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The regulation of iron homeostasis in the fungal human pathogen *Candida glabrata*

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Abstract

Iron is an essential element to most microorganisms, yet an excess of iron is toxic. Hence, living cells have to maintain a tight balance between iron uptake and iron consumption and storage. The control of intracellular iron concentrations is particularly challenging for pathogens because mammalian organisms have evolved sophisticated high-affinity systems to sequester iron from microbes and because iron availability fluctuates among the different host niches. In this review, we present the current understanding of iron homeostasis and its regulation in the fungal pathogen *Candida glabrata*. This yeast is an emerging pathogen which has become the second leading cause of candidemia, a life-threatening invasive mycosis. *C. glabrata* is relatively poorly studied compared to the closely related model yeast *Saccharomyces cerevisiae* or to the pathogenic yeast *Candida albicans*. Still, several research groups have started to identify the actors of *C. glabrata* iron homeostasis and its transcriptional and post-transcriptional regulation. These studies have revealed interesting particularities of *C. glabrata* and have shed new light on the evolution of fungal iron homeostasis.

INTRODUCTION

Fungal pathogens are major sources of nosocomial infections, with annual numbers of deaths close to those of malaria [1]. Candidemias are life-threatening systemic infections caused by *Candida* species that are opportunistic pathogens. They are the most common cause of invasive mycosis [2]. About 20 different clinically relevant *Candida* species have been identified to date. *Candida glabrata* is the second most prevalent cause of candidemia, just behind the extensively studied *Candida albicans*. Its incidence has been continuously increasing over the last 30 years [3, 4]. Like *C. albicans*, *C. glabrata* is a human commensal which is commonly found in the mucosal microbiota of healthy people [5, 6]. In individuals with an impaired immune system or severe dysbiosis of the microbiota, these yeasts can cross the epithelial barriers and provoke systemic infections with high mortality rates. The natural tolerance of *C. glabrata* to azole antifungals and its strong capacity to acquire drug resistance make these infections particularly difficult to cure [7, 8]. Moreover, *C. glabrata* has evolved large families of specific adhesins encoding genes which allow for tight interactions with host cells and medical devices [9]. Genome sequencing projects have shown that *C. glabrata* is much more closely related to the model yeast *Saccharomyces cerevisiae* than to *C. albicans*. In fact, *C. glabrata* and *C. albicans* are more distantly related than humans and fishes. In contrast to *C. albicans*, the common ancestor of *S. cerevisiae* and *C. glabrata* underwent a whole genome duplication which was followed by massive gene loss and significant rewiring of multigenic families [10, 11]. Hence, phylogenetically, *C. glabrata* does not belong to the *Candida* clade but to the genus *Nakaseomyces* [12]. More information on the evolution of the *C. glabrata* genome and the origin of its virulence traits can be found in the review of Gabaldon [13, 14].

The invasive strategy of *C. glabrata* largely differs from other *Candida* species [15]. While the morphogenetic transition from yeast to hyphae is key in *C. albicans* infections [16], *C. glabrata* forms hyphae poorly [17, 18] and only the yeast form has been observed in clinics [19]. *C. glabrata* in fact follows a strategy based on stealth and persistence. Indeed, *C. glabrata* is able to survive and even replicate in the phagolysosomes of cells from the innate immune system, hence using the macrophages as Trojan horses [20–24]. Phagolysosome survival involves many different mechanisms including

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Abbreviations: ALA, 5-aminolevulinate; ARE, AU-rich element; CBC, CCAAT binding complex; ChIP-seq, chromatin immunoprecipitationexperiments followed by deep sequencing; CIA, cytosolic Fe-S clusterassembly machinery; CRD, cysteine-rich domain; UTR, untranslated region; YRE, Yap Response Element.

autophagy, response to nutrient limitations and resistance to oxidative stress [21, 25]. Interestingly, about half of the mutants identified as defective for macrophage survival in a genetic screen also exhibited low growth rates in iron starvation conditions, suggesting that iron uptake is crucial for *C. glabrata* intraphagosomal persistence [25].

Iron is an essential element for nearly all living organisms, due to its incorporation in key metalloenzymes, mostly through iron–sulfur clusters and haems. Hence, iron is involved in a large number of cellular processes, including carbon and nitrogen metabolism, respiration, translation, RNA metabolism and DNA repair. Yet, its bioavailability is very limited because it is mostly present in the form of insoluble ferric oxides. The situation is even worst for microbial pathogens because mammalian organisms have evolved sophisticated high-affinity systems to sequester iron from microbes, such as haemoglobin, transferrin or haemopexin [26, 27]. Within the phagosome of macrophages, iron is sequestered from phagocytosed microorganisms by several intraphagosomal iron scavengers (i.e. lactoferrin and NRAMP1) [28, 29]. Iron sequestration is thus considered as a part of the innate immune system called nutritional immunity [30, 31]. As a consequence, resistance to iron starvation is a key feature for host invasion and the genes involved in iron uptake have been shown to be important for the virulence of almost all fungal pathogens examined to date, including *C. glabrata* [32]. Yet, iron overload is highly toxic to the cells, because of both the generation of reactive oxygen species through the Fenton reaction and its ability to replace copper and zinc in metalloproteins, therefore altering their enzymatic activity. Therefore, the concentration of intracellular iron has to be tightly controlled by iron consumption and storage processes. This is particularly challenging for human commensals and pathogens because iron availability is likely to vary considerably mong the different host niches [33]. To maintain this subtle and critical balance between uptake and consuming mechanisms, fungal pathogens use complex gene expression regulatory networks. In this review, we summarize the knowledge on iron homeostasis and on the responses to iron starvation and iron excess in *C. glabrata*. In the last section, we compare the iron-based transcriptional regulation of *C. glabrata* with those of other fungal pathogens. Most of our primary knowledge on iron homeostasis in *C. glabrata* comes from studies in the model yeast *S. cerevisiae*. So, all the sections of this review, except the last one, will have two sub-parts: a first one summarizing the lessons that can be taken from *S. cerevisiae* and a second one presenting the state of the art in *C. glabrata*. We will start with an overview of the main actors of iron homeostasis in these yeasts.

C. GLABRATA IRON HOMEOSTASIS

Please note that this section is a summary of what is known about iron uptake and iron storage in yeasts. For recent and more comprehensive reviews on the mechanisms of iron homeostasis in fungi, we recommend the following references [32, 34].

Lessons from *S. cerevisiae*

Extracellular iron uptake

In *S. cerevisiae*, three main routes have been identified for iron uptake. The first is high-affinity reductive iron uptake which involves extracellular reduction of ferric iron by ferric reductases encoded by the *FRE* genes [35, 36], followed by reoxidation to its ferric form by the Fet3 multicopper ferroxidase and lastly iron import by the Ftr1 permease [37–39]. The second route is the low-affinity reductive iron uptake system, relying on ferric reductases and on the divalent metal ion transporters Fet4, Smf1 and, possibly, Smf2 [40, 41]. Reductive iron uptake is strongly connected to the metabolism of other metals. Fet4 also transports zinc and copper and Smf1 and 2 are manganese importers [42–45]. Fet3 is a copper-dependent enzyme, post-translational maturation of which depends highly on copper import in the late golgi compartment mediated by the Ccc2 and Atx1 proteins [46, 47]. Accordingly, the vacuolar copper exporter Ctr2, which provides copper to Ccc2 and Atx1, is regulated by iron levels [48].

