The Hog1p kinase regulates Aft1p transcription factor to control iron accumulation

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Summary

Iron acquisition systems have to be tightly regulated to assure a continuous supply of iron, since it is essential for survival, but simultaneously to prevent iron overload that is toxic to the cells. In budding yeast, the low-iron sensing transcription factor Aft1p is a master regulator of the iron regulon. Our previous work revealed that bioactive sphingolipids modulate iron homeostasis as yeast cells lacking the sphingomyelinase Isc1p exhibit an upregulation of the iron regulon. In this study, we show that Isc1p impacts on iron accumulation and localization. Notably, Aft1p is activated in *isc1*Δ cells due to a decrease in its phosphorylation and an increase in its nuclear levels. Consistently, the expression of a phosphomimetic version of Aft1p-S210/S224 that favours its nuclear export abolished iron accumulation in *isc1*Δ cells. Notably, the Hog1p kinase, homologue of mammalian p38, interacts with and directly phosphorylates Aft1p at residues S210 and S224. However, Hog1p-Aft1p interaction decreases in *isc1*Δ cells, which likely contributes to Aft1p dephosphorylation and consequently to Aft1p activation and iron overload in *isc1*Δ cells. These results suggest that alterations in sphingolipid composition in *isc1*Δ cells may impact on iron homeostasis by disturbing the regulation of Aft1p by Hog1p. To our knowledge, Hog1p is the first kinase reported to directly regulate Aft1p, impacting on iron homeostasis.

Keywords: iron; sphingomyelinase; Isc1p; Aft1p; Hog1p; cell signalling

Abbreviations: BPS, bathophenanthrolinedisulfonate; GST, glutathione S-transferase; PDS, post-diauxic-shift; TORC1, Target of Rapamycin Complex 1; V-ATPase, vacuolar H^+-ATPase; YPD, yeast extract peptone dextrose

1. Introduction

Iron is a redox active metal ion essential for survival of almost all organisms since many proteins involved in cellular processes, such as metabolism, energy production, ribosome biogenesis and DNA biosynthesis and repair, require iron cofactors [e.g., heme, iron-sulphur (Fe-S) clusters and di-iron centers]. However, excess labile iron is potentially detrimental because ferrous iron is able to reduce hydrogen peroxide, generating harmful hydroxyl radicals via the Fenton reaction [1]. Thus, iron acquisition systems and iron homeostasis have to be tightly regulated.

In the yeast *Saccharomyces cerevisiae*, the regulation of iron uptake, storage, mobilization and utilization occurs mainly at the transcriptional level [2]. When iron availability is low, the transcription factor Aft1p and its paralog Aft2p are translocated into the nucleus and activate the iron regulon, a set of genes associated with high affinity iron import and mobilization of vacuolar iron [3–5]. Aft1p also upregulates Cth1p and Cth2p, mRNA binding proteins that specifically promote the degradation of mRNAs related to iron storage (*CCC1*) and iron-dependent processes, limiting iron utilization in nonessential pathways [6].

In iron-replete conditions, Aft1p is exported to the cytosol by Msn5p in an iron-dependent manner due to an interaction between Aft1p and the monothiol glutaredoxin Grx3p or Grx4p [7,8]. Proper mitochondrial Fe-S cluster biosynthesis and export to the cytoplasm are required for Aft1p inhibition when iron is available [9]. Grx3p/Grx4p and the iron-regulatory proteins Fra1p/Fra2p form a Fe-S-bridged heterodimeric complex that controls Aft1p transcriptional activity [10,11]. A glutathione-complexed Fe-S cluster, which functions in the reversible cluster exchange with a wide range of Fe-S cluster proteins [12], mediates the key roles of Grx3p/Grx4p in iron trafficking and sensing. Thus, iron insertion into proteins and iron transfer to mitochondria are impaired in cells lacking Grx3p/Grx4p [13]. Under iron replete conditions, the uptake of iron is mediated by a low affinity system [14,15] and Fe-S clusters activate the transcription factor Yap5p, increasing Ccc1p-dependent iron transport into the vacuole [16–19].

Bioactive sphingolipids, such as long chain sphingoid bases (dihydrosphingosine and phytosphingosine in yeast), long chain sphingoid base-1-phosphate derivatives and ceramides, play essential roles in the regulation of numerous cellular processes, including stress responses, cell cycle, apoptosis, vesicular trafficking, autophagy and ageing [20–22]. A cross-

