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Review

Metabolic remodeling in iron-deficient fungi ☆

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ABSTRACT

Eukaryotic cells contain dozens, perhaps hundreds, of iron-dependent proteins, which perform critical functions in nearly every major cellular process. Nutritional iron is frequently available to cells in only limited amounts; thus, unicellular and higher eukaryotes have evolved mechanisms to cope with iron scarcity. These mechanisms have been studied at the molecular level in the model eukaryotes *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, as well as in some pathogenic fungi. Each of these fungal species exhibits metabolic adaptations to iron deficiency that serve to reduce the cell's reliance on iron. However, the regulatory mechanisms that accomplish these adaptations differ greatly between fungal species. This article is part of a Special Issue entitled: Cell Biology of Metals.

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1. Introduction

Although some species of bacteria apparently have no nutritional requirement for iron [1,2], most prokaryotes and all eukaryotes have evolved a substantial dependence on this transition metal. This requirement for iron can pose a challenge to organisms, as bioavailable iron is frequently limiting for growth. Iron deficiency is the most common human nutritional disorder in the world, affecting more than 2 billion individuals [3]. Iron deficiency causes anemia and is associated with increased perinatal mortality in women and infants and impaired cognitive and neurological development in children [4]. Iron deficiency frequently limits the growth of plants, as one third of the world's soils are considered iron deficient due to the limited solubility of iron in an aerobic environment [5]. Despite the prevalence and importance of iron deficiency, little is known about the effects of iron limitation on metabolism at the cellular or organismal level.

Iron is an essential nutrient because it is required for the synthesis of heme and iron–sulfur (Fe–S) clusters and because it directly activates enzymes containing mononuclear or diiron centers [6–8]. Iron cofactors activate enzymes and confer proper structure to proteins involved in nearly every major process performed in cells. Iron-dependent enzymes are required for the synthesis of the major components of the cell: lipids (oxysterols, unsaturated fatty acids, hydroxylated sphingolipids), proteins (multiple amino acids), and nucleic acids (deoxyribonucleotides, purines in higher eukaryotes). Most of the major biochemical processes in the cell contain iron-dependent enzymes: nitrogen fixation, the tricarboxylic acid cycle and respiration, DNA replication and repair, chromatin remodeling, translation, metabolism of xenobiotics, oxygen transport, synthesis of antibiotics and other small molecules. Finally, iron proteins serve as sensors and regulators of gene expression in several pathways. Given this dependence on iron, it is not surprising that the uptake and utilization of iron are carefully controlled processes in cells and organisms.

Iron metabolism has been studied in greatest detail in the model eukaryote, *Saccharomyces cerevisiae*. Iron homeostatic systems have also been elucidated at the molecular level in other fungal species, including *Schizosaccharomyces pombe*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Candida albicans*, and *Cryptococcus neoformans*. The transcriptional responses to iron deprivation in these organisms have been previously reviewed in detail [9–11]. This review will focus on the metabolic adaptations exhibited by fungi in their response to iron deprivation and the diverse mechanisms employed to achieve these responses.

2. The metabolic response to iron deficiency in *S. cerevisiae*

S. cerevisiae can thrive in environments that contain exceeding low as well as very high amounts of bioavailable iron, and the amount of iron accumulated within cells in different environments can vary tremendously. Laboratory strains of *S. cerevisiae* contained approximately 100 ppb of iron when grown in rich media containing yeast extract

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and glucose as a carbon source [12]. In contrast, yeast grown in synthetic glucose medium, supplemented with amino acids and 100 μM iron (an iron-rich medium), contain approximately 38,000 ppb of iron [13]. Yeast cells exhibit only a small (20%) reduction in growth rate in iron-chelated media containing free ferrous iron at a concentration of ca. 10^{-13} M, yet cells grown under these conditions accumulate iron at approximately 20-fold lower levels than yeast grown in standard synthetic media [14]. What adjustments to metabolism occur to accommodate these changes in iron content? Budding yeast respond to iron deficiency by activating the transcription factors Aft1 and Aft2 [15–21]. Aft1 (and to a lesser extent Aft2) activate the transcription of approximately 25 genes involved in the uptake of iron at the plasma membrane, the transfer of stored iron from the vacuole to the cytosol, and the alteration of metabolism to more efficiently use iron.

2.1. Suppression of respiration in iron deficiency

Perhaps the most significant alteration in metabolism that occurs with depletion of cellular iron is the shift to fermentative metabolism. Budding yeast metabolizes glucose, its preferred carbon source, to pyruvate via glycolysis. Pyruvate is converted to ethanol and CO_2 via fermentation or fully oxidized to CO_2 and H_2O via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. If glucose is available, even in the presence of oxygen, budding yeast forgo the greater energy production associated with respiration, metabolizing only 3% of cellular glucose via respiration, while metabolizing 73% via fermentation [22]. Once yeast have depleted all of the available glucose, metabolism shifts from fermentation to respiration. This transition, termed the diauxic shift, is associated with large changes in gene expression that result in the dramatic expansion of the mitochondrial compartment (from ~3% or less of cellular volume to ~13% of cellular volume) and a concurrent increase in the level of TCA cycle enzymes and respiratory complexes in the mitochondria [23,24]. This increase in mitochondrial volume is accompanied by an expansion of the mitochondrial iron pools, as two TCA cycle enzymes, aconitase and succinate dehydrogenase, contain Fe–S clusters and respiratory complexes II–IV contain numerous heme and Fe–S centers. Characterization of the mitochondrial iron pools of respiring vs. fermenting cells using multiple spectroscopic approaches indicates that the overall concentration of iron within mitochondria does not change, but the distribution of iron species changes [25]. The unchanging iron concentration coupled with the increase in mitochondrial volume results in a proportional increase in the mitochondrial iron pool. Iron ions from respiring mitochondria are largely present in respiration-related proteins in the form of heme and Fe–S clusters, while fermenting mitochondria contain lower levels of heme and Fe–S proteins and much higher levels of non-heme, high-spin Fe(II), mononuclear Fe(III), and Fe(III) nanoparticles.

This expansion of mitochondrial iron pools during the shift from fermentative to oxidative metabolism is accomplished by an increase in the expression of several (but not all) genes involved in iron uptake, notably the high-affinity transporter composed of Fet3 and Ftr1, the siderophore transporter Sit1/Arn3, and the cell wall protein Fit2 (and possibly other genes) [26]. This transcriptional upregulation is dependent on both the glucose-regulated kinase of the Snf1–Snf4 complex and the iron-regulated transcription factor, Aft1. The increase in mitochondrial iron pools is absolutely required for respiration, as yeast cannot grow on non-fermentable carbon sources (such as ethanol) when media also contain low concentrations of iron or the strain is lacking genes for high-affinity iron uptake [10].

Metabolite analysis of yeast growing on glucose media containing optimal amounts of iron vs. low amounts of iron reveals that iron depletion is associated with reduced intracellular levels of glucose, glycolytic intermediates, and byproducts of glycolysis, but elevated levels of pyruvate, the product of glycolysis. Levels of triacylglycerol, a neutral lipid synthesized from a glycolytic intermediate, are also reduced in iron-depleted cells [14]. Transcriptome analysis of iron-deficient yeast has consistently demonstrated a down-regulation of genes involved in respiration. These changes in transcript levels are due to a combination of transcriptional and post-transcriptional regulatory mechanisms (see below). The reduced expression of respiratory proteins coupled with the cellular depletion of glucose and glycolytic intermediates and the accumulation of pyruvate indicate that although respiration occurs at low levels in glucose-grown yeast, iron deprivation further represses respiration and cells exhibit an increased reliance on fermentation for energy production.

