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Significance statement - MT-ART-06-2018-000124

The deregulation of metal-related transcription factors may have unexpected cellular consequences. This paper examines the result of using constitutive alleles of the *S. cerevisiae* iron-regulated transcription factor Aft1 to augment yeast iron content. In addition to an increase in endogenous iron, cells become sensitive to the metal, increase oxidative stress and diminish oxygen consumption. Molecular strategies directed to palliate these problems emphasize the connections of iron with lipids and respiration in eukaryotic cells.

Molecular strategies to increase yeast iron accumulation and resistance

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20 **Abstract**

21 All eukaryotic organisms rely on iron as an essential micronutrient for life
22 because it participates as a redox-active cofactor in multiple biological processes.
23 However, excess iron can generate reactive oxygen species that damage cellular
24 macromolecules. The low solubility of ferric iron at physiological conditions
25 increases the prevalence of iron deficiency anemia. A common strategy to treat
26 iron deficiency consists of dietary iron supplementation. The baker's yeast
27 *Saccharomyces cerevisiae* is used as a model eukaryotic organism, but also as a feed
28 supplement. In response to iron deficiency, the yeast Aft1 transcription factor
29 activates cellular iron acquisition. However, when constitutively active, Aft1
30 inhibits growth probably due to iron toxicity. In this report, we have studied the
31 consequences of using hyperactive *AFT1* alleles, including *AFT1-1UP*, to increase
32 yeast iron accumulation. We first characterized the iron sensitivity of cells
33 expressing different constitutively active *AFT1* alleles. We rescued the high iron
34 sensitivity conferred by the *AFT1* alleles by deleting the sphingolipid signaling
35 kinase *YPK1*. We observed that the deletion of *YPK1* exerts different effects on iron
36 accumulation depending on the *AFT1* allele and the environmental iron. Moreover,
37 we determined that the impairment of the high-affinity iron transport system
38 partially rescues the high iron toxicity of *AFT1-1UP*-expressing cells. Finally, we
39 observed that *AFT1-1UP* inhibits oxygen consumption through activation of the
40 RNA-binding protein Cth2. Deletion of *CTH2* partially rescues the *AFT1-1UP*
41 negative respiratory effect. Collectively, these results contribute to understand
42 how the Aft1 transcription factor functions and the multiple consequences derived
43 from its constitutive activation.

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45 Introduction

46 Iron is a vital micronutrient for all eukaryotic cells because it functions as a
47 redox-active cofactor in a wide variety of metabolic processes including energy
48 generation through mitochondrial respiration and the biosynthesis of the main
49 cellular macromolecules: DNA, proteins and lipids. Despite its high abundance, the
50 low solubility of ferric iron at physiological pH highly restricts its availability to
51 living organisms. Thus, iron deficiency has become the most extended nutritional
52 disorder in the world, and one of the few food deficits that are relatively frequent
53 in developed countries. In humans, iron deficiency anemia is estimated to affect
54 about two billion people worldwide, leading to reduced physical capacity, learning
55 problems, and increased morbidity, especially in pregnant women and children.^{1, 2}

56 The baker's yeast *Saccharomyces cerevisiae* is used as a food dietary
57 supplement for human and animal feeding due to its richness in proteins, B-type
58 vitamins and fiber. Multiple studies have explored the potential of this yeast as an
59 efficient source of iron to palliate iron deficiency,³⁻⁵ and have demonstrated the
60 bioavailability of yeast-carried iron used in food fortification.⁶ Baker's yeast is also
61 widely used as a model organism to study the mechanisms that eukaryotic cells
62 use to control cellular iron homeostasis.⁷⁻¹⁴ In response to iron deficiency, the low
63 iron-sensing transcription factor Aft1, and its partially redundant counterpart Aft2,
64 accumulate in the nucleus and activate the expression of a group of genes known
65 as the iron regulon.¹⁵⁻¹⁹ The set of genes activated by Aft1 upon iron scarcity
66 include the FRE family of plasma membrane metalloredutases and inorganic iron
67 transporters (*FRE1-6*, *FET3*, *FTR1*, *FET4*), the ARN family of cell surface iron
68 siderophore transporters, the vacuolar iron exporters (*FET5*, *FTH1*, *SMF3*), and

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69 two factors that reorganize cellular iron metabolism (*CTH1* and *CTH2*).²⁰⁻²⁹ Hence,
70 upon iron starvation, Aft1 activates iron acquisition from the environment, iron
71 mobilization from intracellular storage compartments, and a remodeling of iron
72 metabolism mostly performed by the Cth2 mRNA-binding protein. During the
73 adaptation to iron limitation, Cth2 promotes the degradation of mRNAs that
74 encode proteins with functions in highly iron-consuming pathways such as the
75 mitochondrial electron transport chain and the tricarboxylic acid cycle, to
76 prioritize its utilization in essential iron-dependent processes such as the
77 synthesis of deoxyribonucleotides by ribonucleotide reductase.^{25, 30-32}

78 When yeast cells are iron replete, the monothiol glutaredoxins Grx3 and
79 Grx4 and the cytosolic proteins Fra1 and Fra2 transfer an inhibitory iron signal,
80 which comes from the mitochondrial iron-sulfur cluster biosynthesis, to promote
81 the dissociation of Aft1 from its target promoters and its export to the
82 cytoplasm.^{12, 20, 33-40} The Aft1 residues L99, L102, C291 and C293 are necessary for
83 the interaction between Aft1 and the glutaredoxins Grx3/Grx4, which facilitate the
84 iron-dependent dissociation of Aft1 from its target promoters.⁴⁰ The mutagenesis
85 of any of the C191 and C193 residues abrogates Aft1 association to Grx3/Grx4 and
86 the mitochondrial iron signal, rendering Aft1 insensitive to the iron levels.^{35, 40} In
87 this way, the Aft1-1UP (Aft1-C291F) and Aft1-CA (Aft1-C291A/C293A) mutant
88 proteins constitutively activate the iron regulon regardless of the cytosolic and
89 extracellular iron levels, rendering cells highly sensitive to iron.^{40, 41} Furthermore,
90 the mutagenesis of the Aft1 leucine residues L99 and L102 within its nuclear
91 export signal causes constitutive nuclear retention.⁴² Hence, the Aft1-LA (Aft1-
92 L99A/L102A) protein constitutively expresses the iron regulon genes and causes
93 iron sensitivity.^{40, 42}