The third source of extracellular iron is siderophore uptake. Siderophores are small ferric iron-scavenging organic molecules secreted by some microorganisms to capture iron in their environment [49]. *S. cerevisiae*, like *C. albicans* and *C. glabrata*, does not produce siderophores on its own but it is able to capture xenosiderophores from other fungal and bacterial species [50]. It does so by expressing at its plasma membrane siderophore transporters from the *ARN* gene family, each Arn protein being more or less specific for different classes of bacterial or fungal siderophores [51]. *S. cerevisiae* has four *ARN* genes: *ARN1*, *ARN2*, *SIT1* (*ARN3*) and *ENB1* (*ARN4*) [52–56]. Additionally, three cell-wall proteins (Fit1-3) have been involved in siderophore retention at the cell surface in this species [57, 58].

Of note, other fungal species, such as *C. albicans*, are also able to obtain iron from the haemoglobin of red blood cells. This capacity relies on haem/haemoglobin receptor-encoding genes *RBT5*, *PGA10* (*RBT51*), *PGA7* and *CSA2* [59–61]. Homologues of *RBT5* exist in *S. cerevisiae* but they are involved in cell-wall maintenance and not in iron homeostasis [62].

Intracellular iron metabolism

Besides extracellular iron uptake, a key aspect of iron homeostasis is the circulation, mobilization and consumption of intracellular iron stocks. One of the major iron sequestration sites is the vacuole. The Fet5 and Fth1 proteins are vacuolar homologues of Fet3 and Ftr1, respectively, which act as high-affinity iron exporters to transport iron from the vacuole to the cytosol [63, 64]. Additionally, Smf3, a paralogue of Smf1 and Smf2 located at the vacuolar membrane, was also proposed to transport iron out of the vacuole based on the fact that its expression is induced by iron starvation and that *SMF3* null mutation triggers the iron starvation response [43, 65]. Fre6 is probably the ferric reductase required for these two reductive iron vacuolar export systems [66]. Reciprocally, the import of iron from the cytosol into the vacuole is performed by the Ccc1 transporter, which plays a key role in protecting

the cell from detrimental cytosolic and mitochondrial iron accumulation [67–69].

As mentioned in the introduction, iron is used as a cofactor for metalloproteins, mainly through its incorporation into prosthetic groups such as haem or iron–sulphur clusters (Fe-S clusters). Although being quite simple structures, Fe-S clusters are biosynthesized through a complex series of reactions, beginning in the mitochondria (reviewed in [70–72]). The biogenesis of mitochondrial Fe-S clusters starts with the liberation of sulphur from cysteine by the desulphurase complex Nfs1-Isd11. Iron is provided by the short-term iron storage protein frataxin (encoded by *YFH1*) and the Fe-S cluster is transiently assembled onto the scaffold proteins Isu1 or Isu2. This step involves a short electron transfer chain composed of the ferredoxin Yah1 and the ferredoxin reductase Arh1, probably for sulphur reduction. Transfer of the Fe-S cluster from the Isu1/2 scaffold to mitochondrial recipient apoproteins requires the chaperones Jac1 and Snq1, the co-chaperone Mge1 and the glutaredoxin Grx5. Alternatively, the Fe-S clusters can be exported under a still unknown form from the mitochondria to the cytosol by a process involving the Atm1 transporter and the sulphur-rich tripeptide glutathione. They are then transferred to cytosolic and nuclear recipient proteins by the cytosolic Fe-S cluster assembly machinery (CIA) composed of the electron transfer chain Tah18-Dre2, the scaffold proteins Cfd1, Nbp35 and Nar1, and the CIA targeting complex Cia1-Cia2-Mms19. This process requires the glutaredoxins Grx3 and Grx4, which play a pivotal role in the sensing and distribution of iron in the cytosol and in the nucleus [73, 74]. Some iron-containing proteins need additional Fe-S assembly factors. This is the case for the aconitase Aco1 in the mitochondria, maturation of which requires Isa1, Isa2, Iba57 and Nfu1 [75–77], for the mitochondrial succinate dehydrogenase subunit Sdh2, which requires Nfu1, Bol1 and Bol3 [76, 78], and for the cytosolic and nuclear general translation termination factor Rli1, processing of which involves the Yae1 and Lto1 adaptors [79]. Besides their various contributions to cell metabolism as enzymatic co-factors, Fe-S clusters play key roles in iron sensing and in the iron-dependent regulation of gene expression (see the two next sections)

Haem biosynthesis is a conserved, eight-step pathway [80] which also starts in the mitochondria by the synthesis of 5-aminolevulinate (ALA) from succinyl Co-A and glycine, catalysed by the Hem1 ALA-synthase. Succinyl co-A is provided by the Krebs cycle and glycine is imported into mitochondria by the Ymc1 and Hem15 transporters [81, 82]. ALA is then exported to the cytosol where it is transformed into coproporphyrinogen III by four sequential reactions operated by the Hem2, Hem3, Hem4 and Hem12 enzymes respectively. Coproporphyrinogen III is then transported into mitochondria and converted to Protoporphyrinogen IX by the mitochondrial outer membrane protein Hem13. In the mitochondrial matrix, Hem14 converts protoporphyrinogen IX into protoporphyrin IX, to which iron is added by the ferrochelatase Hem15 to eventually form haem. The rate-limiting steps in this process have been suggested to be the reactions performed by Hem2 and Hem3 [80]. Importantly, yeasts can release iron from haem using an endoplasmic reticulum-located haem oxygenase encoded by the *HMX1* gene [57, 83, 84]. In *S. cerevisiae*, haem has an important role in sensing oxygen [85–87]. However, compared to Fe-S clusters, haem plays a minor role in the transcriptional regulation of iron homeostasis [88, 89].

Thus, the iron chelation steps in the biosynthesis of haem and Fe-S clusters take place in mitochondria [90]. The mitochondrial iron supply is mostly performed by the Mrs3 and Mrs4 carriers [91–95]. In the absence of Mrs3 and Mrs4, the pyrimidine transporter Rim2 is also able to play a role in mitochondrial iron import, but the physiological significance of this activity remains questionable [69, 90, 96, 97]. Additionally, the Mmt1 and Mmt2 cation facilitator transporters have been proposed to function as mitochondrial iron exporters [98, 99], but the elevated mitochondrial iron levels observed in strains overexpressing Mmt1 and Mmt2 suggest that they could also act as iron importers [98, 100].

State of the art in *C. glabrata*

Extracellular iron uptake

C. glabrata has conserved orthologues for most of the *S. cerevisiae* iron homeostasis genes, except for *FIT1-3*. For many of them, the function was not directly addressed in *C. glabrata* and was inferred from the *S. cerevisiae* orthologues (Fig. 1).

Null mutants for orthologues of the high-affinity iron uptake system (i.e. *CgFTR1*, *CgFET3*, *CgCCC2*) are highly sensitive to iron starvation, have low intracellular iron concentrations, show low survival in macrophages and are defective for proliferation in a murine model [101, 102]. The CgFtr1 retrograde trafficking pathway, controlled by the phosphoinositide 3-kinase CgVps34, is also required for survival in both iron excess and starvation and for colonization in mice [103]. These results suggest a pivotal role for the *C. glabrata* high-affinity iron transport system in iron acquisition. In contrast, the low-affinity system orthologue *CgFET4* is dispensable for growth in iron-limited media although its deletion causes a decrease in the intracellular iron content, suggesting a relatively minor role in iron uptake [102]. Accordingly, the deletion of *CgFET4* alters neither survival in macrophages nor proliferation in mice [102].