2.2. Amino acid metabolism in iron deficiency

The synthesis of several amino acids in yeast is dependent on enzymes that contain Fe–S clusters. The enzymes are aconitase (Aco1), glutamate synthase (Glt1), sulfite reductase (Met5), dihydroxyacid dehydratase (Ilv3), isopropylmalate isomerase (Leu1), and homoaconitase (Lys4) [6]. With the exception of Glt1, deletion of any of these enzymes results in amino acid auxotrophy. Thus, one would expect that amino acid metabolism would be affected by iron deficiency. However, metabolite analysis of a yeast strain grown in iron-poor medium demonstrated that although the cells were iron deficient, amino acids were largely present at levels similar to or higher than those of iron-sufficient cells [14]. Only glutamate exhibited a mild, 1.5-fold depletion. Although many laboratory strains of yeast carry mutations in amino acid biosynthetic genes, these experiments were performed in a strain that was prototrophic for amino acids and required no supplementation of amino acids in the medium. This finding does not suggest, however, that iron deficiency does not impact amino acid biosynthesis, but rather, that the amino acid homeostatic mechanisms in yeast are robust. Severe deficiencies in a single or a few amino acids would prove catastrophic to the process of translation. Yeast are protected from these events by the action of Gcn2 kinase [27]. Deficiencies in individual amino acids lead to the accumulation of uncharged tRNAs, which directly bind to and activate Gcn2. Activated Gcn2 phosphorylates eukaryotic initiation factor 2 (eIF2), which is required, in its dephosphorylated form, for the first step of translation initiation. Phosphorylated eIF2 acts as an inhibitor of translation for most mRNAs in yeast. An exception is the mRNA encoding the transcription factor Gcn4, which is translated in the setting of eIF2 phosphorylation and subsequently activates the transcription of many genes involved in amino acid synthesis and other metabolic processes [28]. Amino acid deficiencies also activate pathways of autophagy [29], in which bulk cytosolic proteins and organelles are directed into membrane-bound autophagosomes that are degraded within the vacuole. Amino acids derived from proteins degraded in the vacuole are exported to restore cytosolic amino acid pools. Gcn4 contributes to this process by activating transcription of some genes involved in autophagy [30]. Thus, while iron deficiency may impair amino acid synthesis, yeast coordinate new protein synthesis with available amino acid pools by slowing translation, activating systems of amino acid biosynthesis, and degrading proteins to restore amino acid pools when amino acid levels fall.

Several lines of evidence indicate that iron deficiency has a significant impact on amino acid synthesis in yeast. Transcriptome analysis of yeast prototrophic for amino acids indicates that many transcripts involved in amino acid biosynthesis and uptake are up-regulated during iron deficiency (Table 1) and most of the amino acids affected by these genes have an iron-dependent biosynthetic step [14]. *GLT1* transcripts are down-regulated 6-fold and glutamate dehydrogenase 3 (*GDH3*) transcripts are up-regulated 4.5-fold in iron-deficient yeast. Similarly, the activity of glutamate synthase decreases by 20-fold in iron-starved yeast [19]. What accounts for this shift? All of the nitrogen-containing molecules in yeast derive their nitrogen from glutamine and glutamate; thus, the synthesis of these amino

Table 1
Transcripts related to amino acid metabolism up-regulated in iron deficient cells[a].

Amino acid transcripts up-regulated in iron deficiency			
Gene	Fold change	Function	Amino acid
PTR3	1.75[a]	Amino acid sensor	Amino acids
ARG80	1.61	Transcription factor ARG genes	Arg
ARG3	1.55	Ornithine carbamoyltransferase	Arg
VBA3	3.51	Vacuolar basic amino acid permease	Arg, Lys
ALP1	1.87	Arginine transporter	Arg, Lys
GTO3	11.80	Omega class glutathione transferase	Cys, Met
YCT1	3.98	Cysteine-specific transporter	Cys, Met
ECM4	2.89	Omega class glutathione transferase	Cys, Met
MET2	2.52	L-homoserine-O-acetyltransferase	Cys, Met
MMP1	2.13	S-methylmethionine permease	Cys, Met
BDS1	1.87	Sulfatase	Cys, Met
SUL2	1.77	Sulfate permease	Cys, Met
LAP3	1.74	Cysteine aminopeptidase	Cys, Met
STR3	1.54	Cystathionine beta-lyase	Cys, Met
SNO1	2.60	Glutamine amido transferase	Gln
GDH3	4.52	Glutamate dehydrogenase	Glu
TMT1	4.73	Trans-aconitate methyltransferase	Leu
OAC1	1.72	Mitochondrial carrier, isopropylmalate transport	Leu
BAT2	2.42	Branched-chain amino acid aminotransferase	Leu, Ile, Val
BAP2	12.93	Leucine permease	Leu, Ile, Val
NMA2	2.00	Nicotinic acid mononucleotide adenylyltransferase	Trp
HBN1	5.34	Nitroreductases	Tyr, Phe, Trp
FRM2	4.18	Nitroreductases	Tyr, Phe, Trp

[a] Reproduced from [19].

acids is a critical and highly regulated process in cells. Yeast synthesize glutamate from 2-oxoglutarate and ammonium by either of two mechanisms. One path is via NADPH-dependent glutamate dehydrogenases (Gdh1 and Gdh3) and the other requires glutamine synthetase (Gln1) and Glt1 [31]. Iron deficiency prompts the cell to down-regulate the transcription of the iron-requiring enzyme (Glt1) and up-regulate the transcription of an iron-independent enzyme (Gdh3), thereby diverting iron away from a non-essential pathway. Some of the down-regulation of *GLT1* transcripts is due to post-transcriptional degradation through Cth1 and 2 (see below) [32,33], but much of the regulation is transcriptional. An iron-dependent activation sequence was mapped to a region approximately 500 bp upstream of the open reading frame [19]. This region contains two binding sites for the transcriptional regulator Hap2/3/4/5, raising the possibility that the iron-dependent regulation of *GLT1* is mediated by this regulator [34].

Several investigators have reported a link between iron deficiency and leucine biosynthesis. Leucine synthesis occurs in two phases: 1) the synthesis of α-isopropylmalate (α-IPM) in the mitochondria and 2) the conversion of α-IPM to leucine in the cytosol (Fig. 1) [35]. This biosynthetic pathway is regulated at multiple levels. First, α-IPM synthase (Leu4/Leu9) is allosterically inhibited by leucine and coenzyme A. Second, genes involved in leucine biosynthesis and uptake (*LEU1*, *LEU2*, *LEU4*, *LEU9*, *ILV2*, *ILV5*, *BAT1*, *BAP2*, and *GAP1*) are transcriptionally regulated by the transcription factor Leu3. Leu3 acts as a transcriptional repressor in the absence of α-IPM; but when α-IPM accumulates in cells, α-IPM binds to Leu3 and converts it to a transcriptional activator. Other transcription factors (such as Gcn4) can also act on the leucine biosynthetic genes. Thus, when strains with an intact biosynthetic pathway are grown in medium supplemented with leucine, leucine enters the cell, binds to Leu4 and Leu9, and inhibits the synthesis of α-IPM. In the absence of α-IPM, Leu3 represses the transcription of the leucine biosynthetic genes and the enzymes of the pathway are expressed at very low levels. In medium without leucine supplementation, Leu4/Leu9 is active, α-IPM is produced, Leu3 is activated, and the pathway is expressed at high levels.

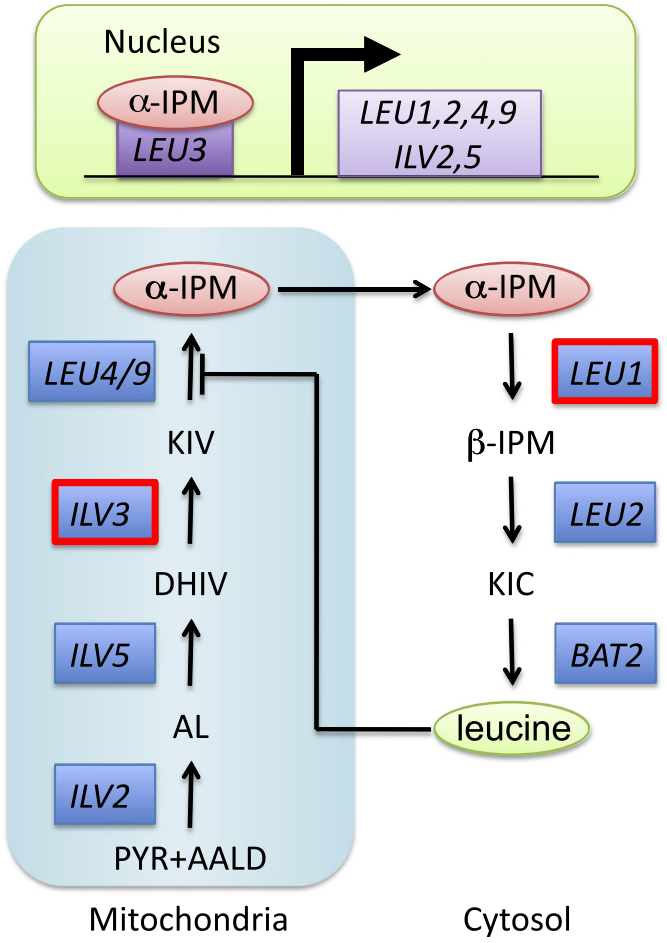

Fig. 1. Leucine biosynthetic pathway of *S. cerevisiae*. α-IPM synthesis occurs in the mitochondrion, although a small amount of Leu4 is present in the cytosol. Leucine allosterically inhibits IPM synthase (Leu4/9). α-IPM activates the transcription factor Leu3. Enzymes boxed in red contain Fe–S clusters. Deletion of Leu2 leads to accumulation of α-IPM. AALD, active acetaldehyde; PYR, pyruvate; AL, acetolactate; DHIV, α,β-dihydroxyisovalerate; KIV, α-ketoisovalerate; α-IPM, α-isopropylmalate; β-IPM, β-isopropylmalate; KIC, α-ketoisocaproate. Based on [35].

