeast strains were grown in YPD medium [1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose] or SD (synthetic defined) medium [6.7 g/l YNB (yeast nitrogen base)] supplemented with auxotrophic requirements, unless indicated. Defined iron medium was prepared using a modified SD-minimal medium containing 0.67 % YNB without iron and copper, 2 % (w/v) dextrose, 25 mM Mes buffer, pH 6.1, and amino acids with auxotrophic requirements. Iron-limited media were prepared by adding 100 μM BPS (bathophenanthroline disulfonate) and ferrous ammonium sulfate at various concentrations. All cultures were incubated at 30 °C unless otherwise stated.

Two-hybrid analysis

Genomic DNA was extracted from cells grown to mid-exponential phase and partially digested with Sau3AI to construct a two-hybrid genomic DNA library. Fragments of 1–2 kb were extracted from agarose gels and ligated into BamHI-digested pGADT7 (Clontech), a two-hybrid vector encoding the GAL4AD (GAL4 activation domain). Ligation mixtures were transformed into \( \textit{Escherichia coli} \) strain DH10B and 2 × 10⁵ independent colonies were collected from LB (Luria–Bertini) agar plates containing ampicillin. Colony PCR showed that 70% of the colonies contained insert DNA. To construct bait for the two-hybrid screen, the hydrophilic N-terminal fragment of the \( \textit{arn3} \) gene was amplified by PCR using the forward primer 5′-TTCCATATGGACCCCTGTTATGG-3′, the reverse primer 5′-ATATATCGTGACCCGTTAGATGC-3′ and yeast genomic DNA as a template. The amplified 228 bp fragment was purified and subcloned into a pGEMT-easy vector (Promega) and the sequence was confirmed by DNA sequencing. The insert was subcloned into a pGEMT-easy vector (Promega) and the resulting plasmid was called pEU-Aft1/1–690, pEU-Aft1/1–177, pEU-Aft1/178–412, pEU-Aft1/413–690 and pEU-Aft1/172–564.

Plasmid construction

Plasmids pGA-Aft1/413–572 and pGA-Aft1/573–690 were constructed by insertion of the DNA fragments obtained by EcoRI digestion of pGBD-Aft1/413–572 and pGBD-Aft1/573–690 (a gift from Y. Yamaguchi-Iwai, Kyoto University, Kyoto, Japan). To investigate protein–protein interactions using SPR (surface plasmon resonance) analysis, the sequences encoding fragments of Arn3 and Aft1 used in the co-immunoprecipitation assays were subcloned into pET23a (Novagen) and pGEX-5X-1 (GE Healthcare) respectively. The resulting subclones were transformed into \( \textit{E. coli} \) BL21 and DH5α cells respectively. Expression was induced by the addition of IPTG (isopropyl β-D-thiogalactoside) and the proteins were purified following the manufacturer’s protocol for each system. To verify the functional interaction between Arn3 and Aft1, β-Galactosidase assay and in vitro co-immunoprecipitation assay were performed as described in [34] using multiscreen filtration plates (Millipore) and 2 μM ⁵⁵Fe (III) (PerkinElmer) and purified. Protein–protein interactions were monitored using a SPR spectroscope.
Sucrose gradient fractionation

Cells expressing Arn3–GFP either with full-length Aft1 or with the fragments of Aft1 were grown in low-iron YNB medium (1 μM FOB) supplemented with 2% (w/v) raffinose and 0.2% galactose at 30°C for 6 h. Cells were harvested by centrifugation and washed once with PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) on to the chip surface at a flow rate of 5 μl/min at 25°C. The chip was regenerated after each experiment using a pulse injection of 45 μl of 0.2 M glycine/HCl buffer, pH 2.0.

