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Iron Sensing and Regulation in *Saccharomyces cerevisiae*: Ironing Out the Mechanistic Details

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Abstract

Regulation of iron metabolism in *Saccharomyces cerevisiae* is achieved at the transcriptional level by low (Aft1 and Aft2) and high iron-sensing (Yap5) transcription factors, and at the post-transcriptional level by mRNA-binding proteins (Cth1 and Cth2). In this review we highlight recent studies unveiling the critical role that iron-sulfur clusters play in control of Aft1/2 and Yap5 activity, as well as the complex relationship between iron homeostasis and thiol redox metabolism. In addition, new insights into the localization and regulation of Cth1/Cth2 have added another layer of complexity to the cell's adaptation to iron deficiency. Finally, biophysical studies on subcellular iron speciation changes in response to environmental and genetic factors have further illuminated the elaborate control mechanisms required to manage iron bioavailability in the cell.

Introduction

The regulation of iron metabolism is a critical function for eukaryotic cells given the diverse and essential roles played by this redox-active protein cofactor. As a genetically-tractable, single-celled eukaryote, the budding yeast *Saccharomyces cerevisiae* provides an excellent model to investigate the cellular and molecular mechanisms of iron sensing and regulation. Recent studies in *S. cerevisiae* continue to tease out the intricate control mechanisms at the transcriptional, post-transcriptional, and post-translational levels that maintain adequate levels of this essential nutrient with changing environmental conditions.

The response to iron deficiency in *S. cerevisiae* is primarily controlled by the low iron-sensing transcriptional activator Aft1 and its paralogue Aft2, which have overlapping and separate DNA targets [1-4]. The list of Aft1- and Aft2-regulated genes includes proteins required for ionic iron and iron-siderophore transport at the plasma membrane, vacuolar iron transport, and mitochondrial iron metabolism [5]. The net result of activation of these genes is the mobilization of iron from extracellular sources and intracellular stores. Although much is known about Aft1/2 target genes and the mechanisms for iron uptake and storage in *S. cerevisiae*, less is known about the molecular mechanisms for controlling Aft1/2 activity and the specific iron signal to which Aft1 and Aft2 respond. We will focus on recent developments that help answer these open questions as well as new studies that directly link thiol redox metabolism with iron sensing and regulation.

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In addition to stimulation of iron uptake and mobilization, Aft1 and Aft2 also activate expression of Cth1 and Cth2, paralogous mRNA-binding proteins that facilitate decay of mRNAs encoding proteins that function in Fe-rich metabolic pathways [6,7]. The relatively small post-transcriptional changes in mRNA levels mediated by Cth1 and Cth2 compared to the dramatic effects of Aft1/Aft2 transcriptional control, suggest that Cth1 and Cth2 function to fine-tune iron metabolism under iron deficiency [8]. Here, we will highlight recent advances that shed light on the mechanisms for achieving these precise adjustments and further our understanding of the regulation and function of Cth1 and Cth2.

At the other extreme of iron deficiency, iron overload is handled by the transcriptional activator Yap5. Yap5 was initially identified as the high iron-sensing transcription factor that activated expression of the vacuolar iron importer *CCC1* [9]. Recent studies have expanded the list of Yap5-regulated genes, providing new information on how cells cope with excess iron. In addition, a study on the intracellular factors controlling iron-dependent Yap5 activity has uncovered a fascinating connection between low and high iron sensing.

Another major advance in understanding iron sensing and regulation in *S. cerevisiae* is characterization of the “iron-ome” in whole cells and organelles in different genetic backgrounds and under varying growth conditions. We will outline recent reports examining the iron-ome in mitochondria and vacuoles upon disruption of mitochondrial iron-sulfur cluster biogenesis and Aft1-regulated iron homeostasis.