How does iron deficiency affect this pathway? Metabolite analysis of iron-deficient yeast grown without amino acid supplementation reveals an accumulation of α-IPM [14]. Leu1 catalyzes the conversion of α-IPM to β-IPM and the accumulation of α-IPM suggests a loss of Leu1 activity. This loss of Leu1 activity in iron-deficient cells has been consistently observed [36,37]; furthermore, yeast grown in iron-poor medium exhibit reduced activities of multiple 4Fe–4S cluster enzymes of the aconitase family: Aco1, Leu1, and Ilv3. In yeast grown without amino acid supplements, changes in transcript levels for these genes are small (2-fold or less) and do not account for the changes in activity [14]. Most of the loss of activity is due to loss of the Fe–S cofactors. Interestingly, when Leu1 is overexpressed, as occurs in strains with mutations in *LEU2*, Aco1 activity is reduced, suggesting a net flux of Fe–S clusters away from Aco1 (in the mitochondria) to Leu1 (in the cytosol) [36]. Essential Fe–S cluster proteins, such as Rli1, also exhibit reduced Fe–S cluster incorporation in iron deficiency. These findings suggest that yeast do not have mechanisms to direct the flow of Fe–S clusters to specific pools of proteins. Only increased or decreased levels of Fe–S protein synthesis, achieved via changes in transcript levels, can alter the delivery of Fe–S clusters to specific proteins in the setting of iron deficiency. Large reductions in Leu1 transcript levels have been observed when yeast carrying mutations in *LEU2* are subjected to iron starvation in medium supplemented with leucine [32,37]. Mutations in *LEU2* also lead to accumulation of α-IPM, which can drive Leu3-dependent transcription

of *LEU1*. This accumulation of α-IPM is consistent with a low level of flux through the pathway despite the inhibitory effects of supplemental leucine. Iron deficiency in this setting likely leads to reduced activity of the upstream Fe–S enzyme, Ilv3. That reduced activity, plus the leucine-mediated inhibition of Leu4 and Leu9, would be predicted to reduce the flux through the pathway and block the accumulation of α-IPM. This would eliminate the stimulatory effect of α-IPM on Leu3 and the subsequent transcription of *LEU1*.

2.3. Pleiotropic effects of heme depletion in iron-deficient yeast

Yeast grown in iron-poor medium exhibit large reductions in cellular heme that appear to be multifactorial in origin [14,37,38]. This decrease in heme is accompanied by a loss of activity in heme-dependent enzymes, which impacts multiple biosynthetic pathways in the cell. The loss of heme- and Fe–S-dependent respiratory complexes in iron deficiency was discussed (see above). Two types of iron-dependent enzymes play important roles in oxysterol and lipid synthesis, heme proteins of the cytochrome P450 family and oxo-diiron enzymes of the fatty acid hydroxylase/sterol desaturase family [39,40]. The ergosterol biosynthetic pathway of yeast contains two heme-dependent enzymes, Erg11 and Erg5, and two oxo-diiron enzymes, Erg25 and Erg3. Metabolite analysis of yeast grown in iron-poor medium reveals the accumulation of ergosterol biosynthetic intermediates, squalene (20-fold) and lanosterol (3.3-fold), accompanied by depletion of the major oxysterol products of the pathway, ergosterol (2-fold) and zymosterol (3-fold) [14]. The accumulation of lanosterol is likely due to loss of activity of Erg11, which catalyzes the first iron-dependent step in the pathway. The cause of the accumulation of squalene is less clear, but suggests a partial inactivation of Erg1, which may be subject to allosteric inhibition by lanosterol. Levels of iron-requiring sterol biosynthetic enzymes exhibited small changes in iron deficiency, with heme proteins Erg11 and Erg5 decreasing, and diiron proteins Erg25 and Erg3 increasing. Many of the transcripts encoding sterol biosynthetic genes are up-regulated by iron deficiency [32]; the lower level of the heme enzymes may be due to instability of the proteins in the absence of their heme co-factor.

Three enzymes involved in fatty acid and sphingolipid metabolism are dependent on iron cofactors: Ole1, Sur2, and Scs7 [39,41,42]. Each of these enzymes contains a desaturase domain with a diiron center. Ole1 and Scs7 also contain heme-binding cytochrome b_5 domains and Sur2 likely relies on a separately encoded cytochrome b_5. Sur2 catalyzes the C4 hydroxylation of dihydrosphingosine to form phytosphingosine and Scs7 catalyzes the α-hydroxylation of the sphingolipid-associated very long chain fatty acids (ceramide). Iron deficiency inhibits both of these enzymes, leading to an accumulation of the unhydroxylated sphingolipid base and ceramide and a depletion of the hydroxylated ceramide [14]. Surprisingly, the metallation of Ole1, the δ-9 fatty acid desaturase, was not significantly inhibited and the abundance of monounsaturated fatty acids was not changed by iron deficiency. Transcription of *OLE1* is up-regulated 8-fold in iron deficient cells and, unlike Sur2 and Scs7, Ole1 protein levels do not fall in iron deficiency. Of these three enzymes, Ole1 is the only one that is encoded by an essential gene. Ole1 is resistant to inactivation during iron deficiency and yeast appear to rely on both transcriptional activation and an enzyme-specific resistance to de-metallation to maintain activity in this setting. Ergosterol and sphingolipids are enriched in microdomains in the outer leaflet of the plasma membrane [43,44]. The decrease in cellular levels of ergosterol and change in composition of sphingolipids would be predicted to have significant effects on the biophysical properties of these microdomains and therefore on the function of integral membrane proteins sensitive to their lipid environment. Ergosterol and sphingolipids affect endocytosis, the localization and activity of transporters, and the resistance to antifungal agents (in *C. albicans*) [40,45].

3. Mechanisms that effect metabolic iron sparing in *S. cerevisiae*

Many of the metabolic changes observed in iron deficient yeast can be explained simply on the basis of an insufficient number of iron co-factors to activate all of the expressed iron-dependent enzymes. However, an inspection of the regulatory responses to iron deficiency reveals that the cell does more than "passively experience" iron deficiency; rather, yeast actively control the utilization of iron through transcriptional and post-transcriptional mechanisms.

3.1. The role of Aft1 in the metabolic adjustment to iron deficiency

Most of the genes that are transcribed by Aft1 and Aft2 during iron deficiency are involved in bringing iron into the cytosol, either from outside the cell or from storage pools in the vacuole. However, a few of the Aft1 target genes are involved in changing the cell's utilization of iron. One method to reduce iron utilization is to shift from an iron-dependent biosynthetic pathway to an iron-independent one. The regulation of glutamate biosynthesis (see Section 2.2) is one example. Another is the regulation of biotin acquisition [19]. Yeast can either synthesize biotin from intermediates in the biotin biosynthetic pathway or take it up from the medium via a high-affinity transporter. The ultimate step in the biotin biosynthetic pathway is catalyzed by biotin synthase (Bio2), a protein of the radical-S-adenosyl methionine family that contains both a 2Fe–2S cluster and a 4Fe–4S cluster [46]. Under conditions of iron deficiency, the high-affinity biotin transporter, Vht1, is actively transcribed under the control of Aft1. Conversely, transcription of the biosynthetic enzymes, Bio3, Bio4, and Bio2, is reduced, thereby converting biotin acquisition from an iron-dependent process (endogenous biosynthesis) to an iron-independent process (uptake from medium). How is this reciprocal regulation of synthesis and uptake achieved? It may require the biotin-regulated transcription factor Vhr1 as well as Aft1. Vhr1 is required for the transcription of Vht1 and Bio5, the transporter for biotin biosynthetic intermediates, under conditions of low biotin [47]. Under conditions of high biotin, Vhr1 is inactive. Whether Vhr1 also regulates the transcription of Bio3, Bio4, and Bio2 is not known, but perhaps the increased Vht1 expression that occurs under conditions of iron deficiency leads to sufficient biotin accumulation to reduce transcription of the biosynthetic genes.