talk between iron and bioactive sphingolipids has emerged in recent years. In yeast, iron deficiency leads to a decrease in the levels of Sur2p [23], a diiron-binding sphinganine C4-hydroxylase that converts dihydrosphingosine into phytosphingosine [24], and to the accumulation of dihydrosphingosine associated with a shortened chronological lifespan [25]. Activation of the protein kinases Pkh1p and Ypk1p by long chain sphingoid bases mediate the toxicity of high levels of iron [26]. *AFT1*-mRNA levels increase in response to heat stress (or overexpression of alkaline dihydroceramidase Ydc1p) by a mechanism associated with the hydrolysis of dihydroceramides [27]. Moreover, we have shown that yeast lacking Isc1p, an orthologue of mammalian neutral sphingomyelinase (nSMase2) that generates phytoceramide by hydrolysis of complex sphingolipids [28], exhibit high levels of iron. Consistently, genes associated with high affinity iron uptake are upregulated whereas *GRX3* is downregulated in *isc1*Δ cells [29]. Thus, iron utilization and sensing seems to be impaired in these mutant cells. Isc1p deficient cells also exhibit mitochondrial dysfunction, oxidative stress sensitivity, and shortened chronological lifespan [29], which has been associated with deregulation of sphingolipid and nutrient signalling pathways including the mitogen-activated protein kinase Hog1p [30–34].

In this study, the mechanism underlying the activation of the iron regulon in *isc1*Δ cells was investigated. Our results show that iron accumulation in *isc1*Δ cells results from an improper activation of the transcriptional regulator Aft1p. Likewise, absence of Hog1p, a kinase with altered activity in *isc1*Δ cells [31], also leads to Aft1p activation and iron accumulation. Notably, Hog1p physically interacts with Aft1p and directly phosphorylates it *in vitro* at S210 and S224 residues, indicating that Hog1p is a negative regulator of Aft1p. However, Hog1p-Aft1p interaction decreases in *isc1*Δ cells. These results implicate for the first time Hog1p in the regulation of Aft1p and suggest that Aft1p activation in *isc1*Δ cells is due to a decrease in Hog1p-Aft1p interaction.

2. Material and Methods

2.1. Yeast strains, growth conditions and plasmids

Saccharomyces cerevisiae strains and plasmids used in this study are listed in Table 1. Yeast cells were grown aerobically at 26°C in an orbital shaker (at 140 rpm), with a 5:1 flask/culture

volume ratio. The growth media used were YPD [(1% (w/v) yeast extract (Conda Pronadisa), 2% (w/v) bactopeptone (LabM), 2% (w/v) glucose (Fisher Scientific)] or synthetic complete (SC) drop-out medium containing, 2% (w/v) glucose (Fisher Scientific), 0.67% (w/v) Difco yeast nitrogen base without amino acids (BD BioSciences) and appropriate amino acids or nucleotides [0.008% (w/v) histidine (Sigma Aldrich), 0.04% (w/v) leucine (Sigma Aldrich) and 0.008% (w/v) uracil (Sigma Aldrich)]. The deletion of *AFT1* or *HOG1* in wild type and *isc1*Δ cells was performed using a deletion fragment containing *HIS3* and the flanking regions of *AFT1* or *HOG1*. The deletion of *AFT1* in *hog1*Δ and *isc1*Δ*hog1*Δ cells was performed using a deletion fragment containing *LEU2* and the flanking regions of *AFT1.* Yeast cells were transformed using the lithium acetate/single-stranded carrier DNA/PEG method as described [35]. Cells were selected in medium lacking the respective selectable marker and gene deletion was confirmed by PCR standard procedures. To generate glutathione S-transferase (GST)-tagged Aft1p, full length of *AFT1* was fused to GST by ligating a 2.1 Kb *Eco*RI-*Not*I fragment carrying *AFT1* into the corresponding sites of the pGEX-4T-2 plasmid. The mutated GST-AFT1-S210A,S224A version, where residues S210 and S224 were replaced by alanines, was generated by site-directed mutagenesis using the NEB Q5 Site-Directed Mutagenesis Kit Protocol (E0554). Primers were designed using the NEBaseChanger tool (FW:TAGAGTACGAGCTACTTATGCGTTAAAGAGGAAAAGATGGAG; RV:AACGGACAGTTATTAAACCTCGCTACACATCTTTTTTTCTTTGG). Mutation was confirmed by Sanger sequencing.

2.2. Preparation of subcellular fractions

Isolation of mitochondria was carried out from yeast cells grown in YPD medium to the post-diauxic-shift (PDS) phase by differential centrifugation of homogenized spheroplasts as previously described [39] and protein content was determined by the Lowry method .

For vacuole extracts preparation, yeast cells were grown in YPD medium to the PDS phase and vacuole isolation was carried out by centrifugation in a Ficoll gradient of homogenized spheroplasts essentially as described [34], except that zymolyase (50 mg per 10 g cells wet weight) was used for spheroplasts formation. Protein content was determined by the Lowry method.

Cell wall extracts were obtained from yeast cells (6×10^8 cells/mL) grown to the PDS phase in YPD medium and washed twice with deionised H_2O. Cells were suspended in digestion buffer [2 M sorbitol, 1 M phosphate pH 7.5, 0.5 M EDTA, 1% (v/v) 2-mercaptoethanol] at a concentration of 10 g cells (wet weight) to 30 mL buffer, and then zymolyase (50 mg per 10 g cells wet weight) was added and the suspension was incubated at 37ºC until most of cells have been converted to spheroplasts. After centrifugation at 7,000 rpm for 10 min, the supernatant was collected.