When present in excess, iron can participate in Fenton type reactions that generate powerful reactive oxygen species (ROS), such as hydroxyl radicals, which ultimately damage cells at the level of lipid peroxidation, protein carbonylation and DNA mutagenesis.⁴³ Thus, iron imbalances underlie multiple human diseases including hereditary hemochromatosis, Friedreich's ataxia, and aceruloplasminemia.⁴⁴ In response to deleterious levels of iron, the yeast high iron-sensing transcription factor Yap5 activates the expression of Ccc1, which imports iron into the vacuole for detoxification and storage.⁴⁵⁻⁴⁷ Unexpectedly, the sphingolipid signaling pathway has been identified as a major mediator of iron toxicity in yeast.⁴⁸ Thus, the inactivation of the sphingolipid signal transduction cascade by deletion of the gene encoding the protein kinases Ypk1 and Pkh1 or the transcription factor Smp1 increases the yeast resistance to high iron conditions.⁴⁸ Additionally, the deletion of *OSH3*, encoding a member of the oxysterol-binding protein family involved in sterol transport between organelles and putatively in sphingolipid homeostasis, also ameliorates growth in high iron conditions through an unknown mechanism.^{49, 50}

In this work, we have studied the consequences of using different constitutively active alleles of the Aft1 transcription factor. In addition to an increase in the endogenous levels of iron, we have observed an enhanced sensitivity to media with high iron content and a decrease in cellular respiration. We test various approaches to counteract these detrimental consequences.

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117 **Experimental procedures**

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119 **Yeast strains, growth conditions and plasmids.** The yeast strains used in this
120 work are listed in Table 1. To delete *AFT1* gene, we generated an *aft1::hphB*
121 cassette as previously described.⁵¹ Liquid yeast precultures were incubated
122 overnight at 30 °C in synthetic complete medium without uracil (SC-ura: 0.17%
123 yeast nitrogen base without amino acids and without ammonium sulfate, 0.5%
124 ammonium sulfate, 2% glucose and Kaiser drop-out supplements without uracil
125 from Formedium), and reinoculated at an optical density at 600 nm (OD₆₀₀) of 0.2.
126 To obtain high iron conditions, ferrous ammonium sulfate (FAS) was added to
127 liquid media at the indicated concentrations. For growth in 96-well plates, yeast
128 cells were inoculated at an OD₆₀₀ of 0.1 in 260 µl of liquid SC-ura medium without
129 or with 10 mM FAS, and the OD₆₀₀ was determined in a Spectrostar Nano
130 absorbance microplate reader (BMGLabtech) every 30 minutes for 3 days at 28 °C.
131 Growth assays in solid media (1.5% agar) were performed as previously
132 described.⁵ Briefly, yeasts were grown to exponential phase, diluted to an OD₆₀₀ of
133 0.1 and spotted directly and after 1:10 and 1:100 dilutions in sterile water.
134 Concentrations from 3.0 to 5.5 mM FeCl₃ were used to obtain solid iron-rich media.
135 YPD (1% yeast extract, 2% peptone, 2% glucose) and YPEG (1% yeast extract, 2%
136 peptone, 2% ethanol, 3% glycerol) media were also used. For the oxidative stress
137 experiments, methylene blue (Sigma) was added to SC-ura plates to a final
138 concentration of 1 mM. Plates were incubated at 30 °C and then photographed. The
139 plasmids used in this work are listed in Table 1.

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141 **RNA analyses.** Total RNA extraction and cellular mRNA levels were determined by
142 reverse transcriptase quantitative PCR (RT-qPCR) as previously described.⁵² The
143 primers used for RT-qPCR are listed in Supplemental Table S1.

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145 **Endogenous iron measurements.** Total intracellular iron levels were determined
146 as previously described.⁵ Briefly, yeast cells that corresponded to a total OD₆₀₀·ml
147 of 5.0 were collected by centrifugation and washed twice with 1 mM EDTA and
148 once with Milli-Q water. Cells were dissolved in 500 µl of 3% HNO₃ and incubated
149 at 98 °C for 16 h with agitation. After digestion, extracts were used to determine
150 iron levels. Iron levels were determined by a previously described colorimetric
151 assay.⁵³ To determine yeast dry weight, the cells that corresponded to a total
152 OD₆₀₀·ml of 30 were collected by centrifugation and washed with distilled water.
153 Then, cells were transferred to a previously weighed Eppendorf tube and were
154 dried at 50 °C for 3 days. Finally, yeast dry weight was obtained by subtracting the
155 Eppendorf tube weight from the total weight. At least two independent biological
156 samples were used.

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158 **Oxygen consumption rates by intact yeast cells.** Oxygen uptake by whole cells
159 was measured for at least 10 min using a Clark-type oxygen electrode (Oxyview
160 system, Hansatech). The cell suspension was placed in an air-tight measuring
161 chamber with continuous stirring. The oxygen consumption rate was expressed as
162 nmol O₂ consumed per minute per OD₆₀₀ (nmol O₂/min·OD₆₀₀) and was taken as an
163 index of the respiratory ability.

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165 **Statistical analyses.** To evaluate the statistical significance, tailed t-student tests
166 were performed. The asterisks indicate statistically significant differences with a
167 p-value indicated in each experiment.

Results

Iron sensitivity of yeast cells expressing different constitutively active *AFT1* alleles.

Previous studies have shown that the *AFT1* alleles *AFT1-1UP* (*AFT1-C291F*), *AFT1-CA* (*AFT1-C291A/C293A*) and *AFT1-LA* (*AFT1-L99A/L102A*) constitutively activate the expression of the iron-regulation, which leads to iron sensitivity.^{15, 20, 40, 42} To dissect the different sensitivity to iron conferred by these *AFT1* alleles, we transformed an *aft1Δ* mutant with plasmids expressing *AFT1*, *AFT1-1UP*, *AFT1-CA* or *AFT1-LA*, and assayed growth in solid and liquid media supplemented with iron. No growth difference was observed when these cells were grown in solid media containing up to 3.0 mM FeCl₃ (Figure 1A). As previously reported,⁴⁰ cells expressing wild-type *AFT1* were able to grow in solid media containing elevated concentrations of iron, whereas this was not the case for any of the hyperactive *AFT1* alleles tested (Figure 1A; 5.5 mM FeCl₃). Moreover, this growth assay allowed distinguishing among the iron sensitivities of the different constitutively active *AFT1* alleles. While *AFT1-LA*-expressing cells still grew on a solid medium with 5.0 mM FeCl₃, no growth was observed for cells transformed with either the *AFT1-1UP* or the *AFT1-CA* allele (Figure 1A). To further study these phenotypes, we assayed growth in liquid media with an alternative iron source, Fe(NH₄)₂(SO₄)₂ (ferrous ammonium sulfate [FAS]). No significant growth differences were observed in the absence of supplemented iron (Figure 1B), whereas all strains displayed a growth delay when 10 mM FAS was added (Figure 1C). Similarly to solid media, cells expressing the *AFT1-1UP* or *AFT1-CA* alleles exhibited a dramatic growth defect in