Immunoprecipitation

Cells precultured in SD medium (lacking leucine and uracil) supplemented with 2% glucose were collected, washed with distilled water, and cultured further in the SD (without leucine and uracil) medium supplemented with 2% (w/v) raffinose and 0.02% galactose until the cell density reached an attenuation of 600 nm (D600) of 0.6. For galactose induction, cells were harvested, resuspended in SD (lacking leucine and uracil) medium supplemented with 2% (w/v) raffinose and 0.02% galactose, and incubated at 30°C for 3 h. To detect ubiquitinated Arn3–GFP, Arn3–GFP was immunoprecipitated under denaturing conditions as described previously in [35]. Briefly, the collected cells were washed with cold TNE buffer (100 mM Tris/HCl, pH 7.5 containing 150 mM NaCl and 5 mM EDTA), were resuspended in TNE supplemented with 1 mM protease inhibitors (aprotinin, pepstatin and PMSF) and 25 mM NEM (N-ethylmaleimide; Sigma–Aldrich), and disrupted with glass beads. Proteins in the cell lysate were precipitated with 10% (v/v) trichloroacetic acid. The protein pellets were resuspended in Laemmli sample buffer without 2-mercaptoethanol and incubated at 37°C for 15 min. After adding 10 volumes of TNE buffer [TNE buffer with 1% (v/v) Triton X-100], the mixture was centrifuged at 13,000 g for 30 min. The supernatant was transferred to a new tube and incubated with anti-GFP antibody (Santa Cruz Biotechnology) overnight with gentle agitation. Beads were collected by centrifugation at 500 g for 5 min and then washed four times with TNET. The immune complex was eluted by incubation at 37°C for 15 min after adding SDS sample buffer. Samples were analysed using SDS/PAGE (8% gels) and immunoblotted with anti-ubiquitin antibody (Santa Cruz Biotechnology).

Site-directed mutagenesis

Three lysine residues located in the N-terminal cytosolic region of Arn3 were replaced by arginine residues using the Quick Change™ site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The template was pEL-Arn3-GFP. The mutagenesis oligonucleotide primers were synthesized by Bioline (Korea) and they had the following sequences (mismatched sites are underlined). The primers used were: for Lys25 substution, sense 5′-GGC TCC TAC TTC CCG AAG GTC-3′ and antisense 5′-GAC TTG GCC TCC AAC CAG-3′; and antisense 5′-CGT TGG CTT GAC ATC GAC CCG GCC TCC TTC CTT TTC-3′; and for Lys58 substution, sense 5′-GGA GAA GTA GGA GCC CCG ATG TGG CCG-3′ and antisense 5′-AGA TGG TAG GGT CGT TCG GGC GAC ATC GAC CTT GGC TCC-3′. The mutations were verified by DNA sequencing.

Microscopy

Cells with pArn3–GFP and pAft derivatives were precultured in SD (lacking leucine and uracil) medium supplemented with 2% (w/v) glucose and collected. The cells were then washed with distilled water and cultured further in the SD (without leucine and uracil) medium supplemented with 2% (w/v) raffinose and 0.02% galactose and incubated at 30°C for 3 h with or without FOB. The cells were examined using a Zeiss fluorescence microscope. DAPI (4',6-diamidino-2-phenylindole) nuclear staining was used as a control.

RESULTS

Physical interaction of Arn3 and Aft1 in vivo and in vitro

The siderophore transporter Arn3 is a membrane protein predicted to have 14 transmembrane domains [36]. To identify the proteins interacting with Arn3, yeast two-hybrid screening was performed using the cytoplasmic N-terminal region of Arn3 (amino acid residues 1–177) and the Aft1 fragments encoding amino acid residues 172–564 (Aft1) and the Aft1 fragments encoding amino acid residues 1–177 and 178–412 were grown with the bait clone on SD (without adenosine, leucine, histidine and tryptophan) plates. To confirm the two-hybrid result and determine the region important for the interaction between Aft1 and Arn3, deletion mutants of Aft1 were constructed in pGBK7T7 (Figure 1A) and co-transformed with the bait into S. cerevisiae AH109. As shown in Figure 1(B), the positive prey clone identified in the two-hybrid screen (amino acid residues 172–564 of Aft1) and the Aft1 fragments encoding amino acid residues 1–177 and 178–412 were grown with the bait clone on SD (without adenosine, leucine, histidine and tryptophan) plates (Figure 1B).

To confirm the results obtained from this plate assay, the β-galactosidase activity of each colony was measured following growth on to mid-exponential phase on YPD medium supplemented with the indicated concentrations of iron (0, 10 or 500 μM FOB). β-galactosidase activities in crude cell lysate was assessed. Cells containing the plasmid combination that did not support growth in the two-hybrid assay (Figure 1B) showed similar or lower β-galactosidase activity than cells containing empty vectors. Cells with the plasmids that did support growth showed higher β-galactosidase activities, independent of the siderophore concentration in the medium (Figure 1C). These results imply that the three fragments of Aft1 containing amino acid residues 1–177, 172–564 and 178–412 interact with the N-terminal cytoplasmic region of Arn3 in vivo.