One striking difference between *C. glabrata* and other yeast species is the relatively low number of ferric reductases encoded in its genome. *S. cerevisiae* and *C. albicans* have nine and 18 FRE genes respectively, while *C. glabrata* has only three of those (*FRE6*, *FRE8* and *AIM14*) [104]. While *CgFRE6* and *CgFRE8* expression is regulated by iron availability [104–106], a double *cgfre6*/*cgfre8* null mutant shows wild type levels of extracellular iron reduction [104]. *C. glabrata,* by contrast, relies on an excreted, non-enzymatic, low-molecular-weight compound, yet to be identified, for reductive iron uptake [104]. Still, growth of a *CgFRE8* mutant is strongly inhibited upon iron limitation and shows lower survival in macrophages compared to the wild type strain [25]. Although a *CgFRE6* mutant showed no growth defect upon iron starvation, it has

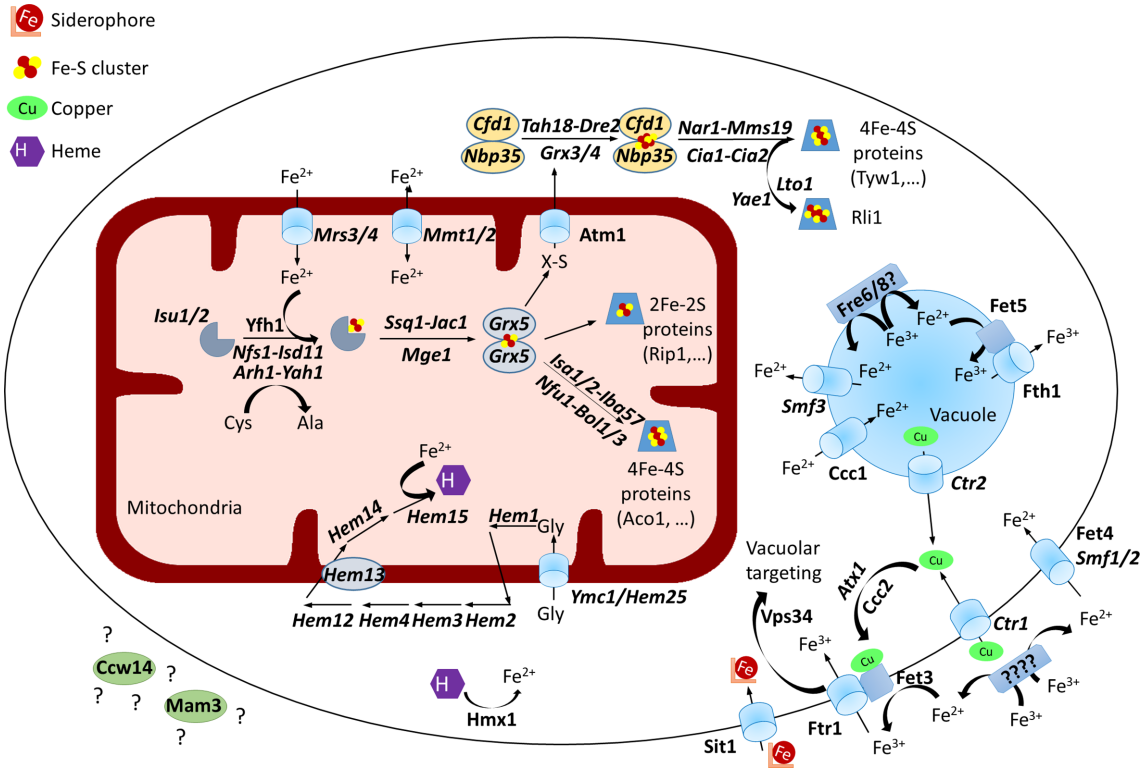

Fig. 1. *C. glabrata* iron homeostasis. The pathways represented on this figure include siderophore uptake (Sit1), extracellular iron reductive uptake (Fet3-Ftr1, Fet4, Smf1/2 and an as yet unknown non-proteic secreted reductant), retrograde trafficking of Ftr1 (Vps34), extracellular and vacuolar copper trafficking (Ctr1/2, Ccc2, Atx1), vacuolar iron trafficking (Smf3, Fet5-Fth1, possibly Fre6/8, Ccc1), mitochondrial iron trafficking (Mrs3/4, Mmt1/2), mitochondrial Fe-S cluster biogenesis and assembly (at the upper part of the mitochondria, X-S stands for an as yet unknown intermediate for Fe-S cluster transport by Atm1), cytoplasmic Fe-S assembly (just above mitochondria), haem biosynthesis (at the bottom part of mitochondria), haem recycling (Hmx1) and the two cell-wall proteins of unknown function Ccw14 and Mam3. The transcriptional regulation of iron homeostasis occurring in the nucleus has been omitted; this is presented in detail in Figs 2 and 3. The names of proteins whose function has not been directly addressed in *C. glabrata* and are inferred from the *S. cerevisiae* orthologue are written in italics.

also been reported to have attenuated virulence in *Drosophila* and mouse [102, 107]. A reasonable hypothesis would be that CgFre8 and/or CgFre6 play other roles in iron homeostasis than extracellular iron reduction, for instance by regulating iron vacuolar export, as ScFre6 does [66, 104].

In contrast to *S. cerevisiae*, *C. glabrata* has only one siderophore transporter-encoding gene in its genome, which was named *CgSIT1*. *CgSIT1* null mutants are unable to grow on media containing the fungal siderophores ferrichrome, ferrirubin or coprogen as sole iron sources [108], strongly suggesting that CgSit1 is indeed a siderophore transporter. In contrast, *C. glabrata* is not able to use the bacterial siderophores enterobactin or desferrioxamine, the latter being a substrate for ScSit1, indicating that CgSit1 does not fulfil all the functions of the four *S. cerevisiae* Arn proteins. Like many genes involved in iron uptake, *CgSIT1* is required for optimal survival within the macrophages [108]. Yet, *Cgsit1Δ* cells have normal proliferation rates in a murine model, which suggests that siderophore uptake is not essential for host invasion [102]. Interestingly, a similar situation has been described in *C. albicans*: this species also has only one *ARN* gene (*CaSIT1*), the deletion of which decreases the ability of *C. albicans* to invade reconstituted epithelium *in vitro* but not its dissemination potential in animals [109, 110].

Like *S. cerevisiae* and unlike *C. albicans*, *C. glabrata* displays very weak haemolysis potential and poorly utilizes extracellular haem as a main iron source [102, 104, 108]. Still, it possesses an Rbt5-like cell-surface protein named Ccw14 and a haemolysin-like protein named Mam3. Mutants for these genes show no growth defect in any conditions tested but exhibit a moderate increase in intracellular iron content [102]. Interestingly, the absence of CgCcw14 or CgMam3 dramatically alters the proliferation potential of the cells in mice. Additionally, the deletion of *CgCCW14* or *CgMAM3* renders *C. glabrata* cells more adherent to mammalian epithelial cells. However, this phenotype is attributed to changes in the cell-wall architecture and the role of these two proteins in *C. glabrata* iron homeostasis remains to be established [102]. Finally, deletion of the haem oxygenase-encoding gene *CgHMX1* has no obvious impact on growth in iron-limited conditions and intracellular iron content [102].

Intracellular iron homeostasis

In *C. glabrata*, deletion of the genes encoding actors in the vacuolar iron high-affinity export system (*CgFTH1* and *CgFET5*) has no impact on growth in iron starvation conditions but the *cgfet5Δ* mutant showed lower proliferation in the kidneys of a murine model [102]. Conversely, deletion of *CgCCC1* renders the cells highly sensitive to iron excess, similarly to what was reported for its *S. cerevisiae* orthologue [68, 101].

The functioning of the Fe-S clusters and haem biogenesis pathways has not been directly addressed in *C. glabrata*. However, a mutant in *CgYFH1* is defective for growth in rich media and exhibits both a dramatic increase in intracellular iron content and a severe decrease in aconitase (an Fe-S cluster-containing enzyme) activity, suggesting that the important role of frataxin in Fe-S cluster biogenesis is conserved in *C. glabrata* [102]. Deletion of *CgATM1* also causes general growth defects [101].