2.3. Iron Levels

Iron levels were quantified using a colorimetric assay as previously described [29]. To quantify Fe^{2+}, sodium ascorbate was replaced by water and samples were purged of oxygen by bubbling nitrogen. Fe^{3+} levels were quantified by subtracting the levels of Fe^{2+} to total iron.

2.4. Fluorescence microscopy

To assess the localization of Aft1p, *aft1*Δ and *isc1*Δ*aft1*Δ cells transformed with pRS426-*GFP-AFT1* (a gift from Jerry Kaplan, University of Utah, EUA) [38] were grown to the exponential phase in YPD medium and treated with 80 μM of bathophenanthrolinedisulfonate (BPS) during 4 h to chelate iron. For nucleus staining, cells were incubated with 4 μg/mL of 4'-6-diamidino-2-phenylindole (DAPI, Molecular Probes, Invitrogen) for 15 min at room temperature, protected from light. After washing with PBS, cells were immobilized in agarose beds and observed by fluorescence microscopy (AxioImager Z1, Carl Zeiss). Output final images were obtained using ImageJ 1.45v software [40].

2.5. β-galactosidase assay

Wild type, *isc1*Δ, *hog1*Δ and *isc1*Δ*hog1*Δ cells transformed with p*CTH2-LacZ* (a gift from Dennis Thiele, Duke University Medical Center, USA) or p*FET3-LacZ* (a gift from Jerry Kaplan, University of Utah, USA) [36] were grown in YPD medium and treated or not with 100 μM BPS for 4 h. β-galactosidase activity was assayed as previously described [29], using 5-15 μg of total protein.

1 **2.6. Detection of Aft1p phosphorylation**

2 To assess Aft1p phosphorylation, *aft1*Δ, *isc1*Δ*aft1*Δ and *hog1*Δ*aft1*Δ cells transformed with
3 pRS416-*AFT1-HA* [37] were grown in YPD medium to the exponential phase. Cells were
4 collected by centrifugation, incubated in 0.1 M NaOH during 5 min at room temperature and
5 harvested by centrifugation at 13,000 rpm (4ºC) during 15 min. The pellet was suspended in
6 modified Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.002%
7 (v/v) bromophenol blue,) and incubated 5 min at 95ºC. After centrifugation at 13,000 rpm (4ºC)
8 for 3 min, the supernatant was collected and the protein content was estimated by the
9 bicinchoninic acid assay (Thermo Scientific). Protein samples (20 µg) were mixed with 20 %
10 (v/v) 2-mercaptoethanol before loading and separation in a 6% SDS polyacrylamide gel.
11 Immunodetection of Aft1p was performed using anti-HA (1:2000; Santa Cruz Biotechnology) as
12 primary antibody and anti-rabbit IgG-peroxidase (1:5000; Sigma Aldrich) as secondary antibody.
13

14 **2.7. Purification of GST proteins and in vitro kinase assay**

15 GST fusion proteins Aft1p, Aft1p-S210A,S224A, Hog1p, and Pbs2EEp were expressed in
16 *Escherichia coli* BL21 RIL and purified with glutathione-Sepharose beads (GE Healthcare) in
17 STET buffer (10 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0, 5% (v/v) Triton X-100, 2
18 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 2 µg/ml leupeptin,
19 2 µg/ml pepstatin). Phosphorylation by Hog1p was monitored as follows: one microgram of
20 GST-Hog1p was activated with 0.5 µg of GST-Pbs2EEp in the presence of kinase buffer (50
21 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol) and 50 µM ATP. After 30 min at 30°C, 1
22 µg of GST-Aft1p or GST-Aft1p-S210D,S224D was added to the Hog1p-Pbs2EEp mixture
23 together with [γ-^{32}P]ATP (0.1 mCi/ml) and the mixture was incubated for 30 min at 30°C. The
24 reaction was stopped by the addition of Laemmli buffer and subsequent boiling. Labeled
25 proteins were resolved by SDS-PAGE, stained with Coomassie brilliant blue, dried, and
26 analyzed by autoradiography [41,42].
27

28 **2.8. Protein co-immunoprecipitation**

29 Protein co-immunoprecipitation was performed using the GFP-Trap beads (Chromotek),
30 according to manufacturer´s instructions, except for the lysis. Cells expressing GFP-tagged

1 Aft1p grown to exponential phase in YPD medium were suspended in lysis buffer (10 mM
2 Tris/HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% (v/v) NP-40) supplemented with 1 mM
3 phenylmethanesulfonyl fluoride and protease inhibitors (Complete, EDTA-free Protease Cocktail
4 Inhibitor Tablets; Roche Applied Science). Cell lysis was performed by vortexing with glass
5 beads followed by centrifugation at 13,000 rpm for 10 min at 4°C. Proteins samples (8 mg) were
6 incubated with GFP-Trap beads for 1 h at 4°C and, after washing, GFP-Trap beads were
7 suspended in 2x Laemmli buffer containing 40 % (v/v) 2-mercaptoethanol and incubated 10 min
8 at 98°C to dissociate immunocomplexes. The supernatant was analysed by Western blotting
9 using anti-GFP (1:200; Roche) or anti-Hog1p (1:2000; Santa Cruz Biotechnology) as primary
10 antibodies and anti-mouse IgG-peroxidase (1:5000; Molecular probes) and anti-rabbit IgG-
11 peroxidase (1:5000; Sigma Aldrich) as secondary antibodies, respectively.
12