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Figure 1 Confirmation of the interaction between Aft1 and Arn3 in a yeast two-hybrid system

(A) Schematic presentation of GAL4BD–ARN3 and Gal4 activation domain GAL4AD–AFT1 fusion proteins, in each vector is represented as a black or hatched box respectively. The numbers in the box denote the corresponding amino acid region of each protein. (B) Various deletion fragments of Aft1 were investigated for their interaction with the N-terminal cytoplasmic region of Arn3. S. cerevisiae AH109 containing the indicated plasmid combinations were spotted in a 10-fold serial dilution series of $2 \times 10^4$ cells, $2 \times 10^3$ cells, $2 \times 10^2$ cells and $2 \times 10$ cells. These cells were spotted on SD plates lacking leucine and tryptophan, and on SD plates lacking adenine, leucine, histidine and tryptophan. The plates were incubated at 30° C for 4 days. (C) The β-galactosidase activities resulting from the interaction of GAL4BD–ARN3 and the indicated GAL4AD–AFT1 fusion protein in S. cerevisiae AH109 were measured. Cells were grown on YPD supplemented with 0, 10 and 500 μM iron as denoted by white, grey and black bars respectively. β-Galactosidase activity was measured as described in the Experimental section. (D) An immunoprecipitation assay performed to confirm the interaction between Aft1 and Arn3 in vitro. The purified, c-Myc-tagged N-terminal region of Arn3 and the three Aft1 fragments found to strongly interact with it in the in vivo assay were subjected to immunoprecipitation with anti-Myc antibody (c-myc) or anti-HA antibody (HA). c-Myc and Aft1 fragments were used as a control to confirm cross-links.

The three clones that showed an interaction with the bait, pGA-Aft1/1–177, pGA-Aft1/172–564 and pGA-Aft1/178–412, were further examined for their in vitro interaction with Arn3 using co-immunoprecipitation of c-Myc-tagged Arn3-(1–90) and HA-tagged Aft1 fragments. Each clone was transcribed and translated in vitro in the presence of [35S]methionine to label the synthesized protein. The c-Myc-tagged Arn3-(1–90) was mixed with buffer or with the HA-tagged Aft1 fragment in the same buffer. Each mixture was subjected to precipitation with the antibody denoted in Figure 1(D). The three Aft1 fragments that supported growth on minimal medium and gave rise to higher β-galactosidase activity also interacted strongly with c-Myc–Arn3 in vitro (Figure 1D).

To provide additional evidence for the in vitro interaction, amino acid residues corresponding to the N-terminal region of Arn3 and the three Aft1 fragments that showed strong physical interactions during co-immunoprecipitation were subcloned into pET23a and pGEX-5X-1 vectors respectively. These plasmids were used for over-expression and protein purification in E. coli. After purification, the proteins were subjected to SPR analysis to investigate in vitro interactions of Aft1 with Arn3 (Figure 2). Binding of His$_6$–Arn3 on to the surface of the CM5 biosensor chip was confirmed by the increase of SPR signal intensity ($\sim 800$ RU (response units)) upon injection of the protein. Purified GST (10 mg/ml) was first applied to the Arn3-bound sensor chip as a control experiment. As expected, GST did not interact with His$_6$–Arn3, eliminating the possibility of non-specific binding. Injection of the protein ligand Aft1-(1–177) at 1 mg/ml in HPS buffer caused a large increase in signal intensity. After being washed with HPS buffer to remove nonspecifically bound proteins, the sensorgram reached a plateau. The total increase in signal intensity was 6000 RU. Injection of the protein ligands Aft1-(178–412) and Aft1-(172–564) also increased the signal intensity by 1800 and 1000 RU respectively. Overall, the SPR analysis demonstrated that Arn3 physically interacts with ligand proteins Aft1-(1–177), Aft1-(178–412) and Aft1-(172–564), but not with GST. The relative strengths of the interactions can be summarized as Aft1-(1–177) > Aft1-(178–412) > Aft1-(172–564).