Finally, iron availability is tightly linked to ergosterol metabolism and azole antifungal resistance in *C. glabrata*. More precisely, iron depletion has been shown to modulate sterol uptake, and fluconazole susceptibility is higher under low iron conditions [111, 112]. Accordingly, mutants for the high-affinity iron uptake machinery show increased fluconazole susceptibility [102]. Notably, sterol biosynthesis requires several iron-containing enzymes (e.g. the cytochrome P450 encoded by *CgERG11* is a haem-binding protein).

To conclude this section, the current knowledge of iron homeostasis in *C. glabrata*, although incomplete, suggests that the reductive pathway is the main actor of extracellular iron uptake in this species. This seems to be true both in laboratory conditions and in the host (Table 1). The existence of only one siderophore transporter Sit1 and the absence of Fit proteins in *C. glabrata* indicate that siderophore uptake is less critical for iron metabolism than it is in *S. cerevisiae*. Moreover, in

Table 1. Iron homeostasis and *C. glabrata* pathogenesis

This table summarizes what is known about the impact of deletions of iron homeostasis genes on growth in iron-limitng conditions, survival in macrophages, dissemination in the host (murine model for most of the publications) and virulence in ***Drosophila***. The cells in grey stand for 'Not Determined'. The corresponding references are indicated.

Strain	Growth defect in iron starvation	Survival in macrophages	Dissemination in host	Virulence in *Drosophila*	Reference(s)
Fet3Δ, Ftr1Δ	Yes	Decreased	Decreased in kidney		[101, 102]
Ccc2Δ	Yes	Decreased	Decreased in kidney and brain		[102]
Cth2Δ, Sef1Δ	Yes	Unchanged	Unchanged	Unchanged	[101, 107]
Aft1Δ	Yes		Decreased in blood		[102]
Sit1Δ	No/yes when siderophores are the sole iron sources	Unchanged/ decreased when pretreated with siderophore	Unchanged		[102, 108]
Fre8Δ	Yes/no	Decreased	Unchanged	Unchanged	[25, 101, 107]
Mam3Δ	No	Decreased	Decreased in kidney, liver, spleen and brain		[102]
Hmx1Δ	No	Increased	Increased in brain		[102]
Fet5Δ	No	Slightly decreased	Decreased in kidney	Unchanged	[101, 102, 107]
Fre6Δ	No	Unchanged	Decreased in kidney/ increased in liver	Decreased	[101, 102, 107]
Ccw14Δ	No	Unchanged	Decreased in liver and spleen		[102]
Fet4Δ	No	Unchanged	Increased in kidney		[102]
Fth1Δ	No	Unchanged	Unchanged	Unchanged	[101, 102, 107]
Aft2Δ	No	Unchanged	Unchanged		[101, 102, 106]
Smf3Δ	No			Unchanged	[101, 107]
Mmt2Δ	No				[101]
Yap5Δ	No, but slightly sensitive to iron excess				[101, 113]
Ccc1Δ	No, but highly sensitive to iron excess			Unchanged	[101, 107]

contrast to *C. albicans*, *C. glabrata* is a poor utilizer of haemin and has weak haemolytic potential. Due to the rarity of free iron in blood, this raises the question of the iron source used by *C. glabrata* when invading the human body.

As mentioned in the introduction, many of these iron homeostasis genes have their expression subjected to tight control. The following two sections compile the available information on the mechanisms controlling this regulation.

TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL RESPONSES TO IRON STARVATION

Lessons from *S. cerevisiae*

The *S. cerevisiae* iron regulon

In a nutshell, upon iron starvation, the expression of iron storage genes is repressed while iron uptake genes are induced. More specifically, in response to iron deficiency, *S. cerevisiae* dramatically increases the mRNA levels of most of the genes encoding proteins involved in the reductive high and low-affinity iron acquisition system (e.g. *FRE1-6*, *FET3*, *FTR1*, *FET4*), copper trafficking towards Fet3 (*CCC2*, *ATX1*, *CTR2*), siderophore uptake (*ARN1-4* and *FIT1-3*), vacuolar iron export (*FTH1* and *SMF3*), mitochondrial iron export (*MMT2*) and haem recycling (*HMX1*). This set of iron-starvation induced genes is called the iron regulon (reviewed in [114]). It also includes a gene of unknown function, *LSO1*, and the post-transcriptional regulator-encoding gene *CTH2* [115–117]. Meanwhile, iron limitation triggers a moderate decrease in mRNA levels of 'iron-consuming genes', i.e. genes involved in vacuolar iron storage (*CCC1*) and in the biosynthesis of haem and Fe-S clusters (with the notable exception of the scaffold protein-encoding genes *ISU1/2* which are induced and belong to the iron regulon per se) [115, 116, 118]. This set of repressed ironconsuming genes also includes a large number of genes encoding iron-containing proteins, which are involved in respiration (*RIP1*, *CYC1*, *CYT1*, *CCP1*), the TCA cycle (e.g. *ACO1*, *SDH2*, *SDH4*, *KGD1*), amino acid metabolism (e.g. genes encoding the glutamate synthase Glt1, the sulphite reductase Met5, the dihydroxyacid dehydratase Ilv3, the isopropylmalate isomerase Leu1, the deoxyhypusine hydroxylase Lia1 and the homoaconitase Lys4), biotin biosynthesis (e.g. *BIO2*), DNA synthesis and repair (e.g. genes encoding the Rnr2 and Rnr4 subunits of the ribonucleotide-diphosphate reductase), RNA processing (e.g. the translation termination factor Rli1), etc. [115, 116].

Transcriptional control by Aft1 and Aft2

Activation of the iron regulon is controlled by the Aft1 and Aft2 transcription factors (reviewed in [119]). *In vitro*, Aft1 and Aft2 recognize the same PyPuCACCC DNA motif in the promoter of their target genes with their Zn^{2+}-containing WRKY-GCM1 domain [120–123]. *In vivo*, Aft1 and Aft2 have slightly distinct iron response elements. Aft1 preferentially binds to the TGCACCC motif while Aft2 prefers the ACACCC consensus [124]. Aft1 is the predominant regulator of iron uptake and the *aft1Δ* mutant has a decreased fitness when iron is limited [125, 126]. In contrast, the *aft2Δ* single mutant has no growth defect. Deletion of the two genes abolishes growth under iron-limited conditions [121, 125]. Aft1 more specifically regulates extracellular iron uptake. For instance, *FET3*, *FTR1*, *FRE1-4*, *CCC2*, *CTR2*, *FET4*, *LSO1*, *ARN1-4* and *FIT1-3* responses to iron starvation are severely diminished in a *aft1Δ* mutant [45, 48, 56, 58, 116, 123–128]. Additionally, Aft1 is responsible for the induction of *FRE5*, *HMX1* and *CTH2* [57, 115]. Aft2 is, by contrast, dedicated to the regulation of intracellular iron trafficking. Aft2-preferential targets are genes encoding the mitochondrial iron importer Mrs4, the Fe-S cluster scaffold protein Isu1, and the vacuolar iron exporters Smf3, Fth1-Fet5 and their associated ferric reductase Fre6 [43, 124, 129]. However, Aft1 and Aft2 have largely overlapping function. In the absence of Aft1, the residual expression of *CCC2*, *HMX1*, *CTH2* and *FTR1* is ensured by Aft2 and the growth defect associated with the absence of *AFT1* is partially complemented by the overexpression of *AFT2* [115, 121, 124, 125]. Moreover, gain-of-function mutants of *AFT1* or *AFT2* similarly activate the whole iron regulon [56, 58, 118, 121, 123–126, 129].