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3 192 liquid medium supplemented with a high iron concentration, whereas *AFT1-LA*
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5 193 cells showed a mild defect as compared to cells expressing wild-type *AFT1* (Figure
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7 194 1C and 1D). These results indicate that the *AFT1-1UP* and *AFT1-CA* alleles confer a
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9 195 higher sensitivity to iron than the *AFT1-LA* allele. Furthermore, no differences
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11 196 were observed between yeast cells expressing either *AFT1-1UP* or *AFT1-CA*.

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13 198 **Deletion of the sphingolipid signaling kinase *YPK1* rescues the toxicity to**
14 199 **high iron conferred by the hyperactive *AFT1* alleles.**

15 200 A previous study has shown that the deletion of various components of the
16 201 sphingolipid signaling pathway, including the serine/threonine protein kinases
17 202 Ypk1 and Pkh1, and the transcription factor Smp1, increases the resistance of wild-
18 203 type yeast to media with high iron levels.⁴⁸ To rescue the iron toxicity of yeast cells
19 204 expressing the constitutive *AFT1* alleles, we deleted *AFT1* gene in a *ypk1Δ*
20 205 background to obtain the *aft1Δypk1Δ* strain. Then we transformed both *aft1Δ* and
21 206 *aft1Δypk1Δ* strains with plasmids expressing either *AFT1* or *AFT1-1UP*, and
22 207 assayed growth in solid media with high iron concentrations (Figure 2). All yeast
23 208 transformants were able to grow up to 3 mM FeCl₃. As previously shown (Figure
24 209 1A), *aft1Δ* cells transformed with the *AFT1-1UP* plasmid were sensitive to iron
25 210 concentrations higher than 3 mM, and no growth was observed between 4 mM and
26 211 5.5 mM FeCl₃ (Figure 2). Importantly, deletion of *YPK1* enhanced the resistance of
27 212 *AFT1-1UP*-expressing cells up to 5 mM FeCl₃ (Figure 2). The most resistant cells
28 213 lacked *YPK1* and expressed *AFT1* (Figure 2; *aft1Δypk1Δ* + *AFT1*). Identical results
29 214 were obtained when the *AFT1-CA* allele was used instead of the *AFT1-1UP*
30 215 (Supplemental Figure S1). We also tested the effect of deleting *YPK1* in cells

transformed with an *AFT1-LA* plasmid. As we have previously observed (Figure 1), the *aft1Δ* cells that expressed *AFT1-LA* were more resistant to iron than the *AFT1-1UP* and *AFT1-CA* containing cells, growing up to 4 mM FeCl₃ (Supplemental Figure S2). Again, deletion of *YPK1* enhanced the resistance, in this case, of *AFT1-LA*-expressing yeast to high iron concentrations, leading to cells that were even more tolerant than wild-type cells (Supplemental Figure S2). Finally, we challenged the resistance of *AFT1-1UP*-expressing cells to iron in liquid medium. As shown in Figure 3A and C, all yeast transformants grew similarly in normal conditions (Scuria). However, the addition of 10 mM FAS decreased the growth rate of all the strains, but especially that of the *aft1Δ* mutant expressing *AFT1-1UP*, which barely grew (Figure 3B-C). Importantly, the deletion of *YPK1* reverted the severe iron sensitivity of *AFT1-1UP* to that of the wild-type cells (Figure 3B-C). Altogether these results demonstrate that the deletion of *YPK1* rescues the iron sensitivity conferred by the constitutively active *AFT1* alleles.

The accumulation of elevated levels of iron generates reactive oxygen species (ROS) that damage lipids, proteins and nucleic acids. To explore how *YPK1* deletion affected the cellular redox state of *AFT1-1UP*-expressing cells, we assayed yeast growth in media that contained the biocompatible redox indicator methylene blue, which loses its blue color when reduced.⁵⁴ We observed that *AFT1-1UP* cells acquired a dark blue color, which is indicative of methylene blue oxidation (Figure 3D). Importantly, *YPK1* deletion decreased the blue coloration displayed by *AFT1-1UP* cells (Figure 3D). These results suggest that the oxidative stress associated with the *AFT1-1UP* allele is reduced in *ypk1Δ* cells.

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Iron accumulated by yeast cells expressing *AFT1-1UP* and lacking *YPK1*.

Previous studies have shown that *AFT1-1UP* cells accumulate high levels of endogenous iron, which favor the oxidative damage of cellular components⁵⁵. Therefore, we decided to explore whether the *YPK1*-mediated rescue of *AFT1-1UP* sensitivity to high iron was due to a decrease in iron uptake. For this purpose, we cultivated *aft1Δ* and *aft1Δypk1Δ* cells expressing either *AFT1* or *AFT1-1UP* in liquid media with two different iron concentrations, and then determined the endogenous iron levels. When yeast cells were grown in iron-sufficient conditions (100 μM FAS), which did not alter growth, we observed that the *AFT1-1UP* cells accumulated up to 4-fold more endogenous iron than *AFT1*-expressing cells (Figure 4). Interestingly, the deletion of *YPK1* did not fully abolish the iron accumulation of *AFT1-1UP* cells, but decreased it to 2.5-fold (Figure 4). At high iron levels (6 mM FAS), only the cells expressing both *AFT1-1UP* and *YPK1* were able to accumulate a slightly higher amount of endogenous iron (Figure 4), although we should consider that these cells hardly grew (Figure 3B-C). In this case, deletion of *YPK1* reduced *AFT1-1UP* endogenous iron to the levels of wild-type cells (Figure 4, 6 mM FAS). These results indicate that, when the iron present in the growth medium is not cytotoxic (100 μM), the deletion of *YPK1* still permits yeast cells to tolerate the increased iron accumulation provided by the *AFT1-1UP* allele. However, at elevated iron concentrations (6 mM FAS), the removal of *YPK1* gene rescues *AFT1-1UP* toxicity by diminishing iron accumulation.