Regulation by High and Low Iron Sensors: Fe-S Clusters are the Key

A number of recent studies have uncovered several key aspects of the regulation mechanisms for responding to both low and high iron in *S. cerevisiae*. Nuclear localization of the low iron regulator Aft1 (and presumably Aft2) is controlled by the cellular iron status: in iron-starved cells, Aft1 accumulates in the nucleus, while under iron-replete conditions, Aft1 is shuttled to the cytosol by the nuclear exportin Msn5 [10-12]. Aft1 and Aft2 were previously shown to interact with the nuclear exporter Msn5 in an iron-dependent manner [10]; however, it was unclear whether nuclear export was necessary to abrogate Aft1/2 transcriptional activity. A new study by Iwai and coworkers has now demonstrated that Msn5-mediated export is not required for inhibition of Aft1 activity, since Aft1 functions normally in an *msn5* strain despite constitutive localization to the nucleus [13]. The role of the monothiol glutaredoxins Grx3 and Grx4 in controlling Aft1 activity was also clarified in this study. Grx3 and Grx4 are known to form [2Fe-2S] cluster-binding complexes that, together with the cytosolic proteins Fra1 and Fra2, transmit an inhibitory signal to Aft1/2 that is dependent on the synthesis of mitochondrial Fe-S clusters ([14-18] and recently reviewed in [19]). However, the specific effect of this signal on Aft1/2 activity was not clear. Ueta et al. show that the Fe-S cluster-dependent Grx3/4-Aft1 interaction specifically promotes dissociation of Aft1 from its target promoters under Fe-replete conditions [13], supporting the model that Fe-S cluster binding to Grx3/4 is a prerequisite for Aft1 inhibition (Figure 1) [19]. Previous work by Lill and coworkers demonstrated that iron binding to Grx3/4 *in vivo* requires components of the mitochondrial Fe-S cluster (ISC) assembly machinery but not cytosolic Fe-S cluster assembly factors [17]. Iwai and colleagues extend these findings by showing that the mitochondrial transporter Atm1 is also required for Fe binding to Grx3/4 and dissociation of Aft1 from DNA during iron sufficiency. Atm1 is proposed to export an unknown sulfur-containing substrate from the mitochondria that is used to build and/or insert Fe-S clusters into cytosolic proteins (recently reviewed in [20]).

Intriguingly, Fe-S clusters are also at the center of the response to high iron mediated by the transcription factor Yap5. Using a novel enzyme assay to monitor bioavailable cytosolic iron levels, Kaplan and coworkers demonstrate that iron-dependent control of both Yap5- and

Aft1/2-regulated genes is independent of changes in cytosolic iron levels or vacuolar/mitochondrial iron sequestration. Instead, both transcriptional responses are tuned to the availability of Fe-S clusters assembled in the mitochondria (Figure 1). It is interesting to note that while Grx3 and Grx4 are required to relay the signal of Fe-S cluster availability to Aft1/2, these cytosolic Fe-S binding proteins do not have an apparent role in controlling Yap5 activity [21]. Thus the molecular mechanism for activating Yap5 in response to Fe-S cluster availability requires further study.

By exploring the Yap5 regulon, researchers have also determined that Fe-S cluster binding proteins play a key role in coping with excess iron. The list of genes regulated by Yap5 is much shorter than the Aft1/Aft2 regulon, limited to genes encoding the vacuolar iron importer Ccc1, the metallothionein Cup1, and the Fe-S cluster binding proteins Grx4 and Tyw1 [22,23]. However, iron-dependent regulation of *CCC1* is not exclusive to Yap5, since *CCC1* expression is still stimulated by an unknown iron-responsive regulator in a *yap5* strain [23]. Activation of *CCC1* functions to sequester excess iron in the vacuole in order to decrease the cytosolic iron pool. Increased Grx4 levels may similarly act as an iron sink via increased Fe-S cluster binding, in addition to ensuring efficient Aft1 inhibition during iron sufficiency. The rationale behind Yap1-dependent upregulation of Tyw1, an enzyme involved in the synthesis of modified tRNA, is less obvious. Kaplan and coworkers examined the role of Tyw1 during iron overload by studying the effects of deletion and overexpression of *TYW1* in different genetic backgrounds [22]. They concluded that Fe-S cluster binding by ER-bound Tyw1, and not its function in nucleoside biosynthesis, helped prevent iron toxicity by sequestering iron as protein-bound Fe-S clusters (Figure 1). Interestingly, they also reported that overexpression of *TYW1* induced the Aft1/2 iron regulon by an unknown mechanism, leading to increased intracellular bioavailable iron. However, this effect was specific to the N-terminal domain of Tyw1 that contains an unusual GGKKGCCSSK repeat sequence and not the catalytic [4Fe-4S] cluster binding domain [22]. The presence of cysteines in this repeat sequence raises the possibility that this domain also binds Fe or Fe-S clusters, although this idea has not yet been tested.