Of note, Aft1 and Aft2 also participate in other, apparently iron-independent, functions. For instance, Aft2 protects the cells from toxic doses of the metalloid selenite by indirectly repressing the low-affinity phosphate transport system, which is a key entry point for selenite [130]. Aft1 has been identified in genetic screens as a partner of the chromosome segregation machinery and as an actor of the DNA damage response [131–134].

Regulation of the regulators: a key role for Fe-S clusters and glutaredoxins

Activity of the Aft transcription factors was initially thought to be mainly controlled by their nucleocytoplasmic localization. Indeed, nuclear localization of Aft1 is strongly dependent on iron status, and in iron-replete conditions Aft1 is actively shuttled to the cytosol by the exportin Msn5 [135, 136]. However, further studies revealed that the Msn5-mediated export is dispensable for the inhibition of Aft1 and Aft2 activity in iron-replete conditions and that this inhibition mostly occurs at the level of their DNA binding efficiency [120, 137]. The Fe-S clusters and the Grx3 and Grx4 glutaredoxins have been shown to be key to this regulation. In the cytosol of iron-replete cells, Grx3 and Grx4 actively interact with the Fe-S clusters exported from the mitochondria by Atm1 [137]. The formation of these Grx3/4-Fe-S cluster complexes requires glutathione. These complexes can interact with the Fra2 (Bol2) protein, which is similar to the Bol1 and Bol3 Fe-S cluster adaptor proteins [138–140]. The Grx3/4-Fe-S-Fra2 complex can then transfer the Fe-S cluster to the Aft transcription factors [120, 137, 141]. It is not clear whether this interaction occurs in the cytoplasm, the nucleus or both [138, 141, 142]. The Fe-S-bridged Aft1 and Aft2 proteins form homodimers with weak DNA affinity and, as a consequence, are efficiently exported from the nucleus [120, 136, 137, 142]. This process also involves the aminopeptidase Fra1 but its

precise role has not yet been established [138]. Upon iron starvation, loading of the Fe-S cluster to Aft1 and Aft2 is less frequent and these factors are in a monomeric form which stably interacts with DNA to activate transcription [120]. Consistent with this model, depletion or null mutations in the genes encoding Grx3, Grx4, Fra2, Fra1, the Fe-S cluster mitochondrial exporter Atm1 or the mitochondrial Fe-S cluster core assembly machinery (e.g. Nfs1, Yah1) leads to constitutive activation of the iron regulon [127, 137, 138, 141–144]. So do mutations in the Grx3/4 active site required for Fe-S cluster capture or in the Aft1 motif required for its interaction with Grx3/4 and Fe-S clusters [136, 137, 139, 141]. Similarly, variations in the concentration of glutathione, which is required for both mitochondrial export of Fe-S clusters and formation of the Fe-S-Grx3/4 complexes, strongly impacts the activity of Aft1 [127, 145]. Conversely, mutations in the downstream Fe-S cluster pathway (CIA machinery and *ISA* genes) have no effect on the regulation of Aft1 [127]. Additional layers of regulation of the Aft transcription factors have been described. For instance, the phosphorylation of Aft1 by the mitogen-activated protein kinase Hog1 is required for nuclear export and the deletion of *HOG1* leads to ectopic activation of the Aft1 regulon [136, 146]. Moreover, negative cross-regulation of Aft1 and Aft2 has been demonstrated [124]. As a consequence, deletion of *AFT1* leads to a slight increase in the expression of the Aft2 targets [124].

Post-transcriptional regulation by Cth2 and Cth1

In addition to the activation of iron uptake genes, Aft1 induces the expression of *CTH2*, encoding a negative post-transcriptional regulator of iron-consuming genes. *CTH2* is required for optimal growth in iron-limited conditions [115]. In the nucleus, Cth2 binds to AU-rich elements (AREs) located in the 3′ untranslated region (UTR) of its target mRNAs. The Cth2-mRNA complex is then exported to the cytoplasm where the mRNAs are degraded by the general mRNA decay machineries [147–149]. The negative effect of Cth2 on the expression levels of its target mRNAs is relatively modest (about two-fold) [115, 150], but it also functions as a translational repressor [151]. According to transcriptome analyses, Cth2 controls the expression of about 100 ARE-containing mRNAs in response to iron starvation, half of them encoding iron-rich proteins or proteins involved in iron-consuming pathways (e.g. *ACO1*, *SDH4*, *CCC1*, *COX10*, *ISA1*, *NFU1*, *HEM1,4,13* and *15*, *GLT1*) [115, 150]. *CTH2* has a paralogue, *CTH1*, which also contributes to the iron starvation response, albeit to a much lesser extent. *CTH1* is dispensable for growth in iron-limited conditions and the deletion of *CTH1* in a *cth2Δ* mutant only slightly increases its growth defect. *CTH1* is transiently induced by Aft1 and Aft2 after iron deprivation and its mRNA levels are unchanged in Atf1 gain-of-function mutants [115, 121, 129, 150]. Cth1 controls the mRNA decay of about ten mRNAs, encoding mostly mitochondrial proteins, in response to iron starvation [115, 150]. *CTH2* and *CTH1* are subjected to auto- and cross-regulation, providing a negative feedback control to their activity [152, 153]. Similarly, Cth2 exerts a modest negative feedback on some genes of the iron regulon [57, 115]. At the protein level, the phosphorylation of Cth2 by the casein kinase Hrr25 promotes its degradation by the proteasome [154]. Impairment of this regulation leads to growth defects upon iron deficiency, indicating that Cth2 levels have to be tightly controlled for an optimal iron starvation response. Only a part of the down-regulation of iron-consuming genes is due to Cth2 and Cth1 and most of them still show a significant decrease in expression in a *cth1Δcth2Δ* double mutant [115, 150]. Of note, many of these genes are connected to respiration and are under the positive transcriptional control of the haem-activated transcription factor Hap1, whose activity is affected by iron deficiency [155]. Hence, down-regulation of those genes may result from the combination of both the negative post-transcriptional regulation by Cth1/2 and from a decrease in the activity of their transcriptional activators.

Iron limitation and the subsequent decrease in the expression of many important enzymes may have a detrimental impact on whole-cell metabolism. These effects are partially buffered either by the overexpression of proteins acting upstream and downstream of these enzymes or by the activation of parallel, iron-independent, metabolic pathways [117]. These compensatory effects will not be addressed here. On this topic, please refer to the review by Philpott *et al*. [156].

State of the art in *C. glabrata*

The *C. glabrata* iron regulon

Several studies have assessed the transcriptomic changes of *C. glabrata* upon iron starvation, using different genetic backgrounds and various protocols to achieve iron deprivation [101, 105, 106, 157]. These analyses revealed that, while the general features of the *S. cerevisiae* iron starvation response are conserved in *C. glabrata*, important quantitative and qualitative differences exist (Fig. 2).