13 **2.9. Oxidative stress resistance**

14 Yeast cells were grown to exponential phase and treated with 1 mM H_2O_2 for 1h. Cell viability
15 was determined by standard dilution plate counts on YPD medium containing 1.5 % (w/v) agar.
16 Colonies were counted after growth for 3 days at 26 ºC and cell viability was expressed as a
17 percentage of colony-forming units.
18

19 **2.10. Statistical analysis**

20 Data were analysed in GraphPad Prism Software v5.01 (GraphPad Software). Values were
21 compared by one-way ANOVA, two-way ANOVA or by Student's *t*-test *, $p< 0.05$; **, $p< 0.01$;
22 ***, $p< 0.001$; ****, $p<0.0001$.
23

24 **3. Results**

25 **3.1. Vacuolar iron accumulation decreases in cells lacking the sphingomyelinase Isc1p**

26 We have previously demonstrated that loss of the sphingomyelinase Isc1p leads to an
27 upregulation of the iron regulon in yeast, resulting in increased intracellular iron levels [29]. In
28 order to characterize the role of Isc1p in the regulation of iron homeostasis, iron levels [total,
29 ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron] were first analysed throughout growth of yeast cells in YPD
30 medium (Fig. 1A-D). Total iron levels increased in wild type and *isc1*Δ cells during growth up to

the diauxic shift (day 2), with both Fe^{2+} and Fe^{3+} contributing to iron accumulation. However, *isc1*Δ cells accumulated higher levels of both Fe^{2+} and Fe^{3+} in all phases of growth. Interestingly, the percentage of Fe^{3+} in cells grown to the PDS phase was higher in *isc1*Δ cells (see also Fig. S1). Moreover, when cells were grown in YPD medium supplemented with 1 mM iron sulphate, both wild type and *isc1*Δ cells accumulated the same levels of iron, however the percentage of Fe^{3+} was significantly higher in *isc1*Δ cells (Fig. S1). This increase in Fe^{3+} content probably occurs due to the higher intracellular oxidation exhibited by *isc1*Δ cells [29]. Mitochondria and vacuoles are major hubs of iron trafficking and homeostasis in yeast, representing important destinations of cellular iron [2,43]. To assess if iron accumulation in *isc1*Δ cells occurs in a specific compartment, iron levels were quantified in intact cells and subcellular fractions, namely cell wall, mitochondria and vacuoles. Despite the upregulation in *isc1*Δ cells of *FIT2* and *FIT3* genes [29], which encode two proteins involved in the retention of siderophore-iron in the cell wall [44], the levels of iron associated with the cell-wall were similar in wild type and *isc1*Δ cells. Consistently, the excess of iron remained associated with *isc1*Δ spheroplasts (Fig. S2). Iron levels were also similar in mitochondria isolated from wild type and Isc1p deficient cells, but vacuolar iron levels were significantly lower in *isc1*Δ cells compared to wild type cells (Fig. 2A-C). The overall results indicate that *isc1*Δ cells exhibits excess iron as well as deregulated iron localization.

3.2. *ISC1* deletion leads to Aft1p dephosphorylation and nuclear accumulation, inducing the iron regulon in iron-replete conditions

As the expression of the iron regulon, including genes associated with iron mobilization from vacuoles, is induced in iron-deficient conditions through Aft1p activation [2,45,46], the involvement of this transcription factor in the accumulation of iron in *isc1*Δ cells was investigated. To assess Aft1p activation, both wild type and *isc1*Δ cells transformed with a plasmid containing the Aft1p binding sequence from *CTH2* promoter fused to a LacZ reporter were grown to the exponential phase and the β-galactosidase activity was determined. As expected, *CTH2*-LacZ was induced in wild type cells treated with the iron chelator BPS. In iron-replete conditions, β-galactosidase activity was significantly higher in *isc1*Δ cells than in parental cells (Fig. 3A). Isc1p-deficient cells expressing a *FET3*-LacZ reporter also exhibited a