Novels Connections Between Iron Regulation and Thiol Redox Metabolism

Several recent reports have revealed functional cross-talk between Aft1/2 low iron signaling and thiol metabolism. Depletion of the thiol-containing tripeptide glutathione (GSH) was previously shown to cause constitutive activation of the Aft1/2 iron regulon [14]. The requirement for GSH may stem from its role as a [2Fe-2S] cluster ligand in Grx3/4 or its participation in export of the unidentified sulfur-containing substrate from the mitochondria used for cytosolic Fe-S cluster biogenesis [19]. Using an engineered yeast strain that accumulates excess GSH, Toledano et al. recently extended these studies by showing that excess GSH has a similar effect on iron metabolism. Furthermore, they establish that GSH's role in thiol redox homeostasis is secondary to its essential role in iron metabolism [24]. A link between iron and redox metabolism was also established in a study on the effect of vacuolar H⁺-ATPase dysfunction on iron regulation. Kane and co-workers discovered that disruption of vacuolar acidification, which leads to lower cytosolic pH, concomitantly induces expression of the Aft1/2 regulon and the cytosolic peroxiredoxin Tsa2. Under these conditions, Tsa2 interacts with Fra1 and may function to limit Aft1 translocation to the nucleus, since deletion of *tsa2* or its paralogue *tsa1* further enhanced induction of the iron regulon [25]. However, the peroxidatic cysteine in Tsa2 was dispensable for its affect of Aft1 regulation, pointing to a peroxidase-independent role in iron homeostasis. Tsa1 and Tsa2 are known to act as molecular chaperones under stress conditions [26], and it is possible this function may be important for inhibition of Aft1/2 when vacuolar function is compromised.

Interestingly, a mitochondrial peroxiredoxin was also implicated in low iron signaling in yeast. In a study by Bárcena and coworkers using redox proteomics and transcriptomics, induction of Aft1/2 regulon genes was detected upon deletion of the mitochondrial peroxiredoxin Prx1. Since mitochondrial Fe-S cluster biogenesis is functional in this strain, these data suggest that *prx1* cells are defective in the export and/or cytosolic transmission of the mitochondrially-derived Fe-S dependent inhibitory signal to Aft1/2. Protein glutathionylation is increased in this strain, which could interfere in the iron signaling mechanism since GSH plays a key role. However, this effect is abolished in the absence of the mitochondrial/cytosolic dithiol glutaredoxin Grx2, which further increases protein glutathionylation levels [27]. Thus, the roles of Prx1 and Grx2 in low iron signaling are unclear and require more study.

Nucleocytoplasmic Shuttling and Negative Feedback Regulation of Cth1/Cth2

In addition to transcriptional activation of iron-related genes by Aft1/2 and Yap5, *S. cerevisiae* also employs mRNA degradation mechanisms to control iron metabolism. The transcriptional and post-transcriptional pathways are directly linked since the mRNA binding proteins Cth1 and Cth2 are induced by Aft1 and Aft2 under iron deficiency. The mRNAs targets of Cth1 and Cth2 encode proteins involved in respiration, the TCA cycle, heme biosynthesis, amino acid, sterol, and fatty acid metabolism, and mitochondrial Fe-S cluster biogenesis [8]. Although the targets of Cth1 and Cth2 and the cellular impact of their function are well-characterized, the details of how and where Cth1/Cth2 recognize and traffic their mRNA targets are nebulous. A recent study by Thiele and coworkers reveals that shuttling of Cth2 between the nucleus and cytosol is important for its function in iron regulation (Figure 2) [28]. They mapped the nuclear import signal to the tandem zinc finger domain that directly binds the target RNAs, and demonstrate that nuclear export of Cth2 is facilitated by nuclear mRNA export pathways rather than nuclear export signal (NES)-dependent transport. Forced localization of Cth2 to the nucleus or cytosol interferes in the mRNA degradation pathway, suggesting that Cth2 must actively move between both compartments for its proper function [28].

Although *CTH1* and *CTH2* genes are both activated by Aft1/2 when iron is low, Cth1 is transiently expressed early in iron deficiency, while Cth2 persists during prolonged iron deficiency [6,7]. The mechanism underlying this phenomenon was recently uncovered by Puig, Thiele and coworkers. They provide evidence that the Cth1/Cth2 regulation system employs auto- and cross-regulation to limit expression of Cth1 and Cth2 [29]. *CTH1* and *CTH2* mRNAs possess AU-rich elements (AREs) that are recognized by both Cth1 and Cth2 and allow targeted turnover of these transcripts (Figure 2). If this autoregulation is disrupted by mutation of the AREs in *CTH2*, cells exhibit a delay in growth recovery after switching from iron limitation to iron sufficiency. Based on their results, they propose that upon iron depletion, Cth1 acts first to downregulate non-essential Fe-rich pathways such as respiration, while Cth2 downregulates other Fe-dependent metabolic pathways and vacuolar iron storage via *CCC1* mRNA turnover to allow adaptation to extended iron depletion. This mechanism thus ensures a rapid response to changing iron bioavailability in order to maximize iron use for optimal growth [29].