As in *S. cerevisiae*, the downregulated genes are mostly involved in Fe-S clusters and haem biosynthesis and assembly (e.g. *CgYAH1*, *CgYFH1*, *CgISA1*, *CgNFU1*, *CgIBA57*, *CgGRX4*, *CgHEM1,3,4,13,14,15; CgYMC1; CgCOX10* and *15; CgCYT2*), iron storage in vacuoles (*CgCCC1*), iron-dependent functions such as respiration (e.g. *CgQCR* and *CgCOX* genes) or encode metalloproteins (e.g. *CgSDH2*, *CgCCP1*, *CgRIP1*, *CgCYT1*, *CgRLI1*, *CgILV3*, *CgLIA1*, *CgCYC1*, *CgGLT1*, *CgMET5*, *CgYHB1*, *CgLEU1*). Conversely, iron starvation induces the expression of genes potentially involved in extracellular iron uptake (*CgFTR1*, *CgFET3*, *CgFET4*, *CgSMF1*, *CgSIT1*), copper transfer towards Fet3 (*CgATX1*, *CgCCC2*, *CgCTR1*, *CgCTR2*), iron recycling from haem (*CgHMX1*), and vacuolar and mitochondrial iron export (*CgMMT2*, *CgFTH1*, *CgSMF3*, *CgFRE6*, *CgFRE8*). Intriguingly, despite its central role in iron uptake, Cg*FET3* is only moderately induced by iron starvation (from 1.3- to 2.6-fold depending on the studies) [101, 105, 106, 157]. This suggests that high levels of *CgFET3* mRNA are already produced in so-called 'iron-replete' conditions. *CgCTH2*, *CgLSO1*, *CgISU1* and *CgISU2* are also induced by iron deficiency, as previously reported for their *S. cerevisiae* orthologues.

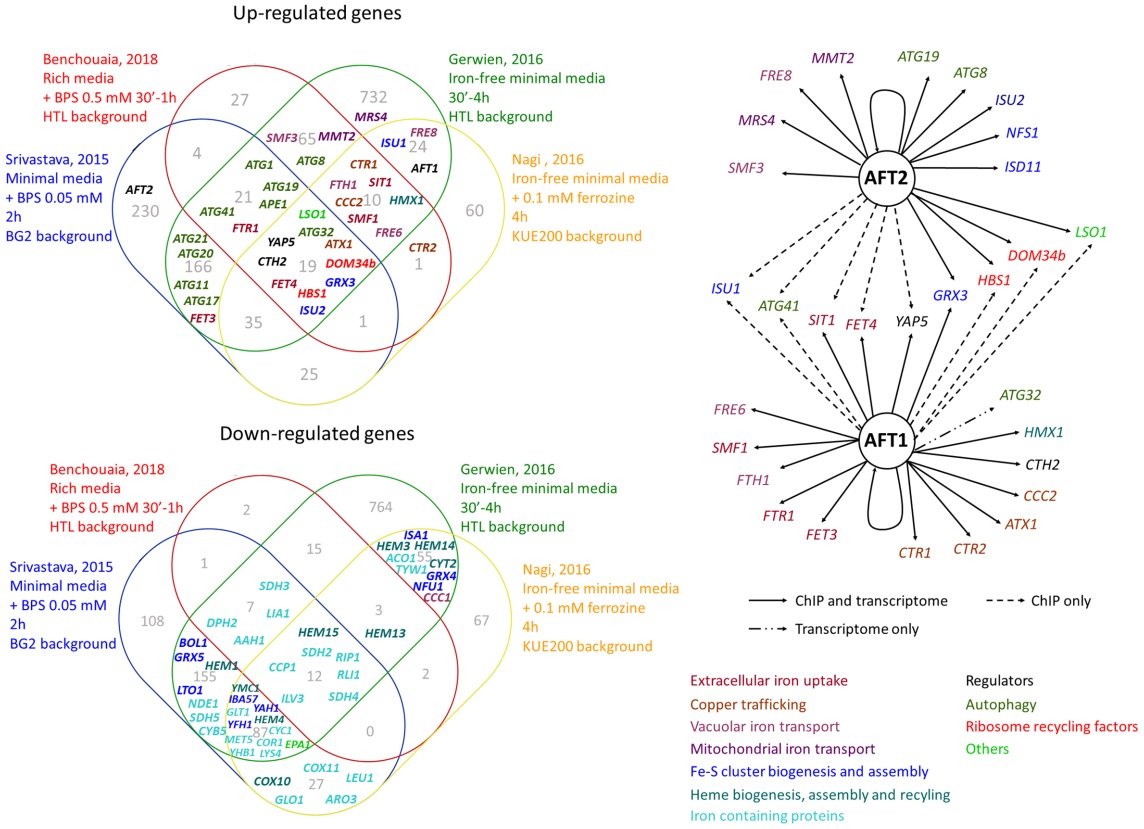

Fig. 2. The iron starvation response of *C. glabrata*. Left panel: overlaps between the four-transcriptome dataset studying the iron starvation response [101, 105, 106, 157]. The same 1.7-fold change cut-off was applied to all studies. The grey numbers are the total number of genes in each category. The names of the genes involved in iron homeostasis have been indicated. The colour code for gene names is at the bottom of the right panel. HTL is a *his3-trp1-leu2- C. glabrata* strain background. KUE200 is a *his3-trp1-* strain, which is also defective for non-homologous end joining. BG2 derives from a clinical isolate and has no auxotrophy. BPS (bathophenanthrolinedisulfonic acid) and ferrozine are chelators of ferrous iron. Right panel: the Aft1/Aft2 network in *C. glabrata*, based on the Aft2 transcriptomic and ChIP-seq data from Benchouaia *et al.* [106], the Aft1 transcriptomic data of Gerwien *et al.* [101] and the Aft1 ChIP-seq data produced by A. Thiebaut (unpublished results). Only the genes involved in iron homeostasis have been indicated. For a more comprehensive list of Aft1/Aft2 targets, please refer to the original publications [101, 106].

A striking feature of the *C. glabrata* iron starvation response is the induction of many genes involved in general autophagy (*CgATG1*, *CgATG17*, *CgATG19*, *CgATG20*, *CgATG21*, *CgATG41*), and mitochondria autophagy (mitophagy) (*CgATG32*, *CgATG8*, *CgATG11*) [105, 157]. This is consistent with the observation that long-term iron starvation (longer than a day) triggers mitophagy in *C. glabrata*, but not in *S. cerevisiae* [105]. Mitophagy has been proposed to maintain the balance between energy production by respiration and toxic ROS accumulation (reviewed in [158]). The role of this phenomenon in *C. glabrata* iron homeostasis needs to be determined. Is it simply a mitochondria recycling process due to the reduced respiratory activity in iron-limited conditions, as suggested by the decrease in expression of many, if not all, respiratory genes? Or does *C. glabrata* use mitochondria as an internal source of iron upon long-term iron starvation? Regardless, this is relevant to *C. glabrata*'s virulence, because *atg32* null mutants show significantly reduced dissemination in mice [105, 107].

Another intriguing observation in *C. glabrata* is the strong induction of *CgGRX3* and of the genes encoding the ribosome recycling factors CgDom34b and CgHbs1 when iron is limiting. A comparative transcriptomics study on eight different yeast species (including *S. cerevisiae*, *C. albicans* and *C. glabrata*) indicated that these regulatory patterns are specific to *C. glabrata* [106]. Moreover, the deletion of *CgDOM34b* or *CgHBS1* led to a clear growth defect in iron-limited conditions, suggesting a particular role, yet to be determined, of the corresponding proteins in *C. glabrata* iron homeostasis [106]. Of note, ribosome recycling requires the essential Fe-S cluster containing protein Rli1, and it was proposed, but has not yet been demonstrated, that the over-expression of CgDom34b and CgHbs1 would compensate for the decreased activity of Rli1 when iron becomes scarce [106]. Due to the role of ScGrx3 in the negative post-translational regulation of ScAft1 and ScAft2 activities, the induction of *CgGRX3* in response to iron limitation is very surprising. This suggests that the role of Grx3 in *C. glabrata* might be different.

Unfortunately, the function of glutaredoxins has not yet been investigated in *C. glabrata*.

The *C. glabrata* transcriptional control of the iron starvation response: CgAft1, CgAft2 … and more?