Interaction with Aft1 is important for Arn3 to take up FOB

Aft1 is a transcriptional activator of the iron regulon in S. cerevisiae. Under conditions of iron depletion, Pse1 interacts with two nuclear localization sequences within Aft1 to transport it into the nucleus. Aft1 then activates the transcription of genes involved in iron transport and metabolism [29]. Conversely, under conditions of excess iron, Aft1 is exported from the nucleus into the cytoplasm by the iron-sensing exportin (nuclear export
Arn3 affects the function of Arn3, the plasmid pEL-Arn3–GFP was used to examine whether the interaction between Aft1 and Arn3 affects the functions of Arn3, full-length Aft1 and the fragments of Aft1 used in the two-hybrid assay were subcloned into pESC-Ura. At the same time, GFP-fused, full-length Arn3 was subcloned into pESC-Leu and called pEL-Arn3–GFP (Figure 3A). Since ARN3 expression is dependent on Aft1, the galactose-inducible promoter in the pESC vector was used to regulate ARN3 expression independently of Aft1 in the S. cerevisiae BY4741/Δaft1Δarn strain. Expression of the protein from an exogenous inducible promoter could lead to mis-localization and abnormal function of the overexpressed protein. To exclude this possibility, cells exogenously expressing Arn3–GFP were examined for their ability to complement the growth deficiency on low-iron medium. pEL-Arn3–GFP and pESC-Leu vector were transformed into the S. cerevisiae YPH499 wild-type strain and the YPH499/Δfet3Δarn1,2,3 deletion mutant strain. Wild-type and mutant cells containing each plasmid were plated by serial dilution on SD (without leucine) and low-iron (0.5 μM FOB) YNB (without leucine) medium supplemented with carbon sources as denoted in Figure 3(B). On the low-iron plate, expression of ARN3 in pESC-Leu was regulated by the supplemented carbon source. Also, as shown in Figure 3(B), cells expressing Arn3–GFP from the galactose-inducible promoter grew comparably with wild-type cells on low-iron YNB plates supplemented with galactose. This result indicated that overexpressed Arn3–GFP functioned properly, without causing, for example, cell toxicity by iron overload.

To examine whether the interaction between Aft1 and Arn3 affects the function of Arn3, the plasmid pEL-Arn3–GFP was co-transformed with each Aft1 fragment into S. cerevisiae BY4741/Δaft1Δarn3. Cells containing a combination of plasmids derived from pESC-Leu and pESC-Ura were grown in SD (without leucine and uracil) with 0.2% galactose and 2% (w/v) raffinose. When the cell density reached a D600 of 0.5, cells were harvested and then analysed by the FOB-uptake assay as described in the Experimental section. FOB uptake by the exogenous Arn3–GFP was 4–5-fold higher when Arn3–GFP was co-expressed with Aft1(172–564) or Aft1(178–412) than when expressed alone. FOB uptake was 2–2.5-fold higher when expressed with Aft1(1–177) or Aft1(413–690) (Figure 3C). Moreover, cells expressing full-length Aft1 showed a high rate of FOB uptake, irrespective of exogenous Arn3–GFP. This result may have been due to the uptake of free iron by endogenous Fet3, which is activated by the exogenous Aft1, as in this assay we used S. cerevisiae BY4741/Δaft1Δarn3, which possesses the wild-type FET3 gene. To test this possibility, free-iron uptake and mRNA levels of the FET3 gene were examined in cells containing the plasmid combinations used in the FOB-uptake experiments. As shown in Figure 4(A), the FET3 gene was expressed when full-length AFT1 was introduced. As expected, cells expressing the exogenous full-length Aft1 showed a relatively high level of free-iron uptake (Figure 4B). To confirm that the Aft1 fragments regulated ARN3 expression, Northern blot analysis of ARN3 was performed. As shown in Figure 5A, a change in the expression of ARN3 was not found in Northern blot analysis, suggesting that most of the FOB uptake depended on Aft1. Consequently, we conclude that Arn3 is activated when co-expressed with Aft1 fragments that interact strongly with Arn3.

High uptake of FOB by Arn3 is consistent with the cytoplasmic membrane localization of Arn3

The Arn transporter family mediates uptake of iron–siderophore chelates in S. cerevisiae. A low concentration of substrate induces Arn to relocalize to the plasma membrane. At high substrate concentrations (1 μM and higher), Arn1 cycles between the plasma membrane and endosomes [22]. As reported previously, binding of the substrate to the high-affinity binding site of the transporter controls the intracellular trafficking of the protein, and blockage of the cytoplasmic trafficking of Arn1 caused a failure to take up siderophore [23]. The control of the intracellular trafficking of transporters is thought to be an important factor for regulating transporter activity [23]. Furthermore, it has been reported that most yeast transporters undergo ubiquitin-mediated endocytosis and degradation in the vacuole in response to elevated levels of their substrate [37,38]. In Figure 3(C), FOB uptake...
Figure 3  FOB uptake activity of Arn3 depends on the interaction with the Aft1 fragment