Total cellular heme is reduced in iron-deficient cells through multiple mechanisms. Although some of this reduction can be attributed to a loss of iron pools available for heme synthesis, lower expression levels of heme biosynthetic enzymes and accelerated heme degradation also contribute to decreased heme pools [38,48]. *HMX1* encodes the yeast heme oxygenase, a heme-degrading enzyme, which is actively transcribed by Aft1 during iron deficiency. Cells lacking Hmx1 exhibit higher intracellular heme levels and grow more slowly in iron-poor medium than cell expressing Hmx1. Thus, the degradation of heme in the setting of iron deficiency confers an adaptive advantage to yeast, perhaps by making heme iron available for other iron-dependent processes.

Heme degradation also has regulatory effects, as heme is required for the activation of the transcription factor Hap1 [49,50]. Hap1 is a binuclear zinc-cluster transcription factor and it depends on heme binding for transcriptional activation of the set of genes involved in respiration and aerobic growth. The dramatic down-regulation of *CYC1* and other genes of respiration during iron deficiency is due in part to the loss of heme-activated transcription by Hap1. Hap4 (in conjunction with the Hap2/Hap3/Hap5 CCAAT-box binding complex, see below) also induces the transcription of a large number of genes involved in respiration and aerobic growth. Hap4 and the Hap2/3/5 complex were recently shown to transcriptionally regulate *CYC1* in response to changes in iron [37]. Thus, the heme deficiency observed in iron-deficient cells is due in part to the regulatory activities of Aft1 and results in the inactivation of transcription factors that control the

expression of a major pathway of iron utilization. Heme is also involved in the Aft1-dependent activation of a subset of the Aft1 regulon [51].

3.2. Transcriptome remodeling through Cth1 and Cth2

Among the open-reading frames that are significantly upregulated by Aft1 under iron deficiency, *CTH2* (Cysteine-Three Histidine 2) is unique in that it does not encode a protein required for iron uptake or mobilization and is not part of an iron-dependent metabolic pathway. Instead, Cth2 is related to the mammalian $Cx_8Cx_5Cx_3H$ (CCCH) tandem zinc finger (TZF) tristetraprolin, which is involved in the targeted destabilization of tumor necrosis factor-α transcripts [52]. Cth2 has a paralogue in yeast, Cth1, and they share 46% overall identity and 79% identity within the TZF domain [53]. Whether *CTH1* expression is iron-dependent is unclear, as it was first reported that *CTH1* mRNA levels are neither increased under low iron conditions [16,19,32] nor in *AFT1-1*^{up} or *AFT2-1*^{up} strains [17,18]. Yet, it was shown that a FLAG-tagged Cth1 fusion protein is transiently expressed early after iron deprivation [33]. *CTH1* and *CTH2* contain two functional Aft1/Aft2 binding sites in their promoter region but, surprisingly, chromatin immunoprecipitation (ChIP) experiments showed that Aft1 occupies the *CTH1* promoter even under iron-replete conditions.

CTH2 expression is required for normal cellular growth on iron-poor medium, and mutants of conserved cysteines in both Cth2 zinc fingers (C190R and C213R) exhibit significant growth defects under the same conditions [32]. *CTH1* expression seems to be dispensable for normal cellular growth under iron deficiency, although the growth defect of a *cth1*Δ*cth2*Δ double mutant in iron-poor media is slightly worse than that of the *cth2*Δ mutant. Interestingly, expressing *CTH1* from the *CTH2* promoter rescues significantly the growth defect of *cth1*Δ*cth2*Δ [33]. Altogether, genetics studies suggest that differences in *CTH1* and *CTH2* expression patterns offer an explanation for the distinct phenotypes of *cth1*Δ and *cth2*Δ mutants although functional disparities between the two proteins cannot be excluded.

3.3. Mechanism of transcriptome remodeling by Cth1 and Cth2

As mentioned above, Cth1 and Cth2 are members of a family of mRNA-binding proteins characterized by a CCCH TZF domain. Experiments using the three-hybrid technique, a method allowing the study of RNA–protein interactions [54], showed that the TZF domains of Cth1 and Cth2 specifically bind directly to AU-rich elements (AREs) located in the 3′ UTR of mRNAs for *ACO1* and *SDH4* and promote their degradation [32]. This degradation mechanism, known as ARE-mediated mRNA decay, involves the recruitment of ARE-containing mRNAs in the nucleus by Cth2, followed by the maturation and export of a messenger ribonucleoprotein complex to the cytosol. Degradation of the mRNA occurs in the cytosol through an iron-independent process that involves a conserved N-terminal region of Cth2 and the DEAD box Dhh1 helicase [55–57].

Because *CTH2* expression is regulated by Aft1 and because *FET3* and *FIT2* mRNA levels are increased in a *cth2*Δ grown in iron-poor medium [58], it was hypothesized that Cth2 and/or Cth1 could be involved in post-transcriptional destabilization of specific mRNAs under low-iron conditions. To test this hypothesis, Puig and coworkers compared transcriptome profiles of *cth1*Δ*cth2*Δ and *cth1*Δ*CTH2*+ cells grown in iron-poor medium and found 80 transcripts to be upregulated [32]. In a subsequent transcriptome analysis, the same group found that *cth1*Δ*CTH2*+ cells grown in iron-poor medium down-regulate the steady-state levels of 223 transcripts compared to *cth1*Δ*cth2*Δ cells and *CTH1* + *cth2*Δ cells down-regulate the abundance of 60 transcripts (at least 1.5-fold decrease) compared to *cth1*Δ*cth2*Δ cells [33]. Among the transcripts down-regulated by *CTH2* expression, approximately half (94 of 223 total, 42%) contain AREs and could be considered direct targets of Cth2. Many of the down-regulated mRNAs do not contain 5′-UAUUUAUU-3′ or 5′-UUAUUUAU-3′ consensus ARE sequences in their 3′UTR, indicating that changes in these transcripts levels are indirect consequences of *CTH1* or *CTH2* expression, or that they contain non-canonical AREs. Some of the ARE-containing transcripts also may not be direct targets of Cth1 or Cth2, as direct interactions have been demonstrated for only a few transcripts.

Of the 94 ARE-containing transcripts down-regulated by Cth2, approximately half encode proteins involved in obvious iron-dependent metabolic pathways, such as cellular respiration, heme and Fe–S cluster biosynthesis, iron homeostasis, fatty acid and ergosterol metabolism, and amino acid metabolism [33]. In contrast, cells expressing only Cth1 exhibited down-regulation of 20 ARE-containing transcripts, mainly encoding mitochondrial proteins involved in respiration. Among the 20 ARE-containing *CTH1* targets, 13 are shared with *CTH2*; leaving only 7 transcripts that appear *CTH1*-specific (Fig. 2).

The mRNA degradation that is mediated by Cth1 and Cth2 results in a change in mRNA abundance of roughly two-fold or less. Indeed, RNA blotting experiments looking at the decay of aconitase (*ACO1*) and succinate dehydrogenase (*SDH4*) mRNAs showed that Cth2 decreases the half-life of these mRNAs by approximately 50% [32]. This correlates well with decreases in transcript levels in the 1.5- to 3-fold range. The relatively mild effect of Cth1 or Cth2 on the abundance of their target mRNAs suggests that Aft1 and Aft2 are the first-line of defense against iron deficiency and are responsible for larger changes in gene expression; Cth1 and Cth2 are involved in fine-tuning iron homeostasis. We will describe several examples of this reprogramming in the following sections.