The deletion of *OSH3* rescues the *AFT1-1UP* toxicity to high iron by decreasing iron accumulation.

A systematic screen using yeast deletion mutants identified *OSH3*, which encodes an oxysterol-binding protein, to be involved in the toxic response to high iron and copper.⁴⁹ Specifically, their data indicated that *osh3Δ* cells displayed the best fitness score in both high-iron and high-copper media.⁴⁹ Therefore, we decided to assay whether *OSH3* deletion rescued the toxicity to elevated levels of iron conferred by the *AFT1-1UP* allele. We deleted the *AFT1* gene in *osh3Δ* cells to obtain the *aft1Δosh3Δ* double mutant, and then we transformed both *aft1Δ* and *aft1Δosh3Δ* yeast strains with plasmids expressing either *AFT1* or *AFT1-1UP*. Again, *aft1Δ* cells expressing *AFT1-1UP* were highly sensitive to excess iron (Figure 5A). Importantly, *OSH3* deletion increased the resistance of *AFT1-1UP* cells up to 5 mM FeCl₃ (Figure 5A). Next, we used the biocompatible redox indicator methylene blue to determine the effect of *OSH3* on the cellular redox state. As shown in Figure 5B, *aft1Δ* cells expressing *AFT1-1UP* acquired a blue coloration when grown in 3 mM FeCl₃, which is characteristic of methylene blue oxidation. Deletion of *OSH3* decreased the blue staining of *AFT1-1UP* cells, which indicates a reduction in oxidative stress.

We also studied the effect of *OSH3* deletion on yeast iron accumulation. We cultivated *aft1Δ* and *aft1Δosh3Δ* cells expressing either *AFT1* or *AFT1-1UP* in liquid media with iron and determined the endogenous iron levels. Under iron-sufficient conditions, *AFT1-1UP*-expressing cells accumulated more iron than *AFT1* cells (Figure 6; 100 μM FAS). However, *OSH3* deletion abolished *AFT1-1UP* iron accumulation (Figure 6; 100 μM FAS). At high iron levels (6 mM FAS), only the cells expressing both *AFT1-1UP* and *OSH3* were able to accumulate a higher amount of endogenous iron (Figure 6). The removal of *OSH3* diminished the accumulation of

iron observed in *AFT1-1UP* cells (Figure 6). Altogether these results indicate that the deletion of *OSH3* gene rescues the *AFT1-1UP* toxicity to high iron by decreasing iron accumulation and oxidative stress.

The deletion of *FET3* but not *FRE6* partially rescues the iron toxicity displayed by the constitutively active *AFT1* alleles.

In response to iron deprivation, Aft1 activates the expression of a set of genes denoted the iron regulon, which includes *FET3* and *FTR1*, two components of the yeast high-affinity iron transport system at the plasma membrane.^{20, 56, 57} To address whether the toxicity conferred by the different *AFT1* alleles was due to excessive iron acquisition, we decided to impair the cell surface high-affinity iron uptake by deleting the *FET3* multicopper ferroxidase gene. We observed that the expression of the *AFT1-1UP* and *AFT1-CA* alleles caused a slight growth defect at 3.5 mM FeCl₃ that was recovered by *FET3* deletion (Figure 7). However, *FET3* removal was unable to rescue the *AFT1-1UP* and *AFT1-CA*-dependent toxicity at higher iron concentrations (Figure 7). A partial rescue of the toxicity provided by the *AFT1-LA* allele was also observed at 4 and 4.5 mM FeCl₃ (Figure 7). These results indicate that the detrimental effect of the constitutively active *AFT1* alleles in high iron media is only partially dependent on the iron acquired by the high-affinity transport system.

In response to iron depletion, yeast cells activate the remobilization of iron from the vacuole, which functions as an iron detoxification and storage organelle. This process is enhanced by Aft1, which activates the transcription of *FTH1/FET5* and *SMF3*, which encode for the high- and low-affinity iron vacuolar exporters,

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3 312 respectively.⁵⁸⁻⁶⁰ Both iron exporters require the previous participation of the
4 313 vacuolar Fre6 metalloredutase, which expression is partially regulated by Aft1.^{21,}
5 314 ^{22, 61} To determine whether the iron toxicity caused by the constitutively active
6 315 *AFT1* alleles was due to an increased vacuolar iron export, we deleted the *FRE6*
7 316 vacuolar metalloredutase gene. However, the deletion of *FRE6* did not alter the
8 317 growth defect in high iron media observed for the yeast cells expressing the
9 318 hyperactive *AFT1* alleles (Supplemental Figure S3). These results suggest that the
10 319 iron toxicity caused by the constitutive activation of the iron regulon is not due to
11 320 vacuolar iron remobilization.
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322 **The *AFT1-1UP* allele promotes a Cth2-dependent repression of respiration.**

13 323 An additional Aft1 target that is activated upon iron scarcity is *CTH2*, an
14 324 RNA-binding protein that promotes the degradation of many transcripts encoding
15 325 iron-dependent proteins or participating in metabolic pathways that rely on iron
16 326 as an essential cofactor.^{25, 30} Therefore, the activation of *CTH2* expression in
17 327 response to iron deficiency coordinates a metabolic reorganization headed to
18 328 spare iron consumption in indispensable processes. As shown in Figure 8A, the
19 329 limitation of iron availability achieved by the addition of 100 μ M BPS for 6 hours
20 330 increased 14-fold the expression of *CTH2*. Consequently, Cth2 activation promoted
21 331 the down-regulation of mRNAs including *SDH4* (a subunit of succinate
22 332 dehydrogenase), *HEM15* (ferrochelatae) and *WTM1* (an inhibitor of
23 333 ribonucleotide reductase) (Figure 8A).^{25, 31, 62, 63} We observed that the hyperactive
24 334 *AFT1-1UP* allele led to a 6-fold increase in *CTH2* mRNA levels under iron-sufficient
25 335 conditions (Figure 8A). The *CTH2* overexpression promoted by the *AFT1-1UP* allele

336 was sufficient to down-regulate the Cth2 mRNA targets *SDH4*, *HEM15* and *WTM1*
337 (Figure 8A). This result indicates that the *AFT1-1UP* allele promotes a Cth2-
338 mediated metabolic remodeling of iron-dependent processes.