(A) AFT1 gene fragments were cloned into the pESC-Ura vector as described in the Experimental section. The indicated numbers correspond to the amino acid residues of Aft1 encoded by each construct. The black bar indicates the GFP fragment fused to the C-terminus of Arn3. (B) To examine whether the exogenous expression of Arn3–GFP (ARN3) could complement the growth deficiency on low-iron medium, pEL-Arn3-GFP was transformed into S. cerevisiae YPH499 wild-type strain (WT) and YPH499/Δfet3Δarn1,2,3 deletion mutant strain (∆fet3∆arn1,2,3). The composition of the medium is indicated above each panel. (C) Subcloned AFT1 fragments were introduced into S. cerevisiae BY4741/Δaft1Δarn3 with the plasmid pEL-Arn3-GFP and subjected to the FOB uptake assay. T (●) and (□) indicate the expression of Arn3 and Aft1 or vector alone, respectively.

analysis showed that the cells exogenously expressing Arn3 have a higher level of FOB uptake when co-expressed with the truncated versions of Aft1. This higher activity of Arn3 may indicate deregulation of the intracellular trafficking of the transporter in these strains. To test the possibility that Aft1 participates in the trafficking of Arn3, we compared the localization of Arn3–GFP when co-expressed with full-length and fragments of Aft1 in S. cerevisiae BY4741/Δaft1Δarn3 using fluorescence microscopy. In cells grown in minimal medium with 1 μM FOB, the localization of Arn3–GFP varied, depending on what fragment of Aft1 was being co-expressed (Figure 5B). Co-expression of full-length Aft1 was associated with localization of Arn3–GFP to the endosomal compartment and mostly to the vacuole lumen. Co-expression of the other fragments of Aft1 caused Arn3–GFP to localize to the plasma membrane and endosomal compartment. Interestingly, we observed stronger fluorescence signals on the plasma membranes of cells in which Arn3–GFP was co-expressed with Aft1-(172–564) or Aft1-(178–412) (Figure 5B). Among the samples observed, only when Arn3–GFP was co-expressed with full-length Aft1 in BY4741/Δaft1Δarn3 did it show the same localization as in wild-type cells in response to external substrate. Our results suggest that, although we could not detect a strong interaction between full-length Aft1 and the N-terminal region of Arn3, Aft1 can functionally interact with Arn3, leading to the proper trafficking pathway.
Aft1 regulates ubiquitination of Arn3

Figure 4 Interaction between Arn3 and Aft1 does not affect the expression of FET3

(A) To confirm the expression of FET3, Northern blot analysis was performed with the same strains used in the FOB uptake assay in Figure 3. Total RNA was extracted as stated in the Experimental section. ACT1 was used as a loading control. (B) To confirm the effect of free-iron uptake on FOB uptake, a free-iron uptake assay was performed with the same strains as in (A).

Trafficking to the cytoplasmic membrane renders Arn3 resistant to degradation

To confirm the Arn3–GFP localization shown in Figure 5(B), we performed sucrose-gradient fractionation analysis for Arn3–GFP co-expressed with various Aft1 fragments (Figure 5C). Cells were grown to mid-exponential phase on minimal medium with 1 μM FOB and then collected. Lysates were prepared, and proteins from the prepared lysates were fractionated by ultracentrifugation and resolved by SDS/PAGE as described in the Experimental section. Arn3–GFP co-expressed with empty vector and with full-length Aft1 was detected in the fraction corresponding to the endosomal compartment. In contrast, the majority of Arn3–GFP was in the plasma membrane fraction when co-expressed with deletion mutants of Aft1, as observed in Figure 5(B). The elevated level of FOB uptake and plasma membrane localization of Arn3–GFP in cells co-expressing Arn3 and Aft1 fragments may result from the inadequate recycling of Arn3, which would normally be degraded in vacuoles. To evaluate this possibility, we used Northern and Western blot analysis respectively to determine the protein and mRNA levels of Arn3–GFP when it was co-expressed with full-length or fragments of Aft1. Cells containing the combinations of plasmids shown in Figure 5(A) were grown to mid-exponential phase in SD (without leucine and uracil) medium and harvested, then divided into two samples: one for total RNA preparation and the other for protein preparation. Although all samples showed similar total signal density for ARN3–GFP mRNA by Northern blot analysis, they showed different protein levels by Western blot analysis. When co-expressed with Aft1 fragments, Arn3–GFP was more abundant than when expressed with an empty vector. Furthermore, when co-expressed with full-length Aft1, the signal of Arn3–GFP was barely detectable (Figure 5A). As we determined above, full-length Aft1 is required for the proper localization and degradation of Arn3, and mis-localization of Arn3 renders the transporter resistant to proteolysis.