1 higher β-galactosidase activity (Fig. 3B). In previous studies, we observed a higher activation of
2 an Aft1p reporter in *isc1*Δ cells but only after BPS treatment [29]. The different growth medium
3 used in the current (YPD) and previous (minimal medium) studies may explain the different
4 results obtained. Notably, iron overload was abolished in *isc1*Δ*aft1*Δ cells (Fig. 3C), indicating
5 that Aft1p activation mediates iron accumulation in *isc1*Δ cells.
6 Since Aft1p activation is accompanied by its translocation to the nucleus, the localization of
7 GFP-Aft1p in *isc1*Δ cells was analysed by fluorescence microscopy. In agreement with previous
8 studies [38,47], Aft1p localized to the cytoplasm in parental cells grown in iron-replete
9 conditions, being translocated to the nucleus when cells were treated with BPS. Consistently
10 with Aft1p activation in *isc1*Δ cells, nuclear Aft1p levels increased in this mutant even in iron-
11 replete conditions (Fig. 4).
12 Aft1p phosphorylation at residues S210 and S224 is essential for the nuclear export of Aft1p in
13 iron-replete conditions. Indeed, cells expressing a phosphoresistant double mutant (Aft1p-
14 SSAA), in which residues S210 and S224 were replaced by alanine, display Aft1p localized in
15 the nucleus even in the presence of iron [37]. It was demonstrated that the phosphorylation of
16 S210 and S224 residues results in the appearance of slower-migrating species of Aft1p [37].To
17 assess if *isc1*Δ cells exhibit a higher Aft1p nuclear localization due to an alteration in Aft1p
18 phosphorylation, the migration pattern of an HA-tagged Aft1p was assessed by Western
19 blotting. Aft1p was detected at a lower molecular weight in *isc1*Δ cells (Fig. 5A), suggesting that
20 Aft1p is less phosphorylated in this mutant, in agreement with the observed Aft1p nuclear
21 accumulation. To assess the functional relevance of Aft1p-S210,S224 dephosphorylation in
22 Aft1p activation and iron accumulation in cells lacking Isc1p, iron levels were quantified in cells
23 expressing a phosphomimetic double mutant (Aft1p-SSDD-HA), in which Aft1p-S210 and S224
24 residues were replaced by aspartate. The expression of Aft1p-HA and Aft1p-SSDD-HA was
25 confirmed by Western blotting (data not shown). Our results show that the expression of Aft1p-
26 SSDD-HA in *isc1*Δ cells abolished iron overload (Fig. 5B), indicating that decreased
27 phosphorylation at S210 and S224 contributes to Aft1p activation and iron accumulation in
28 *isc1*Δ mutant cells.
29 We have previously suggested that iron overload favours the production of reactive oxygen
30 species in *isc1*Δ cells, increasing oxidative damage and cell death by apoptosis[29].

Consistently, the expression of Aft1p-SSDD-HA also suppressed the hydrogen peroxide sensitivity of *isc1*Δ cells (Fig. 5C). However, mitochondrial dysfunction exhibited by *isc1*Δ cells does not seem to result from iron overload since the reduced oxygen consumption rate exhibited by this mutant was not suppressed by Aft1p-SSDD expression (Fig. S3).

3.3. Hog1p phosphorylates Aft1p at residues S210 and S224 and downregulates the iron regulon in iron replete conditions

Although it has been described that Aft1p is regulated by reversible phosphorylation, the protein kinases or phosphatases controlling Aft1p have not been identified. Since Hog1p is deregulated in *isc1*Δ cells [31] and negatively regulates genes involved in iron transport in *Candida albicans* [48] and *Cryptococcus neoformans* [49,50], we investigated the role of this kinase in Aft1p regulation. Our data show that Hog1p regulates Aft1p phosphorylation in vivo and directly targets Aft1p at the nuclear export regulatory residues S210 and S224. Deletion of *HOG1* resulted in Aft1p dephosphorylation (Fig. 6A) and *in vitro* kinase assays showed that Hog1p phosphorylated wild-type Aft1p, but not the phosphoresistant Aft1p mutant (S210A,S224A) (Fig. 6B). Consistently, *hog1*Δ cells exhibited Aft1p activation, as shown by the increase in Aft1p transcriptional activity in *hog1*Δ cells expressing the reporters *CTH2*- or *FET3*-LacZ (Fig. 6C-D), and *hog1*Δ cells exhibited iron accumulation that was suppressed by *AFT1* deletion (Fig. 6E). These results suggest that Hog1p regulates iron homeostasis by an Aft1p-dependent mechanism.

3.4. Hog1p interacts with Aft1p in an Isc1p-dependent manner

Since Aft1p was inhibited by Hog1p-dependent phosphorylation (Fig. 6) and Hog1p is activated in *isc1*Δ cells [31,32], it was puzzling how Aft1p is activated in *isc1*Δ cells (Figs. 3-5). This led us to assess if Hog1p interacts directly with Aft1p and how Isc1p deficiency affects that interaction. For this purpose, parental and *isc1*Δ cells expressing GFP-Aft1p or GFP were grown to the exponential phase, Aft1p was immunoprecipitated using GFP-Trap and the presence of Hog1p was probed by Western blotting. As shown in the Fig. 7A, Hog1p co-precipitated with GFP-Aft1p but not with GFP, revealing that Hog1p interacts with Aft1p. Notably, this interaction was

significantly lower in cells lacking Isc1p (Fig. 7B), which likely impairs Aft1p phosphorylation and leads to Aft1p activation in these mutant cells.