339 The mitochondrial electron transport chain is the main iron-dependent
340 process inhibited by Cth2 in response to iron deprivation.²⁵ Since *AFT1-1UP*
341 activates *CTH2* expression and the down-regulation of Cth2 target mRNAs (Figure
342 8A), we decided to investigate the effect of *AFT1-1UP* on cellular respiration. For
343 this purpose, we grew yeast cells expressing *AFT1* and *AFT1-1UP* in media
344 containing the non-fermentable glycerol and ethanol as the only carbon sources.
345 As shown in Figure 8B, the expression of the *AFT1-1UP* allele notably decreased
346 yeast growth in the ethanol-glycerol medium. Importantly, the deletion of *CTH2*
347 partially rescued the growth of *AFT1-1UP*-expressing cells in ethanol-glycerol. To
348 further explore this observation, we decided to measure respiration by
349 determining the oxygen consumption rate of these strains. Consistent with the
350 growth assays in ethanol-glycerol, the expression of *AFT1-1UP* decreased the
351 oxygen consumption rate, which was partially recovered by the deletion of *CTH2*
352 (Figure 8C). Collectively, these results strongly suggest that *AFT1-1UP* promotes
353 the repression of respiration via Cth2.

Discussion

Yeast iron acquisition requires the coordinated expression of multiple proteins: cell surface metalloredutases (Fre1-6), high-affinity iron transport complexes (Fet3/Ftr1), low-affinity dimetal transporters (Fet4), iron siderophore transporters (Arn1-4), and cell wall manoproteins (Fit1-3).^{7, 10} The genes encoding all these proteins are simultaneously regulated by the iron-sensing transcription factor Aft1, which is only active under iron-deficient conditions.^{20-29, 66} Interestingly, various *AFT1* alleles that do not sense iron and constitutively activate the iron regulon have been identified.^{15, 20, 40, 42} These hyperactive *AFT1* alleles could be potentially used to rapidly increase iron uptake. However, apart from resulting in poor growth in media with elevated concentrations of iron, little was known about their physiological effect. Here, we have studied the consequences of expressing different constitutively active *AFT1* alleles. We have tested two different types of *AFT1* alleles: (i) *AFT1-1UP* and *AFT1-CA*, with mutations at the cysteine residues C291 and C293, which directly bind the iron-sulfur cluster coming from mitochondria, and (ii) *AFT1-LA*, with mutations at the leucine residues within the Aft1 nuclear export signal.^{35, 40, 42} Interestingly, we observed that the cells expressing the *AFT1-1UP* and *AFT1-CA* alleles exhibited a higher sensitivity to iron than those containing the *AFT1-LA* allele (Figure 1). Although both the Aft1-CA and Aft1-LA proteins constitutively localize to the nucleus, chromatin immunoprecipitation experiments suggested that the Aft1-LA protein was partially released from the *FET3* promoter upon iron repletion, while Aft1-CA remained bound ⁴⁰. This is consistent with the Aft1-CA protein being fully insensitive to the mitochondrial iron-sulfur cluster signal, while the Aft1-LA mutant would still bind the cluster and partially dissociate from the DNA. Other

379 *AFT1* alleles such as *AFT1-SA* (AFT1-S210A/S224A) and *AFT1-TA* (AFT1-
380 T421A/T423A/T431A/T435A) localize to the nucleus independently of the iron
381 levels, but were not included in this study because, upon iron supplementation,
382 they are fully released from the promoters shutting down the expression of the
383 iron regulon.⁴⁰

384 Previous studies have reported that a constitutively active *AFT1* allele such
385 as *AFT1-1UP* leads to a cell cycle arrest in high iron media due to an enhanced
386 high-affinity iron uptake.⁴¹ Consistent with these observations, the deletion of the
387 multicopper ferroxidase *FET3*, which is involved in high-affinity iron acquisition,
388 but not the *FRE6* metalloredutase, implicated in vacuolar iron mobilization,
389 partially recovered the growth of cells expressing *AFT1-UP*, *AFT1-CA* or *AFT1-LA*
390 (Figure 7 and Supplementary Figure S3). Various reports have also rescued yeast
391 iron toxicity through the impairment of sphingolipid synthesis and signaling.^{48, 49}
392 In one case, both the inhibition of sphingolipid biosynthesis by either
393 overexpression of its negative regulator *ORM2* or treatment with myriocin, and the
394 impairment of the sphingolipid signaling pathway by *PKH1*, *YPK1* or *SMP1* deletion
395 provide resistance to high iron media.⁴⁸ Interestingly, this study suggested that the
396 overexpression of *ORM2* suppressed iron toxicity without reducing cellular iron
397 uptake.⁴⁸ Taken this background into account, we decided to explore whether the
398 deletion of the sphingolipid-activated kinase *YPK1* was able to rescue the toxicity
399 to high iron caused by the constitutively active *AFT1* alleles. Indeed, *YPK1* deletion
400 rescued the iron sensitivity due to all the hyperactive *AFT1* alleles tested (Figures
401 2 and 3; Supplemental Figures S1 and S2). However, when iron accumulation was
402 determined, we observed a different effect of *ypk1Δ* on iron accumulation
403 depending on the levels of metal in the medium (Figure 4). The deletion of *YPK1* in

404 *AFT1-UP*-expressing cells still permitted a significant increase in iron accumulation
405 in iron-sufficient (100 μ M FAS), but not in high iron (6 mM FAS) conditions. Given
406 that iron could be entering yeast cells through different mechanisms at iron-
407 sufficient and high iron conditions,⁶⁷ these results suggest that the lack of *YPK1*
408 could be influencing these processes in a different manner. In the case of deleting
409 the *OSH3* gene, which encodes an oxysterol-binding protein, iron resistance was
410 achieved by decreasing iron accumulation (Figure 6). In both cases, assays with the
411 redox indicator methylene blue indicated that the deletion of either *YPK1* or *OSH3*
412 reduced the oxidative stress sensed by the *AFT1-1UP* expressing cells (Figures 3
413 and 5). Related to these observations, we have recently explored the resistance of
414 many wild and domestic strains of *S. cerevisiae* isolated from different
415 geographical origins and sources.⁵ We obtained yeast strains particularly resistant
416 or sensitive to high iron levels (in the mM range), both in solid and liquid media.
417 We observed that the strains that were resistant to high iron media accumulated
418 less intracellular iron than the iron-sensitive strains. The drop observed in
419 intracellular iron correlated with a decrease in oxidative stress⁵. The biophysical
420 characterization of the iron accumulated by yeast cells renders similar
421 conclusions.^{55, 67, 68} These analyses show that great changes in environmental iron
422 (up to 250-fold) only lead to minor increases in endogenous iron.⁶⁷ These results
423 suggest that, when extracellular iron levels increase, yeast cells limit iron import as
424 a common strategy to prevent toxicity. Although the underlying mechanism is
425 unknown, a potential explanation could involve alterations in sphingolipid and
426 sterol synthesis and signaling. Interestingly, the yeast Osh proteins are involved in
427 the transport of sterols between organelles and in sphingolipid homeostasis.^{50, 69}

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428 These findings highlight the significance of an adequate balance in the lipid
429 composition of the cell membranes to appropriately respond to an iron excess.