Mutation of a cytoplasmic residue (Lys25) leads to degradation of Arn3 in the ER (endoplasmic reticulum)

Previous papers reported that trafficking of Arn1 from the MVB (multivesicular body) to the vacuole is dependent on ubiquitination, and this ubiquitination is regulated by an extracellular substrate, FC [23,26]. Recently, Erpapazoglou et al. reported that Arn3, like Arn1, participates in a substrate- and ubiquitin-dependent trafficking pathway [25]. Ubiquitination plays a role in diverse cellular processes, including cell division, signal transduction, differentiation, protein trafficking and quality control. In particular, mono-ubiquitination has important functions in the various steps of protein sorting, including membrane protein internalization, sorting of internalized proteins into MVBs, and sorting of secretory proteins into early endosomes/vacuoles from the secretory pathway.

In the experiment described above, we observed that Aft1 induced the correct localization and degradation of Arn3. We therefore investigated whether the different patterns of intracellular trafficking and degradation of Arn3 caused by Aft1 are associated with ubiquitination. Ubiquitination patterns of Arn3 when expressed with Aft1 derivatives were examined by immunoprecipitation with an anti-GFP antibody, followed by immunoblotting with an anti-ubiquitin antibody as described in the Experimental section. As shown in Figure 6(A), when Arn3–GFP was co-expressed with full-length Aft1, the level of ubiquitinated Arn3–GFP was higher than when co-expressed with the fragments of Aft1. Rsp5-dependent ubiquitination of Arn3 occurs during Golgi exit and/or sorting from the early endosome.
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Figure 5 Aft1 induces correct localization and degradation of Arn3

(A) S. cerevisiae BY4741/Δaft1/Δarn3 carrying the indicated plasmid combinations derived from pESC-Leu and pESC-Ura were grown in low-iron YNB at 30°C for 6 h. Cell lysates prepared from the strains were subjected to Western blot analysis (upper panels). Pgk1 was used as a loading control. Total RNA was extracted as described in the Experimental section and subjected to Northern blot analysis (lower panels). CMD1 was used as a loading control. (B) GFP fluorescence was visualized in living cells grown in low-iron YNB (+FOB; 1 μM FOB) or iron-deficient YNB (−FOB). The media in both cases was supplemented with 2 % raffinose as a carbon source for cell growth and 0.2 % galactose for induction of protein expression. (+) and (−) indicate the expression of Arn3–GFP and the numbers represent the amino acid residues of the Aft1 fragment. (C) Cells expressing ARN3–GFP with full-length Aft1 or with the indicated fragments of Aft1 were grown in low-iron YNB (1 μM FOB) supplemented with 2 % raffinose and 0.2 % galactose at 30°C for 6 h. Cell lysates were prepared and loaded onto 20–60 % sucrose gradient cushions. After ultracentrifugation, the fractions collected from each sample were subjected to Western blot analysis using anti-GFP antibody. CPY, Pma1 and Dpm1 were used as the control proteins for the vacuole, plasma membrane and ER respectively.

Figure 5 Aft1 induces correct localization and degradation of Arn3

(A) S. cerevisiae BY4741/Δaft1/Δarn3 carrying the indicated plasmid combinations derived from pESC-Leu and pESC-Ura were grown in low-iron YNB at 30°C for 6 h. Cell lysates prepared from the strains were subjected to Western blot analysis (upper panels). Pgk1 was used as a loading control. Total RNA was extracted as described in the Experimental section and subjected to Northern blot analysis (lower panels). CMD1 was used as a loading control. (B) GFP fluorescence was visualized in living cells grown in low-iron YNB (+FOB; 1 μM FOB) or iron-deficient YNB (−FOB). The media in both cases was supplemented with 2 % raffinose as a carbon source for cell growth and 0.2 % galactose for induction of protein expression. (+) and (−) indicate the expression of Arn3–GFP and the numbers represent the amino acid residues of the Aft1 fragment. (C) Cells expressing ARN3–GFP with full-length Aft1 or with the indicated fragments of Aft1 were grown in low-iron YNB (1 μM FOB) supplemented with 2 % raffinose and 0.2 % galactose at 30°C for 6 h. Cell lysates were prepared and loaded onto 20–60 % sucrose gradient cushions. After ultracentrifugation, the fractions collected from each sample were subjected to Western blot analysis using anti-GFP antibody. CPY, Pma1 and Dpm1 were used as the control proteins for the vacuole, plasma membrane and ER respectively.