3.3.1. Cth2p down-regulation of CCC1

Cth2 inhibits vacuolar iron storage under iron deficiency by down-regulating *CCC1* mRNA. *CCC1* encodes a vacuolar iron transporter that mediates the transfer of iron from the cytosol into the vacuole under conditions of iron sufficiency. *CCC1* transcription is controlled by the iron-sensing transcription factor Yap5. Under iron-replete conditions, Yap5 binds iron and activates the transcription of *CCC1*, promoting iron storage in the vacuole. Under iron deficiency, apo-Yap5 does not activate *CCC1* expression [59]. The *CCC1* transcript contains two AREs in its 3′UTR and is down-regulated two-fold in the presence of Cth2 under low iron conditions. RNA blotting experiments have confirmed the down-regulation of *CCC1* transcripts [32,33]. Thus, while Yap5 increases the expression of Ccc1 under iron sufficient conditions, Cth2 decreases the expression of *CCC1* under iron-deficient conditions, preventing the vacuolar storage of iron and making it available for iron-dependent processes.

3.3.2. Cth2 down-regulates the TCA cycle and respiratory chain

Cth1 and Cth2 target many transcripts encoding proteins involved in the respiratory metabolism. Cth1 and Cth2 decrease the expression of several TCA cycle transcripts in response to iron deprivation. Down-regulated, ARE-containing mRNAs include those encoding succinate dehydrogenase, but also those encoding the 4Fe–4S cluster-containing aconitase (*ACO1*), isocitrate dehydrogenase (*IDH2*), and alpha-ketoglutarate dehydrogenase (*KGD1* and *KGD2*). Levels of mRNAs encoding aconitase (*ACO1*, *ACO2*), alpha-ketoglutarate dehydrogenase (*KGD1*) and succinate dehydrogenase (*SDH1-4*) are also decreased under low iron conditions [19,32], and metabolomic studies support the idea that TCA cycle function is slowed down under iron deficiency (see section 2.1). Alpha-ketoglutarate dehydrogenase is made of three subunits but is not an iron-containing enzyme. Yet the subunit encoded by *KGD2* requires lipoic acid as a cofactor and the lipoic acid synthase, Lip5, contains two 4Fe–4S clusters. *LIP5* mRNA itself contains two AREs and is down-regulated by Cth2 [32]. This may explain why the cell reduces expression of alpha-ketoglutarate dehydrogenase under low iron conditions. The transcripts encoding all four subunits of respiratory complex II/succinate dehydrogenase (*SDH1-4*) are down-regulated by Cth1 and Cth2. Given that

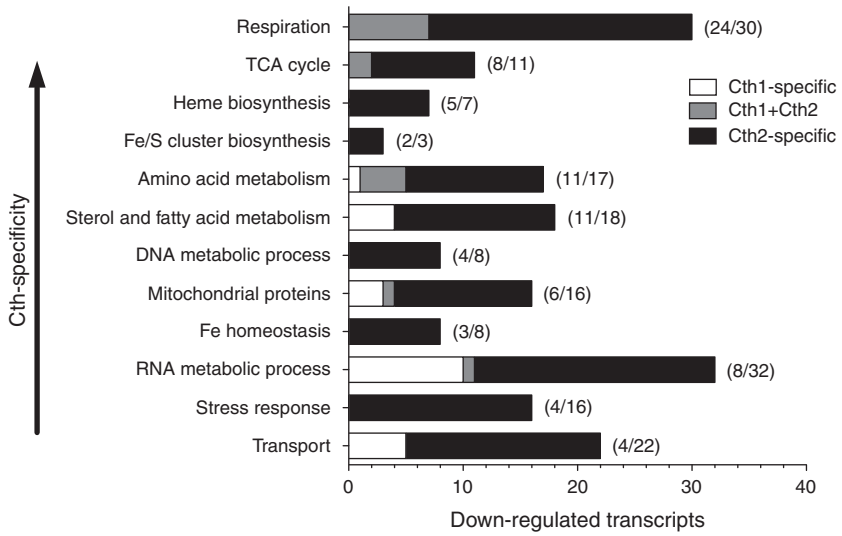

Fig. 2. Cth1 and Cth2 targets clustered by gene function and ordered according to the proportion of ARE-containing transcripts. Microarray data from are combined together in this chart to illustrate which cellular processes are primary targets of Cth1 and Cth2 [32,33]. Cellular processes are ordered from top to bottom according to the ratio of AREs-containing transcripts to the total number of downregulated transcripts (numbers are indicated in parenthesis). Categories with higher ratios are more Cth-specific than categories with lower ratios (Respiration vs. Transport for example). Cth1 and Cth2 targets with functions labeled as "unknown" or "others" in the microarray data were not included in this graph. Cth1-specific targets are in white, targets common to Cth1 and Cth2 are in gray, and Cth2-specific targets are in black.

this enzyme complex requires three Fe–S clusters and one heme *b*, the cell conserves significant amounts of iron in this regulatory step. Complexes III and IV contain several Fe–S cluster and heme cofactors, and many of the mRNAs encoding complex III and IV subunits appear down-regulated by Cth1 or Cth2: *RIP1*, encoding the Rieske Fe–S protein; *CYT1*, encoding cytochrome *c*1; and *CYC1*, encoding cytochrome *c*, are a few examples. The vast majority of these transcripts (80%) contain at least one ARE in their 3′UTR, suggesting that down-regulating the expression of respiration-related genes is a primary function of the Cth1 and Cth2 regulatory system.

3.3.3. Cth1 and Cth2 significantly down-regulate transcripts involved in heme biosynthesis but spare the 2Fe–2S cluster assembly machinery

Several steps of heme biosynthesis are targets of the Cth1/2 regulatory system: mRNAs encoding Hem1, Hem4, Hem13, and Hem15 are all down-regulated by Cth2 expression and contain AREs in their 3′ UTR. Down-regulation of the later steps of heme biosynthesis in the setting of iron deficiency would benefit the cell by preventing the accumulation of porphyrins, which are toxic. Interestingly, *COX10* transcript levels are also down-regulated by Cth2. *COX10* encodes the protoheme IX farnesyltransferase involved in the formation of heme *a*, which is only found in complex IV of the respiratory chain. Cth2 down-regulates only two transcripts encoding proteins involved in the biosynthesis of Fe–S clusters [6]: *ISA1*, which has been confirmed by RNA blotting, and *NFU1* [32]. Isa1 has been described as specifically involved in the maturation of mitochondrial 4Fe–4S cluster-containing enzymes [60,61]. Nfu1 has also been linked to impaired activities of the Fe–S enzymes succinate dehydrogenase and lipoic acid synthase [62]. This suggests that cells react to low iron availability by down-regulating genes involved in the maturation of some 4Fe–4S cluster-containing mitochondrial enzymes. It is noteworthy that none of the enzymes that are essential to mitochondrial Fe–S assembly nor any of the enzymes involved in the cytosolic Fe–S cluster assembly machinery are targets of Cth1 or Cth2.

3.3.4. Cth1 and Cth2 targets in amino acid biosynthesis

The biosynthesis of several amino acids strictly relies on Fe–S cluster-containing enzymes (see above). With the exception of *MET5*, transcripts encoding those proteins contain AREs in their 3′ UTRs and microarray analysis suggests that they are down-regulated by Cth1 or Cth2. Control of *GLT1* transcript levels appears partially Cth1- and Cth2-dependent because seven AREs are found in the 3′ UTR of *GLT1* mRNA. Interestingly, *GLT1* is one of the few ARE-containing transcripts that is targeted by both Cth1p and Cth2p [32,33].

3.3.5. Role of Cth1 and Cth2 in ergosterol and fatty acid synthesis

As mentioned above, some enzymes involved in sterol, fatty acid, and sphingolipid synthesis contain iron cofactors. While transcripts encoding several of these enzymes are up-regulated under conditions of iron deficiency, they also contain AREs and are subject to Cth2-mediated down-regulation. These contradictory changes may reflect more of the regulatory "fine tuning" that is attributed to Cth1 and Cth2, or they may indicate that some of the changes in transcript stability attributed to Cth proteins are not significant for cellular metabolism.

3.3.6. Cth1- and Cth2- mediated regulation of ribonucleotide reductase

Ribonucleotide reductase (RNR) is an essential enzyme that catalyzes the conversion of ribonucleotides to the corresponding deoxyribonucleotides, which are used for synthesis and repair of DNA. RNR is composed of two subunits, R1, the catalytic subunit, and R2, which contains an essential diiron center. In *S. cerevisiae*, the R1 subunit consists of an Rnr1p homodimer and the R2 subunit consists of an Rnr2–Rnr4 heterodimer. RNR activity is partially regulated by controlling the localization of the Rnr2–Rnr4 heterodimer. Except during the S phase of the cell cycle and in responding to DNA damage, Rnr1 is normally localized mainly in the cytoplasm, while Rnr2–Rnr4 is predominantly localized in the nucleus [63]. Wtm1, a WD40 protein, anchors the Rnr2–Rnr4 complex in the nucleus, limiting its export to the cytoplasm and limiting the activity of the holoenzyme.