430 In response to iron deficiency, the Aft1 transcription factor activates the
431 expression of Cth2, which promotes the degradation of many mRNAs encoding
432 components of the mitochondrial electron transport chain (Figure 8).²⁵ Although
433 to a minor extent, we observed that cells expressing the constitutive allele *AFT1-1UP*
434 also activated *CTH2* and down-regulated genes important for respiration, such
435 as *SDH4*, under iron-sufficient conditions (Figure 8). By measuring growth in
436 media with non-fermentable carbon sources (ethanol and glycerol) and oxygen
437 consumption, we could conclude that the expression of the *AFT1-1UP* allele
438 represses respiration (Figure 8). The ability to respire is an important property for
439 any yeast strain to be efficiently produced. The industrial production of yeast
440 biomass includes a fed-batch phase in low sucrose conditions that forces the
441 respiratory utilization of the carbon to ensure the highest biomass yields.
442 Therefore, to rescue the respiratory capacity of *AFT1-1UP* cells, we decided to
443 delete *CTH2*. Importantly, the deletion of *CTH2* improved the growth in
444 ethanol/glycerol and the oxygen consumption exhibited by the *AFT1-1UP* cells
445 (Figure 8). These results demonstrate that the repression of respiration displayed
446 by *AFT1-1UP*-expressing cells is due to the up-regulation of *CTH2*, and indicate that
447 any strategy including constitutively active *AFT1* alleles should consider the
448 respiratory inhibition effect produced by Cth2 protein.

449 Iron bioavailability can greatly vary depending on the source of iron
450 (inorganic, heme, ferritin) and the components of the diet, which could to a greater
451 or lesser extent influence iron solubility and intestinal absorption.⁷ Exponentially
452 growing yeast cells contain the majority of its iron in mitochondria and vacuoles,

although the total amount and species can change according to the yeast genotype and the growth conditions. However, post-exponential and metabolically inactive cells accumulate ferric iron in the cell wall.⁷⁰ Biophysical analyses indicate that iron-deficient yeasts contain little iron stored in vacuoles.⁶⁷ Instead, most iron in deprived cells is equally distributed between mitochondria (in the form of Fe-S clusters and heme) and non-heme high-spin Fe²⁺ species.⁶⁷ Under iron-sufficient conditions, yeast cells accumulate vacuolar non-heme high-spin Fe³⁺ species and mitochondrial Fe³⁺ nanoparticles.⁶⁷ The speciation of iron in wild-type cells grown under high iron conditions is similar to the distribution observed under iron sufficiency, except that vacuoles are overloaded with iron.⁶⁷ *AFT1-1UP* cells grown in low iron media accumulate mononuclear high-spin Fe³⁺ ions in the vacuole as compared to wild-type cells.⁵⁵ However, when *AFT1-1UP* cells are cultivated in high iron media, additional iron accumulates as vacuolar high-spin Fe³⁺ species and mitochondrial Fe³⁺ phosphate nanoparticles, a subcellular distribution and speciation similar to wild-type cells overloaded with iron.⁵⁵ Although the supply of iron-enriched yeasts have been proved to recover animals from iron deficiency,³ it would be of interest to further explore how the form of iron present in baker's yeast, as well as the presence of potential enhancers or inhibitors of iron absorption, influence its efficient utilization as an iron supplement.⁶

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473 Conclusions

474 The expression in baker's yeast of constitutively active *AFT1* alleles, such as
475 *AFT1-1UP*, leads to the accumulation of intracellular iron. This molecular strategy
476 could be considered in order to obtain iron-enriched yeasts. However, in the

current study we have observed that the hyperactive *AFT1* alleles also cause iron sensitivity probably due to the oxidative stress produced by the high levels of endogenous iron achieved. These detrimental effects can be rescued to some extent by deleting the *YPK1* sphingolipid kinase suggesting the existence of connections between iron and lipid metabolism, which should be explored. Furthermore, we showed that the *AFT1-1UP* allele decreases the consumption of oxygen in a process mediated by the Cth2 mRNA-binding factor, which down-regulates multiple mRNAs implicated in mitochondrial electron transport.²³ In conclusion, the constitutive activation of the yeast iron regulon not only increases iron acquisition but provokes oxidative stress and alters cellular pathways including mitochondrial respiration and lipid metabolism. Therefore, further studies would contribute to fully understand the multiple effects of activating the yeast Aft1 transcription factor and of accumulating high levels of intracellular iron.

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491 **Conflicts of interest**

492 The authors declare no conflicts of interest.

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494 **Acknowledgements**

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Figure legends

Figure 1. Sensitivity to high iron media of yeast cells expressing different hyperactive *AFT1* alleles. The *aft1* Δ (SPY28) yeast strain was transformed with pRS416-AFT1-12HA (*AFT1*), pRS416-AFT1-C291F-12HA (*AFT1-1UP*), pRS416-AFT1-C291A/C293A-12HA (*AFT1-CA*) and pRS416-AFT1-L99A/L102A-12HA (*AFT1-LA*) plasmids. (A) Yeast transformants were cultivated to the exponential phase and then spotted in 10-fold serial dilutions on SC-ura and SC-ura supplemented with 3.0 to 5.5 mM FeCl₃. One representative plate of two biological replicates is shown. (B and C) Yeast transformants were inoculated at an OD₆₀₀ of 0.15 in liquid SC-ura medium supplemented or not with 10 mM FAS for 3 days at 28 °C, and then the OD₆₀₀ was determined every 30 minutes with a Spectrostar Nano absorbance 96-plate reader. The average curve of 2-4 biologically independent assays is shown. (D) The maximal rate growth (represented as doubling time) at the exponential phase is shown for the growth assays performed in panels B and C. Data represent the average and standard deviation of 2-4 biologically independent experiments (n=4 for *AFT1* and *AFT1-1UP* in 10 mM FAS; n=3 for all SC-ura samples; n=2 for *AFT1-CA* and *AFT1-LA* in 10 mM FAS). The asterisk (*) indicates statistically significant differences (p-value < 0.02) between cells growth in SC-ura and 10 mM FAS. The double asterisk (**) also indicates statistically significant differences (p-value < 0.05) between the *aft1* Δ + *AFT1* cells and the rest of yeast cells grown in 10 mM FAS.