4. Discussion

Iron accumulation has been demonstrated in many metabolic disorders affecting ceramide metabolism and mitochondrial function, as well as during ageing and in neurodegenerative disorders [51]. Sphingolipids have been implicated in ageing and age related diseases [52] as well as in iron homeostasis and toxicity. Studies performed in yeast cells have shown that iron deprivation leads to an increase of the long chain sphingoid base dihydrospingosine [25] and that upregulation of yeast dihydroceramidase (Ydc1p), which generates the dihydrospingosine by hydrolysis of dihydroceramides, leads to Aft1p activation [27]. In both conditions, yeast exhibit a shortened lifespan [25,53]. We have previously shown that yeast cells lacking the neutral sphingomyelinase Isc1p exhibit high levels of iron associated with the upregulation of the iron regulon and a shortened lifespan [29]. However, the mechanisms by which sphingolipids and Isc1p modulate Aft1p, a master regulator of iron homeostasis, remains poorly characterized.

In this study, we show that Aft1p is dephosphorylated and activated in cells lacking Isc1p, leading to the accumulation of both Fe^{2+} and Fe^{3+} in all phases of growth, with a higher % of Fe^{3+} at the PDS phase. Notably, *isc1*Δ cells exhibited lower levels of iron in vacuoles, which play a key role on iron storage predominantly as Fe^{3+} ions [54,55]. This decrease may also result from Aft1p activation since it regulates the expression of genes associated with the mobilization of iron from the vacuole (*FRE6*, *FET5* and *FTH1*) [3,55]. In *isc1*Δ cells, the increase of vacuolar pH due to a reduction of vacuolar H^+-ATPase (V-ATPase) activity [34] may also impair iron accumulation in this organelle and iron signalling. Indeed, the acidic environment of the vacuole is required for proper iron accumulation and the loss of V-ATPase activity generates an iron deficiency signal [56].

We found that Aft1p transcriptional activity and nuclear accumulation were higher in *isc1*Δ cells in iron replete conditions and that *AFT1* deletion abolished iron accumulation. Aft1p activation seems to be due to a decrease in Aft1p phosphorylation at S210/S224, residues with a critical role in Aft1p regulation. In iron-replete conditions, S210/S224 phosphorylation is required for the

1 nuclear export of Aft1p mediated by Msn5p [37], but not for the dissociation of Aft1p from the
2 promoter of target genes [9]. Likewise, expression of Aft1p-S210A,S224A results in Aft1p
3 nuclear accumulation but not in induction of the iron regulon in iron-replete conditions. As such,
4 dephosphorylation of Aft1p in *isc1*Δ cells may not be sufficient to induce Aft1p activation.
5 Previous studies have shown that the interaction of Aft1p with the glutaredoxins Grx3/4p is
6 crucial to the dissociation of Aft1p from target promoters under iron-replete conditions [9].
7 Because *GRX3* transcription was observed to be decreased in *isc1*Δ cells [29], it may also
8 contribute to the activation of the iron regulon in cells lacking Isc1p (Fig. 8).
9 In wild type cells, Aft1p is translocated to the nucleus and activates the iron regulon under low
10 iron conditions [2], but also in response to other stimuli independently on the iron levels in the
11 medium. An example of this is the Aft1p phosphorylation and activation upon glucose
12 exhaustion (at the diauxic shift) to support the iron demands of mitochondrial respiration [57].
13 Activation of Aft1p at the diauxic transition is dependent on the AMP kinase homologue Snf1p
14 [58]. Conversely, the protein kinase A (PKA) is required for transcriptional repression of the iron
15 regulon when cells are grown on fermentable carbon sources [59]. However, Aft1p
16 phosphorylation is Snf1p- or PKA-independent [57,58].
17 In this study we identified for the first time a protein kinase that directly regulates Aft1p, namely
18 Hog1p (human p38 MAPK homologue). We show that *hog1*Δ cells exhibited lower Aft1p
19 phosphorylation associated with an increase of Aft1p transcriptional activity and intracellular iron
20 levels. Moreover, Hog1p interacts directly with Aft1p and phosphorylates this transcription factor
21 at residues S210 and S224. Hog1p phosphorylation at these aminoacid residues was
22 unexpected as they are not present in a typical Hog1p consensus phosphorylation site (Ser/Thr-
23 Pro) [60]. Although Hog1p is activated in *isc1*Δ cells [31], in this report we show that Hog1p-
24 Aft1p interaction and Aft1p phosphorylation decreased in this mutant. Moreover, Aft1p
25 dephosphorylation mediates the induction of the iron regulon in *isc1*Δ cells since the expression
26 of an Aft1p-S210D,S224D mutant protein abolished iron overload and oxidative stress
27 sensitivity. Thus, we propose that Aft1p phosphorylation is reduced in *isc1*Δ cells because the
28 interaction of Hog1p with Aft1p is diminished. More studies will be required to understand the
29 regulation of Hog1p-Aft1p interaction and the role of Isc1p and sphingolipids in this process.