RNR2 and *RNR4* transcripts were identified as potential Cth1 and Cth2 targets in transcriptome studies and they exhibit AREs in their 3′UTR. *WTM1* mRNA was also identified as a Cth1 and Cth2 target in the same transcriptome studies and it harbors two AREs in its 3′UTR [32,33]. In trying to decipher how RNR activity is affected under iron deficiency, Sanvisens and coworkers observed that, upon iron depletion, levels of dATP and dCTP increase two-fold, indicating that RNR function is maintained, even though iron availability is decreased [64]. The authors could explain this counterintuitive observation by showing that Rnr2–Rnr4 translocates from the nucleus to

the cytoplasm during iron deficiency. This relocation event was traced to a Cth1- and Cth2-dependent decrease in Wtm1 protein levels, which allowed the Rnr2-Rnr4 complex to leave the nucleus. The authors also showed that down-regulation of the *WTM1* transcript is mediated by a direct interaction between the Cth1 and Cth2 TZFs and the *WTM1* AREs. Surprisingly, *RNR2* and *RNR4* transcripts were also shown to be direct targets of Cth1 and Cth2. Altogether, these experiments showed that under low iron conditions, Cth1 and Cth2 regulate yeast RNR function at multiple levels by 1) enhancing R2 translocation to the cytosol and thereby increasing RNR activity and by 2) down-regulating *RNR2* and *RNR4* transcripts and thereby limiting the synthesis of new R2 subunits. The net effect of these regulatory steps would be to maintain RNR activity in the face of iron deficiency while minimizing the flow of iron into newly synthesized R2.

4. Adaptation to iron deficiency in other ascomycetes

S. cerevisiae relies on the major iron-dependent transcription factors Aft1 and Aft2, the heme-dependent transcriptional activator Hap1, Hap4, and the post-transcriptional regulators Cth1 and Cth2 to coordinate the metabolic responses to iron deficiency. Other species of fungi must also respond to changes in the availability of extracellular iron, and those species that have been studied in detail also respond to iron deficiency by expressing systems devoted to iron acquisition and altering cellular metabolism in ways that conserve iron. Surprisingly, other species of fungi have evolved a common regulatory mechanism, which is completely different from that of *S. cerevisiae*, to achieve these responses to iron deficiency. With the exception of *Kluyveromyces lactis* and *C. albicans*, Aft1 orthologs have not been identified in other fungi, nor has a functional ortholog of Cth1 or 2 been identified [65,66]. Instead, iron homeostasis is mediated by two negative-acting, iron-sensing transcriptional repressors, a GATA-type factor and a CCAAT-binding factor. The coordinated action of these two transcriptional repressors is critical for the adaptive response to iron deficiency.

4.1. Identification of GATA-type transcription factors

The first hint that other fungi relied on regulators unlike Aft1 came from studies in the basidiomycete *Ustilago maydis*, using a genetic screen for mutants that constitutively produced the siderophore ferrichrome [67]. Siderophores are small molecules, synthesized and secreted by most species of fungi, which bind ferric iron with high affinity and specificity. Fungi rely on the uptake of ferric siderophores to meet their metabolic iron requirements. Although *S. cerevisiae* does not synthesize siderophores, it can take up a variety of siderophores secreted by other unicellular organisms. It was subsequently shown that the negative-acting *U. maydis regulator of biosynthesis of siderophores*, or Urbs1, repressed the transcription of the ornithine N^5-oxygenase-encoding *sid1* in response to high iron [68]. Promoter analysis of *sid1*, which catalyzes the first committed step in siderophore biosynthesis, revealed the presence of the conserved motif 5′-(G/T)GATAA-3′, the cognate binding site for the GATA transcription factor family. Subsequent studies showed that GATA factors orthologous to Urbs1, including SreA in *Aspergillus fumagatus* and *A. nidulans*, Fep1 in *S. pombe*, Sre1 in *Histoplasma capsulatum*, Sfu1 in *C. albicans*, SRE in *Neurospora crassa*, and Cir1 in *C. neoformans*, repress genes involved in iron acquisition and mobilization [69–74].

In addition to multiple genes involved in siderophore production, iron-responsive GATA-type factors also control expression of genes involved in siderophore iron uptake, reductive iron uptake, and mobilization of stored iron from the vacuole. For example in *S. pombe*, Fep1 represses the transcription of the ferric reductase *frp1*$^+$, the ferrous iron oxidase *fio1*$^+$, and the iron permease *fip1*$^+$ [73,75]. Other iron-responsive GATA targets are involved in mobilization of iron from cellular storage sites, such as *abc3*$^+$, the Fep1 target that encodes a vacuolar iron efflux pump [76].

Insight into the mechanism of iron-responsive regulation of GATA-type factors initially came from studies of Fep1 [9]. Similar to other atypical iron-responsive GATA-type repressors, Fep1 contains two Cys_2Cys_2-type zinc fingers (N-terminal ZF1 and C-terminal ZF2), interconnected by a 27-amino acid linker region containing four highly conserved cysteine residues. *In vitro* and *in vivo* data indicate that, although ZF2 is primarily responsible for the DNA-binding activity of Fep1, ZF1 also contributes to the affinity for GATA-containing DNA. Conserved cysteine residues in the linker region appear to be required for both high-affinity DNA interactions and iron-dependent activation. *In vitro* evidence suggests that Fep1 binds iron directly, possibly through the conserved cysteine residues of the linker region. Together, these results indicate that the iron-responsive GATA factors share an evolutionarily conserved mechanism of directly binding iron to mediate repressor function. Under conditions of iron deficiency, the GATA factor does not bind iron and does not interact with GATA elements in the promoter regions of the iron-repressed genes. In the absence of the GATA-mediated repression, the genes involved in iron acquisition are highly expressed.

4.2. Identification of CCAAT-binding complexes in A. nidulans and S. pombe

In analyzing the transcriptomes of iron-deficient and iron-replete *A. nidulans* and *S. pombe*, investigators noted that several genes involved in iron-dependent processes were down-regulated during iron-deficiency [77–79]. Initially, the identity of the *trans*-acting factor(s) controlling expression of these genes was unclear. Sequence analysis revealed the presence of the 5′-CCAAT-3′ motif in one or more copies in the regulatory promoters of these genes. This motif suggested the involvement of the CCAAT-binding complex (CBC) in the transcriptional down-regulation of the iron-requiring genes. The first CBC identified in fungi was the Hap complex of *S. cerevisiae*, which is responsible for inducing genes involved in oxidative phosphorylation in response to growth on non-fermentable carbon sources [80]. The *S. cerevisiae* Hap complex consists of four subunits, Hap2, Hap3, Hap4, and Hap5. Hap2/3/5 are found pre-assembled in a complex that is sufficient to confer DNA binding activity, but is transcriptionally incompetent [81,82]. Association of Hap4, which contains activation domain function, with Hap2/3/5 results in a transcriptionally active complex [83]. Because Hap2/3/5 protein levels are constitutive, Hap activator function is ultimately regulated by the availability and activity of Hap4, which is transcriptionally controlled by carbon source availability. Recent studies also indicate that Hap4-dependent transcription can also be activated by iron [37].

Evidence that a homologous CBC in *A. nidulans* was involved in iron regulation came from a yeast two-hybrid analysis, which suggested that the Hap2 ortholog, HapB, physically interacted with a newly identified protein, HapX [84]. Initially, *hapX* did not appear to function in a CBC context, as it failed to complement a *hap4*Δ mutant strain in *S. cerevisiae* [85]. Moreover the orthologous genes induced by the Hap4/CBC activator complex in *S. cerevisiae*, such as cytochrome c, aconitase, and 5-aminolevuilnate synthase, are repressed in response to iron starvation in other ascomycetes, while they are induced by the *S. cerevisiae* Hap CBC. Thus, it initially appeared that the *A. nidulans* CBC complex did not play a role in repressing these genes. HapX also demonstrated no homology to *S. cerevisiae* Hap4, with the exception of a small 17 amino acid N-terminal motif. It was later shown, however, that this motif was required for the physical interaction of *S. cerevisiae* Hap4 with the CBC, again raising the possibility that *hapX* encoded the putative fourth subunit of the CBC in *A. nidulans* [81]. Moreover, deletion of *hapB*, *hapC*, or *hapE* resulted in a strong growth defect in iron-limiting media, which strongly suggested a role of the CBC and HapX in iron metabolism in *A. nidulans* [77]. Transcriptome analysis of SreA target genes revealed that *hapX*

was repressed during growth under iron-replete conditions and derepressed in a strain lacking *sreA*, suggesting a model in which *hapX* expression is repressed by SreA in iron-sufficient cells, but is actively expressed in iron-deficient cells, where it can associate with the CBC, resulting in the functional transcriptional repressor complex. Additionally, it was shown that iron abolishes the physical interaction between HapX and HapB, both *in vivo* and *in vitro*, providing a mechanism for HapX/CBC inactivation when iron levels rise [77].