Iron-ome Studies Provides New Insights into Iron Trafficking and Regulation

As a complement to the numerous genetic and biochemical studies focused on iron-related genes and proteins, an innovative, metal-centric approach has provided a new perspective on

yeast iron metabolism. Using a combination of Mossbauer, EPR, and UV-visible absorption spectroscopies that allows determination of the iron concentration and speciation in subcellular compartments, Lindahl and coworkers have revealed key insights into the mechanisms of metal trafficking and storage and the regulation of these processes [30-33]. In one intriguing study, the “iron-ome” in a strain expressing a mutant Aft1 that is constitutively active (*AFT1-I^{up}*) was compared to a strain that is defective in mitochondrial Fe-S cluster biogenesis via downregulation of the mitochondrial ferredoxin Yah1 (*GAL-YAH1*) [33]. Although *AFT1-I^{up}* and *GAL-YAH1* strains both accumulate iron due to constitutive activation of the Aft1 regulon, the subcellular iron distribution and speciation in each is markedly different. In *AFT1-I^{up}* cells, excess iron accumulates in the vacuole as high spin Fe³⁺ with some iron deposited in mitochondria as Fe³⁺-phosphate nanoparticles in addition to Fe-S clusters and heme. In *GAL-YAH1* and other mitochondrial ISC mutants, vacuolar iron is virtually absent with massive iron import into mitochondria where Fe³⁺-phosphate nanoparticles accumulate at the expense of hemes and Fe-S clusters [33-35]. These results thus further uncover the pathways for communication between mitochondria and vacuoles, the two main hubs of iron trafficking [36]. In *AFT1-I^{up}* cells, transport of iron to vacuoles compensates for constitutive iron uptake at the plasma membrane preventing toxic accumulation of cytosolic iron. However, in *GAL-YAH1* cells, the lines of communication are disrupted and mitochondria bear the brunt of the iron overload. These phenotypes can now be explained given the discovery that both Aft1/2 and Yap5 use Fe-S clusters as sensors of cellular iron status (Figure 3). In the *AFT1-I^{up}* strain, only Aft1 function is disrupted while Yap5 is responsive to increased iron levels. Yap5 activation thus facilitates this iron sequestration in the vacuole via activation of *CCC1* encoding the vacuolar iron importer. Although increased *CTH1/2* expression by Aft1/2 promotes *CCC1* mRNA turnover, Yap5 transcriptional activation of *CCC1* likely overcomes this effect. On the other hand, in *GALYAH1* cells, both Aft1/2 and Yap5 are unable to sense the cellular iron status. Consequently, the Aft1/2 regulon is constitutively on while the Yap5 regulon is constitutively off. Thus, the Aft1/2-regulated vacuolar iron exporters Fet5/Fth1 are expressed, while the Yap5-regulated vacuolar iron importer Ccc1 is not. Indeed, previous DNA microarray analysis of *GAL-YAH1* cells confirms this result [37]. The net outcome is decreased vacuolar iron uptake leading to mitochondrial iron overload (Figure 3).

Conclusions and Future Directions

The model eukaryote *S. cerevisiae* has a complicated system of checks and balances to ensure adequate levels and efficient use of the essential nutrient iron. Extensive cross-regulation exists between transcriptional and post-transcriptional control pathways to allow appropriate and rapid responses to both high and low iron conditions. The new studies outlined here have uncovered several key aspects of the cellular and molecular mechanisms required to carefully orchestrate these responses. First, new mechanistic details about post-transcriptional control by Cth1 and Cth2 have been revealed, demonstrating auto- and cross-regulation of these mRNA binding proteins and nucleocytoplasmic shuttling of Cth2 to allow efficient and rapid mRNA recognition and degradation. Preliminary studies suggest different post-translational modifications for nuclear vs. cytosolic Cth2 [28], thus future studies will focus on how these modifications impact Cth2 function and regulation.