to the late endosome [25]. Therefore, we suggest that Aft1 plays a role, directly or indirectly, in the ubiquitination of Arn3 and its subsequent trafficking in the cell.

Finally, we investigated possible ubiquitination sites on the N-terminal cytoplasmic region of Arn3, which is the region that interacts with Aft1. There are three lysine residues at positions 25, 30, and 34 (Figure 6B). We replaced these lysine (K) residues with arginine (R) using site-directed mutagenesis PCR. Each mutant clone was co-transformed with Aft1 derivatives as indicated in Figure 6(B), and fluorescence microscopy was used to observe the localization. We failed to detect any effect on Arn3 localization due to the K30R or K34R mutations. However, as shown in Figure 6(B), the K25R mutation caused the Arn3 mutant protein to be degraded rapidly compared with wild-type Arn3. In addition, the Arn3K25R protein accumulated in the ER compartment in spite of the presence of full-length Aft1 (Figure 6B).

DISCUSSION

Aft1 acts as a transcriptional activator of the iron regulon in S. cerevisiae [27]. In fact, Aft1 regulates most of the genes involved in high-affinity iron metabolism at the transcriptional level. The activity of Aft1 itself is regulated by a change in its localization from the cytosol to the nucleus [39] in response to cellular iron concentration [28]. However, the details of how Aft1 is regulated
Aft1 regulates ubiquitination of Arn3

Figure 6 Role of Aft1 in Arn3 ubiquitination

(A) Immunoprecipitation was performed to investigate the ubiquitination patterns of Arn3 co-expressed with various Aft1 derivatives as described in the Experimental section. Immunoprecipitates were subjected to Western blot (WB) analysis using anti-ubiquitin antibody (anti-Ub), then anti-GFP antibody (anti-GFP). Western blot images were used for the quantification of the ratio between ubiquitinated Arn3–GFP and total Arn3–GFP. This ratio is denoted as Arn3-GFP-UB/Arn3-GFP between the upper and lower panels. (B) The sequence of residues 1–100 of the N-terminus of Arn3 is presented in the upper panel. Lysine residues substituted with arginine are indicated by arrows. The first transmembrane region is underlined and the extracellular loop is denoted by italics. Plasmids encoding wild-type (WT) and mutant (K25R) Arn3–GFP were co-transformed into S. cerevisiae BY4741/Δaft1/Δarn3 with pESC-Ura encoding full-length Aft1 or an Aft1 fragment containing residues 178–412. Control cells were mock-transformed. Cells were grown at 30°C for 6 h in low-iron YNB (1 μM FOB) or iron-deficient YNB media; in both cases, the media were supplemented with 2 % raffinose and 0.2 % galactose. GFP fluorescence was imaged in living cells using fluorescence microscopy.

remain unknown. ARN3, which encodes the membrane-associated FOB transporter of S. cerevisiae [19], is one of the iron regulon genes regulated by Aft1 [14] at the transcriptional level and the mechanism by which it takes up FOB has been studied in detail [22]. Recently, both the post-transcriptional regulation and the ubiquitin- and substrate-dependent proteolytic degradation of Arn3 has been reported [25].

To characterize FOB uptake, and how it is regulated by Arn3, in greater detail, we conducted a two-hybrid screen using the N-terminal 90 amino acids of Arn3 as bait and the yeast genomic DNA library as prey. We found that specific fragments of Aft1, but not full-length Aft1, interact with the N-terminal region of Arn3. The reason why full-length Aft1 does not interact may be a result of the protein folding structure [40]. Protein interaction is based on folding structure and there will be no interaction if the protein folds incorrectly, even though it includes the strongly interacting domain. To identify the functional importance of the interaction between these proteins, FOB-uptake assays were performed using S. cerevisiae BY4741/Δaft1Δarn3 expressing full-length Arn3 with the Aft1 derivatives. To minimize the effect of the endogenous promotor in the expression of AFT1 and ARN3, given ARN3 expression is dependent on Aft1, a galactose-inducible promoter was used. The possibility of mis-localization and abnormal function of Arn3 and Aft1 is one of the important problems encountered in the over-expression system. However, Figures 3(B) and 4 showed that the expression of Aft1 and Arn3 by the Gal4 promoter works properly because the reductive iron uptake activity was recovered by introducing full-length Arn3 without causing cell toxicity by iron overload.