Studies in *S. pombe* also indicate that a CBC complex is involved in the repression of iron-requiring genes in iron deficient cells [78]. *S. pombe* Php4 exhibits only limited homology to *S. cerevisiae* Hap4, but it is, similar to HapX, repressed in iron-sufficient cells by Fep1 and required for the repression of multiple iron-requiring proteins in iron-deficient cells. The transcriptional repression of Php4 also requires the CBC proteins Php2, Php3, and Php5 and increased cellular iron can disrupt the repressing activity of Php4.

Among the genes repressed by HapX/Php4 during iron deficiency is the GATA-family repressor SreA/Fep1, indicating that HapX/Php4 and SreA/Fep1 are interconnected by a negative regulatory feedback loop (Fig. 3): Under iron-sufficient conditions, SreA/Fep1 is active and represses expression of *hapX*/*php4*$^+$, while cellular iron disrupts the activity of HapX/Php4 protein with the CBC. Under iron-deficient conditions, SreA/Fep1 protein loses Fe-dependent repressor activity and *hapX*/*php4*+ are expressed. Active HapX/Php4 protein then represses transcription of *sreA*/*fep1*$^+$. Inactivation of both SreA and HapX in the same strain results in lethality, underscoring the critical role of both *Aspergillus* regulators in maintaining precise intracellular iron concentrations [77]. Interestingly, this synthetic lethality is not observed between *fep1*$^+$ and *php4*$^+$ of *S. pombe* [86].

4.3. HapX/Php4-dependent adaptation to iron deficiency

Identification of HapX/Php4 target genes in *A. spp.* and *S. pombe* suggests that these fungi, similar to *S. cerevisiae*, also down-regulate iron-consuming pathways as an adaptive response to iron deficiency (Table 2). While comprehensive metabolite analysis of iron-starved cells is limiting from other fungi, gene expression profiling suggests that *A. nidulans*, *A. fumigatus*, and *S. pombe* also down-regulate genes encoding iron-dependent proteins in a HapX/Php4-dependent manner. For example, all three species repress expression of genes encoding the 4Fe–4S containing enzymes aconitase, biotin synthase, α-IPM isomerase, and glutamate synthase [77–79,87]. Multiple enzymes of the TCA cycle and the respiratory chain are repressed by HapX/Php4. Genes required for the synthesis of iron-containing cofactors are also repressed by HapX/Php4, such as the 5-aminolevulinate synthase of heme biosynthesis and the Isa1 iron–sulfur cluster assembly protein. Consistent with the repression of these genes in response to iron deficiency by CCAAT-binding proteins, a proteomic analysis of an iron-limited *A. nidulans hapX*Δ strain independently verified that protein levels fell in accordance with the transcriptional regulation by HapX [77]. In total, over 31% of genes that are derepressed in a *hapX*Δ mutant grown in iron-poor medium encode proteins localized to the mitochondria, suggesting a major regulatory role of HapX on mitochondrial function in response to iron starvation. Thus, while fungi employ a common strategy of down-regulating genes involved in iron-consuming pathways, it is accomplished by fundamentally distinct mechanisms.

4.4. SreA–HapX- and Fep1–Php4-mediated adaptation to iron deficiency plays a major role in siderophore production

Siderophore biosynthesis constitutes a substantial portion of the metabolic response to iron deficiency in siderophore-producing fungi. *A. fumigatus*, for example, produces the hydroxymate-type siderophores fusarinine C (FsC) and its acetylated derivative triacetylfusarinine C (TAFC), which are responsible for mobilizing extracellular iron [88]. In all siderophore-producing fungi, the first committed step of biosynthesis is the hydroxylation of ornithine by ornithine-N^5-oxygenase (SidA in *Aspergillus sp.*), which is under negative control by GATA repressors [70,74,89]. Following the generation of N^5-hydroxyornithine, FsC- and TAFC-destined N^5-hydroxyornithine is transacylated by the SreA target gene product SidF, which transfers an anhydromevalonyl acyl group [70,90].

A link between siderophore production and remodeling of ergosterol biosynthetic pathways was recently demonstrated in *A. fumigatus*. Similar to *S. cerevisiae*, cellular ergosterol content is reduced in

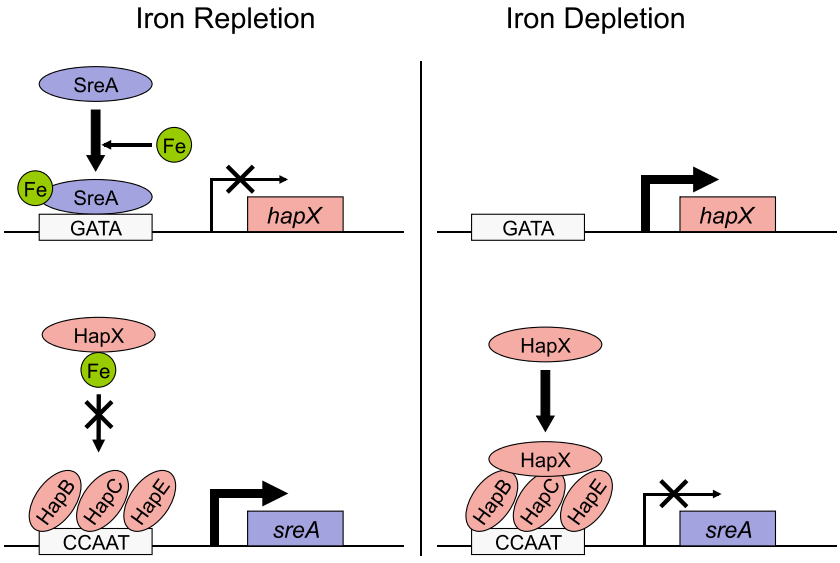

Fig. 3. Coordinated regulation of iron-responsive GATA-type and CBC repressors. The shown schematic is representative of the iron-responsive transcriptional regulatory network in *A. fumigatus*. Under iron-replete conditions, iron binding to SreA increases DNA binding affinity of the repressor for GATA elements found in the promoter of its gene targets, including *hapX*. Iron also inhibits the association of HapX with HapB/C/E. These events result decreased activity of HapX, allowing for increased expression of genes normally targeted by the HapX-CBC. Loss of iron bound by SreA results in dissociation of SreA from its target promoters. Under iron depletion, loss of SreA promoter occupancy increases expression of *hapX*. The concomitant increase in HapX protein levels promotes binding of HapX to HapB/C/E at CCAAT-containing promoters, resulting in repression of HapX targets, including *sreA*. The presence of iron inhibits interaction of HapX with HapB/C/E.

Table 2
Cth1/2, HapX, and Php4 down-regulate similar genes involved in iron-consuming metabolic pathways.