Second, it is clear that the transcriptional machineries that control the responses to both iron deficiency and iron overload are specifically tuned to the ability of the cell to assemble Fe-S clusters. These versatile and ancient cofactors are a logical choice for this role since their assembly and trafficking requires integration of iron homeostasis and redox control pathways. This point is further emphasized by the numerous studies demonstrating that altered thiol redox metabolism disrupts iron regulation. The underlying cause for these disruptions may be the inability to assemble and/or traffic Fe-S clusters. The strong link

between Fe-S clusters and iron regulation is further revealed by iron-omics studies. By comparing the cellular consequences of defective mitochondrial Fe-S cluster biogenesis vs. Aft1 upregulation, these studies suggest that communication between cytosolic, mitochondrial, and vacuolar iron pools is dependent on Fe-S cluster biogenesis and support the finding that both Yap5 and Aft1/2 use Fe-S clusters as sensors of cellular iron status. Despite these recent discoveries, key details in the sensing and regulation mechanisms for Aft1/2 and Yap5 are lacking, including the identity of the specific Fe-S-dependent signal exported from the mitochondrial and the molecular mechanisms for inhibiting Aft1/2 and activating Yap5 in response to this signal. Uncovering these mechanistic details will provide a more complete picture of yeast iron regulation at the molecular level.

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Highlights

- Iron-dependent Aft1 nuclear export is not required for iron regulon deactivation
- High and low iron sensing depends on mitochondrial iron-sulfur cluster biogenesis
- Disruptions in thiol redox metabolism activate the Aft1/2 iron regulon
- Cth1/2 cross- and auto-regulation enhances the response to iron deficiency
- Iron-ome studies highlight iron trafficking between mitochondria and vacuoles

Figure 1. Fe-S clusters are essential for transcriptional control by Aft1/2 and Yap5 during iron sufficiency

(1) During conditions of iron sufficiency, Fe-S clusters are synthesized in mitochondria via integration of iron, sulfur, and redox control pathways. (2) An unknown substrate produced by the mitochondrial ISC machinery is exported out to the cytosol by the transporter Atm1. Glutathione (GSH) is also required for export of this signal. (3) Grx3 and Grx4, which form GSH-ligated, Fe-S-bridged homo- and heterocomplexes, are proposed to relay this signal to Aft1/2 in conjunction with Fra2 and Fra1. (4) Interaction of Grx3/4 with Aft1 promotes dissociation of the transcriptional activator from its target DNA, leading to deactivation of Aft1/2-regulated genes. (5) Meanwhile, the mitochondria ISC-dependent signal exported by Atm1 is also relayed to Yap5 via as-yet-unidentified factor(s). (6) This signal stimulates Yap5 activation of its target genes including *CCC1*, *GRX4*, and *TYW1*. (7) Increased expression of *CCC1* stimulates iron import and sequestration in the vacuole, (8) while increased expression of *TYW1* promotes iron sequestration as protein-bound Fe-S clusters. Increased Grx4 protein levels may also act as an iron sink in addition to ensuring Aft1/2 inhibition.

Figure 2. Cth1/2 nucleocytoplasmic shuttling and auto- and cross-regulation during iron deficiency

When iron is limited, the Yap5 regulon is off, while Aft1/2 activates expression of iron uptake, trafficking, and storage genes in addition to the mRNA binding proteins Cth1 and Cth2. Cth2 (and presumably Cth1) shuttle into the nucleus where they bind the AU-rich elements (AREs) of target mRNAs encoding proteins involved in iron-rich metabolic pathways. *CTH1* and *CTH2* mRNAs also possess AREs that are recognized by both Cth1 and Cth2, thus allowing tight control of Cth1/2 protein levels. Nuclear export of Cth2 with its target mRNA is facilitated by nuclear mRNA export pathways. In the cytosol, Cth1/2 promotes decay of the target mRNA.

Figure 3. Effects on mitochondrial Fe-S cluster defects on intracellular iron trafficking and storage

In yeast strains with defective mitochondrial Fe-S cluster biogenesis and export, Aft1/2 is constitutively activated while Yap5 is constitutively deactivated regardless of the cellular iron status. Constitutive activation of Aft1/2 leads to increased iron uptake at the plasma membrane and iron export from the vacuole. As a result, cytosolic and mitochondrial iron levels increase. *CTH1* and *CTH2* levels also rise, causing increased degradation of *CCC1* mRNA and consequently decreased vacuolar iron import. Constitutive deactivation of the Yap5 regulon further leads to reduced *CCC1* expression and reduced vacuolar iron levels.