Although the post-translational regulation of CgAft1 and CgAft2 activity and their nucleocytoplasmic localization has not been addressed, their target genes and their impact on the iron starvation response have been investigated by several groups (Fig. 2) [101, 102, 106]. As in *S. cerevisiae*, deletion of *CgAFT2* has no impact on growth, even upon iron starvation [101, 102, 106]. Moreover, *cgatf2Δ* cells have wild-type levels of macrophage survival and proliferation rates in mice [102]. In contrast, the deletion of *CgAFT1* is highly detrimental to growth and it was, at first, hypothesized to be essential [102]. Actually, *cgaft1Δ* mutants must be supplied with 50 times the normal iron concentration of yeast culture media to grow [101]. This suggests that the requirements in extracellular iron are higher in *C. glabrata* than in *S. cerevisiae*, at least in laboratory conditions. As in *S. cerevisiae*, the deletion of *CgAFT1* led to a severe decrease in the expression of the genes involved in the reductive iron uptake pathway (*CgFTR1*, *CgFET3*, *CgFET4*, *CgSMF1*), copper homeostasis (*CgCTR1*, *CgCTR2*, *CgCCC2*, *CgATX1*), siderophore uptake (*CgSIT1*), vacuolar iron export (*CgFRE6*, *CgFTH1*) and iron uptake from haem (*CgHMX1*) [101]. The iron starvation response of *CgCTH2* was also nearly abolished in the *cgaft1Δ* cells. Chromatin immunoprecipitation experiments followed by deep sequencing (ChIP-seq) confirmed the binding of CgAft1 to the promoters of these genes (A. Thiebaut, unpublished results). Consistently, the TGCACCC motif is enriched in the promoters of genes associated with iron uptake and recycling [101, 157] and in the CgAft1 ChIP peaks (A. Thiebaut, unpublished results). This is in accordance with previous bioinformatics analyses which predicted that the binding preferences of CgAft1 should be largely conserved in the *Saccharomycetaceae*, with the sole exception of *Kluyveromyces lactis* [159, 160]. As described for its orthologue in *S. cerevisiae*, CgAft2 binds and regulates the iron starvation response of genes involved in intracellular iron trafficking (*CgSMF3*, *CgMMT2*, *CgMRS4*) [106]. It also controls the expression of several genes involved in Fe-S cluster biosynthesis (*CgISU2*, *CgNFS1*, *CgISD11*) and of the iron-responsive gene of unknown function, *CgLSO1*. The CgAft2 DNA binding motif predicted from ChIP-seq experiments is ACACCC, as for ScAft2 [106].

The *C. glabrata*-specific members of the iron regulon described in the previous section are also under control of the CgAft transcription factors. CgAft2 binds and controls the expression of the autophagy genes *CgATG8* and *CgATG19* [106]. CgAft1 controls the induction of the mitophagy-specific receptor encoding gene *CgATG32* in late iron starvation response [101]. No binding of CgAft1 was detected by ChIP-seq on this gene but a CgAft1 binding motif is present 400 bp upstream of the *CgATG32* start codon (A. Thiebaut, unpublished results). Also, the induction of the ribosome recycling factor-encoding genes *CgDOM34b* and *CgHBS1* is directly controlled by CgAft2 [106]. CgAft1 and CgAft2 are both required for the proper up-regulation of *CgGRX3* under iron-limited conditions [101, 106]. Phylogenetic analyses of the promoters of *CgDOM34b*, *CgHBS1* and *CgGRX3* suggest that their regulation by Aft transcription factors arose after the whole genome duplication and was secondarily lost in the *S. cerevisiae* lineage [106].

In contrast to the situation in *S. cerevisiae*, the relative specificities of CgAft1 and CgAft2 have not been properly addressed in *C. glabrata* because neither double mutants, nor gain of function mutations of *CgAFT1* and *CgAFT2* have yet been obtained. Still, there is evidence of cross-regulation and functional overlap between these two transcription factors in *C. glabrata* too (Fig. 2). As mentioned above, both CgAft1 and CgAft2 are required for the regulation of *CgGRX3*. Several CgAft2 targets are bound by CgAft1 (*CgDOM34b*, *CgHBS1*, *CgLSO1*) (A. Thiebaut, unpublished results). Reciprocally, *CgSIT1* and *CgFET4* are bound by CgAft2 [106]. Finally, *CgATG41* and the Fe-S cluster scaffold protein-encoding gene *CgISU1* are bound by CgAft1 and CgAft2, although a single deletion of either regulator has no impact on their expression [101, 106]. A negative effect of CgAft1 on the expression of some CgAft2-specific targets (e.g. *CgSMF3*, *CgMMT2*, *CgFRE8*) has also been reported [101, 104], which suggests that, as in *S. cerevisiae*, CgAft1 exerts a negative feedback on CgAft2 activity. The mRNA levels of *CgAFT2* increase in the absence of *CgAFT1* [101]. However, this regulation is not likely to be direct, because no binding of CgAft1 on the promoter of *CgAFT2* has been detected to date and no CgAft1 binding motif is present in the CgAft2 promoter (A. Thiebaut, unpublished results). ChIP-seq experiments have shown binding of CgAft1 and CgAft2 to their own promoters, indicating that auto-regulatory loops may exist [106] (A. Thiebaut, unpublished results). Of note, *CgAFT1* and *CgAFT2* have been reported to be induced upon iron starvation in some of the transcriptomics studies reported above.

In addition to the Aft regulators, the CgSef1 zinc finger transcription factor has been proposed to play a role in the regulation of the iron starvation response, based on the increased susceptibility to iron limitation of *cgsef1Δ* cells compared to the wild type and on the regulation of CgSef1 mRNA levels by CgCth2 [101]. Interestingly, while Sef1 has no known role in *S. cerevisiae* and while the *Scsef1Δ* mutant has no growth defect in iron-limited conditions [101, 161], it is a key activator of iron uptake genes in response to iron starvation in *C. albicans* [161]. This led to the proposal that *C. glabrata* relies on a hybrid Aft1/Sef1 transcriptional network to cope with iron-limited conditions [101]. Surprisingly, the deletion of *CgSEF1* has no impact on the *C. glabrata* iron regulon. It results in a decrease of the expression of a limited number of genes encoding iron-consuming proteins (e.g. *CgACO1*, *CgISA1*), suggesting that CgSef1 acts antagonistically to the post-transcriptional repression of these genes by Cth2 [101]. This observation is difficult to reconcile with the iron starvation susceptibility of the *cgsef1Δ* mutant, and the role of

this factor in *C. glabrata* iron homeostasis needs to be more thoroughly investigated [34].

Furthermore, deletion of the MAPK kinase encoding gene *CgHOG1* has been shown to result in perturbation of iron homeostasis and down-regulation of iron uptake genes (*CgFTR1*, *CgFET3*, *CgAFT1*, etc.) [157]. This observation is opposite to the situation described in *S. cerevisiae* in which ScHog1 is a negative regulator of ScAft1 (see previous section). The mechanism of action of Hog1 in *C. glabrata* has not yet been deciphered.

Post-transcriptional regulation: one Cth protein to rule them all

C. glabrata has only one homologue to *ScCTH1* and *ScCTH2*, which is named *CgCTH2* and which is induced by iron starvation under the control of CgAft1. The impact of CgCth2 on mRNA stability has not been directly addressed. Still, the role of this protein in the post-transcriptional down-regulation of iron-consuming genes in response to iron starvation is very likely to be conserved. Indeed, the *cgcth2Δ* mutant exhibits a growth defect in iron-limited conditions and an increase in the mRNA levels of many genes whose orthologues are Cth1/2 targets in *S. cerevisiae* (e.g. *CgCYT1*, *CgCCP1*, *CgACO1*, *CgHEM15*, *CgCCC1*) [101]. Moreover, the 3′UTRs of mRNA down-regulated upon iron starvation are enriched in AREs. Like *ScCTH1/2*, *CgCTH2* seems to exert a moderate negative feedback on the expression levels of iron-uptake genes [101]. Of note, the increase in the expression of several iron-consuming genes (e.g. *CgCYC1*, *CgCYT1*, *CgCCP1*, *CgISA1*, *CgACO1*, *CgHEM15*) observed in the *cgaft1Δ* mutant is probably an indirect effect due to the sharp decrease of *CgCTH2* expression in this mutant [101].