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Figure 2. The deletion of *YPK1* rescues the sensitivity of *AFT1-1UP*-expressing cells to high iron in solid media. The *aft1Δ* (SPY28) and *aft1Δypk1Δ* (SPY602) yeast strains transformed with pRS416-AFT1-12HA (*AFT1*) and pRS416-AFT1-C291F-12HA (*AFT1-1UP*) plasmids were cultivated to the exponential phase and then spotted in 10-fold serial dilutions on SC-ura and SC-ura supplemented with 3.0 to 5.5 mM FeCl₃. One representative plate of two biological replicates is shown.

Figure 3. Effect of *YPK1* deletion on iron sensitivity and oxidative stress. The *aft1Δ* (SPY28) and *aft1Δypk1Δ* (SPY602) yeast strains transformed with pRS416-AFT1-12HA (*AFT1*) and pRS416-AFT1-C291F-12HA (*AFT1-1UP*) plasmids were cultivated to the exponential phase and then inoculated at an OD₆₀₀ of 0.2 in liquid SC-ura medium without (A) or supplemented with 10 mM Fe(NH₄)₂(SO₄)₂ or FAS (B) for 3 days at 28 °C, and then the OD₆₀₀ was determined every 30 minutes with a Spectrostar Nano absorbance 96-plate reader. The average curve of 3-4 biologically independent assays is shown. (C) The maximal rate growth (represented as doubling time) at the exponential phase is shown for the growth assays performed in panels A and B. Data represent the average and standard deviation of 3-4 biologically independent experiments (n=4 for *aft1Δ* + *AFT1-1UP* in SC-ura, and *aft1Δ* + *AFT1* and *aft1Δypk1Δ* + *AFT1* in 10 mM FAS; n=3 for the rest of samples). The asterisk (*) indicates statistically significant differences (p-value < 0.04) between cells growth in SC-ura and 10 mM FAS. The double asterisk (**) also indicates statistically significant differences (p-value < 0.02) between the *aft1Δ* + *AFT1-1UP* cells and the rest of yeast cells grown in 10 mM FAS. (D) The yeast

transformants used in Figure 2 were grown on SC-ura plates containing 3.5 mM FeCl₃ and 1 mM of the redox state indicator methylene blue.

Figure 4. Endogenous iron accumulation of *ypk1Δ* cells expressing *AFT1-1UP*.

The yeast transformants used in Figure 2 were cultivated for 24 hours in SC-ura supplemented with 0.1 or 6 mM Fe(NH₄)₂(SO₄)₂ or FAS. Then, cells were collected and digested, and endogenous iron was determined as indicated in the Experimental procedures section. Data and error bars represent the average and standard deviations of various independent biological samples (n=4 for *aft1Δ* + *AFT1* in 0.1 and 6 mM, and *aft1Δypk1Δ* + *AFT1* in 6 mM; n=3 for *aft1Δ* + *AFT1-1UP* in 0.1 mM, and *aft1Δypk1Δ* + *AFT1-1UP* in 6 mM; n=2 for the rest of samples). The asterisk (*) indicates statistically significant differences (p-value < 0.02) from the corresponding *aft1Δ* + *AFT1* cells.

Figure 5. The deletion of *OSH3* rescues the sensitivity of *AFT1-1UP*-

expressing cells to high iron. The *aft1Δ* (SPY28) and *aft1Δypk1Δ* (SPY602) yeast strains transformed with pRS416-AFT1-12HA (*AFT1*) and pRS416-AFT1-C291F-12HA (*AFT1-1UP*) plasmids were cultivated to the exponential phase and then spotted in 10-fold serial dilutions on SC-ura and SC-ura supplemented with 3.0 to 5.5 mM FeCl₃ without (A) or with (B) 1 mM of the redox state indicator methylene blue. One representative plate of two biological replicates is shown.

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800 **Figure 6. Endogenous iron accumulation of *osh3Δ* cells expressing *AFT1-1UP*.**

801 The yeast transformants used in Figure 5 were cultivated in SC-ura supplemented
802 with 0.1 or 6 mM FAS, and iron determined as indicated in the Experimental
803 procedures section. Data and error bars indicate the average and standard
804 deviations of independent biological samples (n=4 for *aft1Δ* + *AFT1* and *aft1Δ* +
805 *AFT1-1UP* in 6 mM; and n=2 for the rest of samples). The asterisk (*) represents
806 statistically significant differences (p-value < 0.01) from the corresponding *aft1Δ* +
807 *AFT1* cells.

808

809 **Figure 7. The deletion of *FET3* partially rescues the sensitivity of *AFT1-1UP*-**

810 **expressing cells to high iron.** The *aft1Δ* (SPY28) and *aft1Δfet3Δ* (SPY605) yeast
811 strains transformed with pRS416-AFT1-12HA (*AFT1*), pRS416-AFT1-C291F-12HA
812 (*AFT1-1UP*), pRS416-AFT1-C291A/C293A-12HA (*AFT1-CA*) and pRS416-AFT1-
813 L99A/L102A-12HA (*AFT1-LA*) plasmids were cultivated to the exponential phase
814 and then spotted in 10-fold serial dilutions on SC-ura and SC-ura supplemented
815 with 3.5 to 4.5 mM FeCl₃. One representative plate of two biological replicates is
816 shown.