Cell function	Gene product	*S. cer.*[a]	*A. fum.*[b]	*S. pombe*[c]
Respiration	Cytochrome c1, heme protein precursor	*CYT1*	*Afu1g02070*	*cyt1*
	Cytochrome b5	*CYB5*	*Afu2g04710*	*oca8*
	Cytochrome c	*CYC1*	*cycA*	*cyc1*
	Electron transfer flavoprotein-ubiquinone oxidoreductase	*CIR2*	*Afu3g10110*	*SPAC20G8.04C*
	Cytochrome c oxidase subunit V	*COX5A*	*Afu5g10560*	*Cox5*
Amino acid biosynthesis	NADH-dependent glutamate synthase (Glu)	*GLT1*	*Afu1g07380*	*glt1*
	α-Isopropylmalate isomerase (Leu)	*LEU1*	*Afu2g11260*	*leu2*
	Dihydroxy acid dehydratase (Ile/Val)	*ILV3*	*Afu2g14210*	*SPAC17G8.06C*
	Homoaconitase (Lys)	*LYS4*	*lysF*	*lys2*
Fe–S cluster biosynthesis	Iron sulfur assembly protein	*ISA1*	*Afu4g10690*	*isa1*
TCA cycle	Succinate dehydrogenase, flavoprotein subunit	*SDH1*	*Afu3g07810*	*sdh1*
	succinate dehydrogenase, cytochrome b560	*SDH3*	*Afu5g09680*	*sdh3*
	subunit succinate dehydrogenase,	*SDH2*	*Afu5g10370*	*sdh2*
	iron sulfur protein aconitase	*ACO1*	*Afu6g12930*	*acoA*
Biotin synthesis	Biotin synthase	*BIO2*[d]	*Afu6g03670*	*bio2*
Translation	RNase L inhibitor of the ABC superfamily	*RLI1*	*Afu1g10310*	*SPAC24C9.06C*

[a] Targets of Cth2 were genes whose expression was elevated >1.5-fold in an iron-deficient *cth1Δcth2Δ* strain expressing an empty vector compared to *cth1Δcth2Δ* expressing endogenous levels of plasmid-derived Cth2. Targets also contained 3′-UTR ARE sequences [33].
[b] HapX targets demonstrate a. >1.5-fold increase from iron starvation to iron replete growth medium in a wild type strain, b. >1.5-fold decrease from iron starvation to iron sufficiency in *hapX*-deleted strain versus wild type, and c. >1.5-fold increase during iron-starved growth in *hapX*-deleted strain versus wild type [79].
[c] Php4 targets show a. >1.5-fold increase under iron repletion versus iron-starved in wild type cells and b. >1.5-fold increase in *php4*$^{+}$-deleted cells versus wild type [87].
[d] Down-regulated, but not identified as a target of Cth1 or 2.

iron-starved *A. fumigatus*, in part due to reduced expression of genes encoding iron-dependent enzymes in the ergosterol biosynthetic pathway, such as the heme-dependent lanosterol 14-α-demethylase and sterol C-5 desaturase [90]. Paradoxically, the gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting step in ergosterol production, is induced during iron deficiency. This induction is due to the requirement of mevalonate, an intermediate in the sterol biosynthetic pathway, as a precursor for transacylation of anydromevalonyl-CoA to N^5-hydroxyornithine in the extracellular siderophore biosynthetic pathway. Disruption of siderophore biosynthesis by inactivation of *sid*A partially restores ergosterol levels to those of iron-replete cells. This observation suggests that sterol production is largely controlled by mevalonate levels, which are increased by iron deficiency. Both mevalonate levels and intracellular iron levels are affected by the production of extracellular siderophores.

Siderophore-producing fungi also exhibit alterations in ornithine biosynthetic pathways in response to iron-deficiency. Cells require a sustained flux of ornithine, a non-proteinogenic amino acid, to ensure continued siderophore synthesis under iron-limiting conditions. Ornithine can be generated in fungi by the cytosolic degradation of arginine, although this pathway does not appear to be a major contributor to cellular ornithine pools. Mitochondrially-derived glutamate is the main precursor for the biosynthesis of ornithine and is produced through two alternate pathways similar to those found in *S. cerevisiae*. In the first pathway, the ATP-dependent glutamine synthetase (Gln1) catalyzes the incorporation of ammonium into glutamate to form glutamine. The amino group from glutamine is then transferred to 2-oxoglutarate by the 4Fe–4S-containing glutamate synthase (Glt1), with the net production of one molecule of glutamate [91]. Alternatively, glutamate can be directly produced from ammonium and 2-oxoglutarate by glutamate dehydrogenase (Gdh1), which is not dependent on iron for function [92]. Under iron-replete conditions, Fep1 represses expression of *gdh1+* and cells rely on the iron-dependent Glt1 and Gln1 for glutamate synthesis. Conversely, when cells are grown under iron-limiting conditions, Php4 represses expression of *gln1*$^{+}$ and *glt1*$^{+}$, and cells shift to an iron-independent mode of glutamate production. Thus, both *S. pombe* and *S. cerevisiae* remodel glutamate biosynthesis in an iron-dependent manner, which allows for continued glutamate production while sparing Glt1-bound iron. Moreover, shifting to the iron-independent mode of synthesis also spares ATP consumption, which may be adaptive in the setting of reduced respiration and ATP synthesis.

Mitochondrially-derived ornithine appears to be important for siderophore production in other ascomycetes as well. For example, expression of the mitochondrial ornithine efflux pump, *amcA+*, is derepressed in iron-deficient *A. nidulans* and *A. fumigatus* in an SreA-dependent manner [74,93]. This observation is consistent with a model in which ornithine synthesized from mitochondrial pools of glutamate is important for siderophore production. This diversion of glutamate to siderophore production likely impacts pools of other amino acids. Unlike *S. cerevisiae*, *A. fumigatus* grown in iron-poor medium exhibits relatively large changes in the levels of several amino acids, including glutamate [79]. Together, these findings suggest that fungal species coordinate ornithine and mevalonate production in a manner that spares iron without compromising siderophore biosynthesis.

5. Conclusions

Fungi respond to the depletion of iron in the extracellular environment by increasing the expression of systems devoted to iron acquisition, increasing the expression of systems devoted to mobilization of stored iron, and decreasing the expression of systems that require iron for function. The latter response requires changes in the abundance of a large number of proteins, many of which perform critical, essential functions for cells. By selectively reducing expression of iron-requiring enzymes, cells can more efficiently distribute iron to other essential pathways. The importance of this metabolic remodeling to cells is illustrated by the reduced growth under conditions of iron deficiency demonstrated by *S. cerevisiae* strains lacking *CTH1* and *CTH2* and by *A. nidulans* strains lacking *hapX* [32,77]. The impact of these regulators is somewhat surprising given that many, but not all, of the regulated genes exhibit relatively small changes in expression. Transcripts of Cth1 and Cth2 target genes generally exhibit about a two-fold decrease in abundance that is attributable to accelerated mRNA degradation. Changes in the mRNA levels of many, but not all, HapX- and Php4-repressed genes are similarly in the two-fold range. A two-fold reduction in the expression of a single iron-dependent protein may not significantly alter cellular iron pools, but multiplying that two-fold reduction over a large number of genes would result in a substantial “iron savings” for the cell.

The remarkable similarity in the responses to iron deficiency observed in different species of fungi becomes very surprising when one considers that completely different regulatory mechanisms are employed by *S. cerevisiae* and the other fungal species. *S. cerevisiae* relies on transcriptional activators Aft1 and 2 to increase the expression of iron-deficiency proteins, then relies on the regulatory actions of some of these proteins (Cth1, Cth2, Hmx1) to effect metabolic remodeling. Additional regulators, such as the heme-dependent Hap1, also contribute to the metabolic remodeling. Other fungi rely on a pair of transcriptional repressors SreA/Fep1 and HapX/Php4 to coordinate the up-regulation of iron-deficiency genes and down-regulation of iron-utilization genes in the setting of iron deficiency. The differences between these systems may reflect the tolerance these fungi exhibit for disruptions of iron homeostasis. *S. cerevisiae* tolerates huge changes in both environmental and intracellular iron pools, and strains deleted for *Aft1* and *Aft2* grow well under laboratory conditions. Arguably, the dual repressor system of *Aspergillus spp.* and *S. pombe* allows for tighter control of iron homeostasis. Notably *Aspergillus* strains deleted for both *sreA* and *hapX* are not viable, highlighting the need of both interconnected regulatory networks to control iron levels. Perhaps the added metabolic burden of siderophore biosynthesis necessitates this dual repressor system. Surprisingly, monothiol glutaredoxins appear to be involved in iron sensing for all of these transcription factors [13,86,94–97]. Other regulatory mechanisms, such as iron-dependent transcriptional activation through the *S. cerevisiae* Hap4-CBC, remain to be examined, and other regulatory mechanisms are likely involved for *S. pombe*, as well. Iron uptake and utilization are coordinated with oxygen availability in each of these fungal species, as heme synthesis is an oxygen-dependent process and many heme proteins function in pathways that consume oxygen (such as respiration and ergosterol biosynthesis). Yet again, the regulatory mechanisms that effect this coordination differ between *S. cerevisiae* [98,99] and other fungal species [100–102].

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