Interestingly, FOB uptake was higher in cells expressing Arn3 with Aft1 fragments than in cells expressing Arn3 with full-length Aft1. This increased FOB uptake may reflect enhanced Arn3 activity or its resistance to proteolysis. Fluorescence microscopy analysis showed that more Arn3 was directed to the plasma membrane when the protein was co-expressed with the Aft1 fragments than with full-length Aft1. In siderophore-deficient YNB medium, Arn3 was mislocalized to the plasma membrane, despite the strong interaction between Arn3 and Aft1 fragments. Taken together, these results suggest that Aft1 in the cytosol may have a novel function related to the intracellular trafficking of Arn3. The increase in FOB uptake observed with the fragments of Aft1 may indicate that they have lost the function of the full-length protein, even though they interact with Arn3 more strongly than the full-length protein. The domain
important for this novel function of Aft1 needs to be investigated further.

The regulation of metal transport has been described at both the transcriptional and post-translational levels [4]. Proper trafficking of membrane-bound metal transporters is an important aspect of post-translational regulation and is essential for protein function and activity in order to maintain metal ion homeostasis. Aft1, a major participant in transcriptional regulation of the iron regulon, is located in the nucleus in the absence of iron, where it activates the transcription of target genes. As iron enters the cell, Aft1 is exported to the cytosol and the expression of its target genes is reduced.

In the present study, we found that exogenous Aft1 can induce the correct localization of Arn3 in response to substrate, and we propose a novel role for Aft1 in the cytosol. Fluorescence microscopy and sucrose-density gradient analysis suggest that cytosolic Aft1 regulates the localization of Arn3 (Figure 5). Normal degradation and ubiquitination of Arn3 could be detected only when intact Aft1 was co-expressed with Arn3 (Figures 5 and 6). From these results, we predict that Aft1 interacts with Arn3 through domains of Arn3. These interactions may affect ubiquitination of specific sites through an unknown mechanism, thereby regulating the intracellular trafficking and degradation of Arn3. To confirm the result in Figure 6(A), we mutated lysine residues at positions 25, 30, and 34 in the same cytosolic N-terminus region of Arn3 that had been used in the yeast two-hybrid experiment. The mutation Arn3K25,K30,K34 caused rapid degradation of Arn3, such that its level was barely detectable, in contrast with the non-degraded wild-type protein. Furthermore, this mutant accumulated in the ER compartment, despite the presence of full-length Aft1.

The cellular localization of Arn3–GFP and the function of Aft1 might be affected by the over-expression system, so we needed to confirm whether or not Arn3 and Aft1 carry out their normal function. However, the cellular localization of Arn3–GFP expressed by the galactose-inducible promoter coincided with the localization reported previously in [26]. The results presented in Figures 5(B) and 6(B) also indicated that Arn3 localized correctly and that cellular localization of Arn3 was affected by cellular FOB. Furthermore, the amino acids in the N-terminus affected the cellular trafficking of Arn3 as reported previously [24,26]. The ubiquitination of Arn3 also might be affected by over-expression, because proteins found at high levels will be targeted by ubiquitination. However, as shown in Figure 6(A), ubiquitination of Arn3 was specific only to co-expression of either full-length Aft1 or the fragments of Aft1 which showed relatively low iron-uptake activity.

We had predicted that mutation of the candidate ubiquitinated lysine residues would render the mutant protein more resistant to proteolysis than the wild-type protein. The unexpected ER accumulation of the mutant protein observed in the present study merits further research.

**AUTHOR CONTRIBUTION**

Mi-Young Jeong performed most of the experiments. Chang-Min Kang, Ji-Hyun Kim, Dong-Hyuk Heo and In-Joon Baek performed some of the experiments. Hyeon-Su Ro performed the SPR experiment. Miwha Chang and Il-Dong Choi and Tae-Hyoung Kim gave critical experimental advice. Cheol-Won Yun designed the study and wrote the manuscript.

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