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818 **Figure 8. The *AFT1-1UP* allele represses respiration in a *CTH2*-dependent**

819 **manner** (A) Expression of *AFT1-1UP* up-regulates *CTH2* and down-regulates
820 various Cth2 mRNA targets. The *aft1Δ* (SPY28) yeast cells transformed with the
821 pRS416-AFT1-12HA (*AFT1*) and pRS416-AFT1-C291F-12HA (*AFT1-1UP*) were
822 cultivated for 6 hours in SC-ura and SC-ura + 100 μM of the Fe²⁺ chelator BPS to
823 exponential phase. Then, total RNA was extracted and the *CTH2*, *SDH4*, *HEM15* and

824 *WTM1* mRNA levels were determined by RT-qPCR as described in the
825 Experimental procedures section. *ACT1* transcript levels were used as a control.
826 (B) Deletion of *CTH2* partially recovers the growth defect of *AFT1-1UP*-expressing
827 cells in non-fermentable carbon sources. The *aft1Δ* (SPY28) and *aft1Δcth2Δ*
828 (SPY972) yeast cells transformed with the pRS416-AFT1-12HA (*AFT1*) and
829 pRS416-AFT1-C291F-12HA (*AFT1-1UP*) were cultivated to exponential phase, and
830 spotted in 10-fold serial dilutions on YPD (Glucose) and YPEG (Ethanol-glycerol)
831 media. (C) Deletion of *CTH2* partially rescues the oxygen consumption defect of
832 *AFT1-1UP*-expressing cells. The yeast transformants used in panel B were grown
833 for 6 hours in SC-ura, and then used to determine oxygen consumption. The
834 average and standard deviations from at least two independent biological samples
835 is shown. The asterisk (*) represents statistically significant differences (p-value <
836 0.03).

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837 **Supplemental Figure legends**

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839 **Supplemental Figure S1. The deletion of *YPK1* rescues the sensitivity of *AFT1*-**
840 ***CA*-expressing cells to high iron.** The *aft1Δ* (SPY28) and *aft1Δypk1Δ* (SPY602)
841 yeast strains transformed with pRS416-AFT1-12HA (*AFT1*) and pRS416-AFT1-
842 C291A/C293A-12HA (*AFT1-CA*) plasmids were cultivated to the exponential phase
843 and then spotted in 10-fold serial dilutions on SC-ura and SC-ura supplemented
844 with 3.0 to 5.5 mM FeCl₃. Plates were incubated at 30 °C and photographed.

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846 **Supplemental Figure S2. The deletion of *YPK1* rescues the sensitivity of *AFT1*-**
847 ***LA*-expressing cells to high iron.** The *aft1Δ* (SPY28) and *aft1Δypk1Δ* (SPY602)
848 yeast strains transformed with pRS416-AFT1-12HA (*AFT1*) and pRS416-AFT1-
849 L99A/L102A-12HA (*AFT1-LA*) plasmids were cultivated to the exponential phase
850 and then spotted in 10-fold serial dilutions on SC-ura and SC-ura supplemented
851 with 3.0 to 5.0 mM FeCl₃. Plates were incubated at 30 °C and photographed.

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853 **Supplemental Figure S3. The deletion of *FRE6* does not rescue the sensitivity**
854 **of *AFT1-1UP*-expressing cells to high iron.** The *aft1Δ* (SPY28) and *aft1Δfre6Δ*
855 (SPY607) yeast strains transformed with pRS416-AFT1-12HA (*AFT1*), pRS416-
856 AFT1-C291F-12HA (*AFT1-1UP*), pRS416-AFT1-C291A/C293A-12HA (*AFT1-CA*)
857 and pRS416-AFT1-L99A/L102A-12HA (*AFT1-LA*) plasmids were cultivated to the
858 exponential phase and then spotted in 10-fold serial dilutions on SC-ura and SC-
859 ura supplemented with 3.5 to 4.5 mM FeCl₃. Plates were incubated at 30 °C and
860 photographed.

861 **Table 1. List of yeast strains and plasmids used in this study.**

Strain or plasmid	Description	Source/Reference
<u><i>Yeast strains</i></u>		
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Research Genetics
SPY28	BY4741 <i>aft1::KanMX4</i>	Research Genetics
SPY600	BY4741 <i>ypk1::KanMX4</i>	Research Genetics
SPY602	BY4741 <i>ypk1::KanMX4 aft1::hphMX4</i>	This study
SPY753	BY4741 <i>osh3::KanMX4</i>	Research Genetics
SPY903	BY4741 <i>osh3::KanMX4 aft1::hphMX4</i>	This study
SPY23	BY4741 <i>fet3::KanMX4</i>	Research Genetics
SPY605	BY4741 <i>fet3::KanMX4 aft1::hphMX4</i>	This study
SPY25	BY4741 <i>cth2::KanMX4</i>	Research Genetics
SPY904	BY4741 <i>cth2::KanMX4 aft1::hphMX4</i>	This study
SPY598	BY4741 <i>fre6::KanMX4</i>	Research Genetics
SPY607	BY4741 <i>fre6::KanMX4 aft1::hphMX4</i>	This study
<u><i>Plasmids</i></u>		
pAFT1	pRS416-AFT1-12HA	71
pAFT1-UP	pRS416-AFT1-C291F-12HA	40
pAFT1-CA	pRS416-AFT1-C291A/C293A-12HA	40
pAFT1-LA	pRS416-AFT1-L99A/L102A-12HA	40
pAG32	pFA6-hphMX4	51

862

863 **Supplemental Table S1. Oligonucleotides used in this study**

Name	Sequence (from 5' to 3')
CTH2-qPCR-F	GCAGTTTCATTCTCTCCAC
CTH2-qPCR-R	TAGGTGCCGTGCTATTCAGG
SDH4-qPCR-F	GCACTCCCAATGATGCCTAC
SDH4-qPCR-R	AATGGAACGACGGACAAGG
HEM15-qPCR-F	CCAAAGTTGATGGCCTAATG
HEM15-qPCR-R	TATTCCGATTCCCCAATGAC
WTM1-qPCR-F	TCCTGACGATACCATTGCTC
WTM1-qPCR-R	TCTTCTGCTTCCACCCTTGT
ACT1-qPCR-F	TCGTTCCAATTTACGCTGGTT
ACT1-qPCR-R	CGGCCAAATCGATTCTCAA
AFT1-50-hph-F	GACGATTCATGCTATACCGTAGGTTTTTGATACAAGTCTACAGCTGAAGCTTCGTACGC
AFT1-hph-R	ATGGACGAGAGATACGTCTAAGTTTGATTTCATCTATATGTAGGCCACTAGTGGATCTG
AFT1-200F	TCGGTGTCATTGACAAACCT
TEFpromR	GGGCGACAGTCACATCAT

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Figure 1

Fig 1

190x254mm (300 x 300 DPI)

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Figure 8