1 Hog1p is the core signalling effector of the high osmolarity glycerol (HOG) signalling pathway
2 involved in the adaptation to high osmolarity [61]. Upon osmotic shock, Hog1p is activated and
3 transported into the nucleus where regulates gene transcription and cell cycle, although Hog1p
4 also regulates targets in the cytoplasm. Hog1p is also moderately activated in response to a
5 number of non-osmotic stresses, such as cold stress, heat stress, hypoxia, arsenite, acetic acid,
6 and low pH [32,61], as well as upon inhibition of sphingolipid syntesis or exposure to ceramide
7 [32,61,62]. Here we propose that Hog1p is also involved in the regulation of iron homeostasis in
8 *S. cerevisiae* by negatively regulating Aft1p. A link between Hog1p and iron homeostasis have
9 also been reported in other models. *HOG1* deletion results in increased expression of the iron
10 regulon in *C. albicans* and *C. neoformans* [48–50]. Furthermore, Hog1p is phosphorylated upon
11 exposure of *C. albicans* to high iron concentrations [48] and p38, the mammalian homologue of
12 Hog1p, is phosphorylated in iron depleted human cell lines [63,64].
13 In summary, our results show that Hog1p interacts directly with Aft1p and phosphorylates this
14 transcription factor, negatively regulating iron uptake. The interaction between Hog1p and Aft1p
15 decreases in yeast lacking Isc1p, and this alteration correlates with a decrease in Aft1p
16 phosphorylation that favours Aft1p accumulation in the nucleus, upregulation of iron regulon
17 genes and iron overload (Fig. 8).
18

Competing interests

No competing interests declared.

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FIGURE LEGENDS

Fig.1. Cells lacking Isc1p accumulate iron in all phases of growth. (A) Wild type (wt) and *isc1*Δ cells were grown in YPD medium and OD_{600} was measured over time. Cells were collected at different time points for the quantification of (B) total iron, (C) Fe^{3+} and (D) Fe^{2+} levels. Data are mean ± SEM of at least three independent experiments.

Fig. 2. *ISC1* deletion results in decreased levels of vacuolar iron. Wild type (wt) and *isc1*Δ cells were grown in YPD medium to the PDS phase and iron levels were quantified in (A) total cellular extracts, (B) mitochondria, and (C) vacuolar extracts. Data are mean ± SEM of at least three independent experiments. *, $p< 0.05$; **, $p< 0.01$ (Student's *t*-test).

Fig. 3. Iron accumulation in *isc1*Δ cells is Aft1p-dependent. (A, B) Wild type (wt) and *isc1*Δ cells expressing the Aft1p binding sequence from *CTH2* promoter (A) or *FET3* promoter (B) fused to a LacZ reporter were grown to the exponential phase and β-galactosidase activity was determined. Wild type cells treated with the iron chelator BPS (wt + BPS) were used as control. Values are mean ± SEM of at least three independent experiments; *, $p< 0.05$; **, $p< 0.01$ (Student's *t*-test). (C) Iron levels were quantified in wt, *isc1*Δ, *aft1*Δ and *isc1*Δ*aft1*Δ cells grown to the PDS phase. Data are mean ± SEM of three independent experiments; *, $p<0.05$ (one-way ANOVA).

Fig. 4. Aft1p accumulates in the nucleus of *isc1*Δ cells in iron replete conditions. To assess the localization of Aft1p, *aft1*Δ and *isc1*Δ*aft1*Δ cells expressing *GFP-AFT1* were grown to the exponential phase, incubated with DAPI (for nuclear staining) and examined by fluorescence microscopy. As a control, *aft1*Δ cells expressing *GFP-AFT1* cells were treated with 80 μM of BPS during 4 h (iron-starved conditions). Representative images are shown along with the quantification of the cells displaying nuclear Aft1p (at least 500 cells were analysed). Data are mean ± SEM of three independent experiments; *, $p<0.05$ (Student's *t*-test).

Fig. 5. The decrease in Aft1p phosphorylation mediates iron overload in *isc1*Δ cells. (A) To assess changes in Aft1p phosphorylation, protein extracts from *aft1*Δ and *isc1*Δ*aft1*Δ cells

expressing Aft1p-HA grown to the exponential phase were separated by 6 % SDS-PAGE and analysed by immunoblotting, using anti-HA. A representative image of three independent experiments is shown. (B) Iron levels were determined in *aft1*Δ and *isc1*Δ*aft1*Δ cells expressing Aft1p-HA or Aft1p-SSDD-HA, a phosphomimetic version of Aft1p (Aft1p-S210D,S224D-HA), grown to the PDS phase. Values are mean ± SEM of at least three independent experiments; *, $p< 0.05$ (one-way ANOVA). (C) Oxidative stress resistance was determined in *aft1*Δ and *isc1*Δ*aft1*Δ cells expressing Aft1p-HA or Aft1p-SSDD-HA treated with 1 mM H_2O_2 for 1 h. Values are mean ± SEM of at least three independent experiments; **, $p<0.01$ (Student's *t*-test).

Fig. 6. The Hog1p kinase negatively regulates Aft1p in iron-replete conditions. (A) Protein extracts from *aft1*Δ and *hog1*Δ*aft1*Δ cells expressing Aft1p-HA grown to the exponential phase were separated by 6% SDS-PAGE and analysed by immunoblotting, using anti-HA. A representative image of three independent experiments is shown. (B) In vitro kinase assay. GST-Aft1p or GST-Aft1p-S210A,S224A (GST-Aft1p-SSAA) were incubated with both GST-Hog1p and the constitutively active GST-Pbs2EEp allele (or GST-Pbs2EEp only) in the presence of [γ-32P]ATP. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography (upper panel). GST-tagged proteins were detected by staining with Coomassie brilliant blue (lower panel). A representative image of three independent experiments is shown. (C, D) Wt, *isc1*Δ, *hog1*Δ and *isc1*Δ*hog1*Δ cells expressing the Aft1p binding sequence from *CTH2* promoter (C) or *FET3* promoter (D) fused to a LacZ reporter were grown to the exponential phase and β-galactosidase activity was determined. Wild type cells treated with the iron chelator BPS (wt + BPS) were used as control. Values are mean ± SEM (n>3); *, $p< 0.05$ (one-way ANOVA). (E) Iron levels were quantified in indicated strains grown to PDS phase. Values are mean ± SEM (n>3); *, $p< 0.05$; ***, $p< 0.001$ (one-way ANOVA).

Fig. 7. The kinase Hog1p regulates Aft1p. (A) *aft1*Δ and *isc1*Δ*aft1*Δ cells expressing GFP-Aft1p were grown to exponential phase and GFP-Aft1p was immunoprecipitated using GFP-Trap. Wild type cells expressing GFP were used as control. The presence of Hog1p and Aft1p in the precipitates (and in cell lysates – input) was probed by immunoblotting using anti-Hog1p and anti-GFP, respectively. A representative image is shown, out of three independent experiments

1 with similar results. (B) The intensity of the bands was quantified by densitometry and the signal
2 of co-precipitated Hog1p was normalized to the signal of immunoprecipitated GFP-Aft1p.
3 Values are mean ± SEM of at least three independent experiments; **, p<0.001 (Student's *t*-
4 test).
5
6 **Fig. 8.** Hog1p negatively regulates iron uptake by phosphorylating Aft1p at residues S210 and
7 S224. In iron replete conditions, the monothiol glutaredoxins Grx3/4p bind to Aft1p in an iron
8 dependent manner leading to its dissociation from the promoter of target genes (iron regulon).
9 Aft1p is phosphorylated by Hog1p at residues S210 and S224, recognized by Msn5p and
10 exported to the cytosol as a complex Aft1-Grx3/4.
11 In the absence of the sphingomyelinase Isc1p, Aft1p is found dephosphorylated due to a
12 decrease in Hog1p-Aft1p interaction, leading to its accumulation in the nucleus. *GRX3*
13 transcription is decreased in *isc1*Δ cells [29], which likely favours Aft1 mediated transcription of
14 the iron regulon, leading to iron overload.
15
16

1 **Table 1.** *Saccharomyces cerevisiae* strains and plasmids used in this study.

S. cerevisiae	**Genotype**	**Source**
BY4741	Mata, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0	EUROSCARF
*isc1*Δ	BY4741 *isc1*::*KanMx4*	EUROSCARF
*aft1*Δ	BY4741 *aft1*::*HIS3*	This study
*isc1*Δ*aft1*Δ	BY4741 *isc1*::*KanMx4 aft1*::*HIS3*	This study
*hog1*Δ	BY4741 *hog1*::*HIS3*	This study
*isc1*Δ*hog1*Δ	BY4741 *isc1*::*KanMx4 hog1*::*HIS3*	This study
*hog1*Δ*aft1*Δ	BY4741 *hog1*::*HIS3 aft1*::*LEU2*	This study
*isc1*Δ*hog1*Δ*aft1*Δ	BY4741 *isc1*::*KanMx4 hog1*::*HIS3 aft1*::*LEU2*	This study
Plasmids		
p*CTH2-LacZ*	pCM64-*CTH2-FeRE-CYC1-LacZ*	[6]
p*FET3-LacZ*	pYEp354-*FET3-FeRE-LacZ*	[36]
p*AFT1-HA*	pRS416-*AFT1-HA*	[37]
p*AFT1-SSDD-HA*	pRS416-*AFT1-S210D,S224D-HA*	[37]
p*GFP-AFT1*	pRS426-*GFP-AFT1*	[38]
p*GST-AFT1*	pGEX-4T-2-*GST-AFT1*	This study
pGST-AFT1-SSAA	pGEX-4T-2-*GST-AFT1-S210A,S224A*	This study

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