To conclude, the predominant role of Aft1/2 and Cth proteins in the iron starvation response is globally conserved between *S. cerevisiae* and *C. glabrata*. Yet, *C. glabrata* has some intriguing specificities, such as the induction of mitophagy and ribosome recycling factors, or the involvement of the Sef1 transcription factor. The exact functioning and physiological meaning of these specificities remain to be elucidated.

TRANSCRIPTIONAL RESPONSE TO IRON EXCESS

Lessons from *S. cerevisiae*

The high iron response and the Yap5 regulon

To cope with iron overload, yeast cells decrease iron uptake and increase iron storage in both vacuole and proteins. Hence the transcriptomic response to excessively high iron concentrations is basically the opposite of that described for iron starvation: the genes of the iron regulon are down-regulated while the genes encoding iron-containing proteins, the vacuolar iron importer Ccc1 and Fe-S clusters and haem biogenesis factors are up-regulated [162]. Part of these gene expression changes can be attributed to the inhibition of Aft1/2 DNA binding by glutaredoxins and the related decrease in Cth2 activity, which are described in the previous section. However, a qualitatively very important part of the transcriptional response to iron excess is handled by the iron-responsive regulator Yap5. Yap5 is a transcription factor of the bZip family which recognizes the Yap Response Element (YRE) TTA(C/G)TAA in the promoter of its target genes [163–165]. Cells deleted for *YAP5* are particularly sensitive to iron excess [163]. Yap5 plays an important role in the overexpression of *CCC1*, which is the main route for iron detoxification [163]. It was also shown to induce the expression of *TYW1*, a gene encoding an Fe-S cluster-containing protein involved the synthesis of modified tRNA. *TYW1* overexpression helps to prevent iron toxicity by sequestering it in a protein-bound form [166]. Finally, Yap5 was shown to exert an indirect negative feedback on iron uptake pathways by inducing the expression of *GRX4*, whose product inhibits the activity of Aft1/2, and *CUP1*, encoding a metallothionein which may limit copper availability to Fet3 [162]. Accordingly, abnormal nucleocytoplasmic localization of Aft1 has been described in a *yap5Δ* strain [162].

Regulation of the regulator: Fe-S clusters again

Unlike Aft1/2, Yap5 is constitutively localized to the nucleus and binds to its target promoters in an iron-independent manner [163]. However, its transactivation potential is regulated by iron [163]. As for Aft1/2, mitochondrially generated Fe-S clusters are the main determinants of this regulation. The activation of *CCC1* by Yap5 is impaired in mutants for the mitochondrial Fe-S cluster biogenesis pathway (e.g. *SSQ1*, *YFH1*, *ISU1*, *NFS1*, *ATM1*), but not in mutants for the CIA complex (e.g. *DRE2*, *NAR1*, *CFD1*, *NBP35*, *MMS19*) [167, 168]. This regulation does not involve the glutaredoxins Grx3/4, as a *grx3Δgrx4Δ* double mutant shows normal Yap5 activity and normal Yap5 iron incorporation [167, 168]. Actually, Yap5 was shown to directly bind Fe-S clusters *in vivo* through conserved cysteine-rich domains (CRDs) in its C-terminal transcription activation region. Fe-S cluster binding to Yap5 triggers a conformational change of the protein, which turns it into a transactivator [168]. Hence, mutagenesis of the cysteines in the CRD causes defects in the Yap5 iron-mediated activation [163].

Although early studies reported that *YAP5* deletion totally abolishes *CCC1* induction by high iron concentrations [163], more recent results have indicated that a residual activation of *CCC1* is still observed in *yap5Δ* cells [162, 168]. The kinase Snf1 and its targets, the general stress transcription factors Msn2 and Msn4, are responsible for this residual activity [169].

State of the art in *C. glabrata*

The CgYap5 regulon

To the best of our knowledge, only two data sets are available for the genome-wide gene expression changes to excessively high iron concentrations in *C. glabrata* [106, 157]. The global response of *C. glabrata* to iron overload is very similar to the *S. cerevisiae* response. The iron regulon is strongly down-regulated while the iron-consuming genes and respiratory genes are up-regulated (Fig. 3). Additionally, the *C. glabrata*-specific

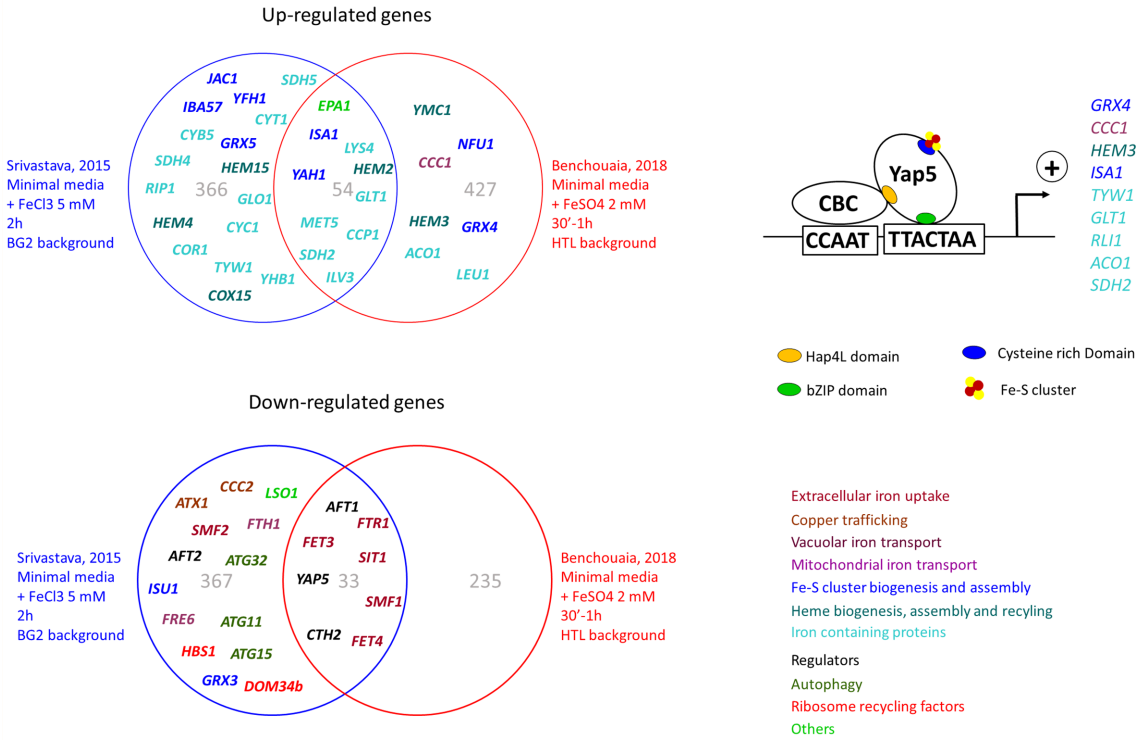

Fig. 3. The iron excess response of *C. glabrata*. Left panel: overlaps between two transcriptome datasets studying the iron starvation response [106, 157]. The same 1.7-fold change cut-off was applied to all studies. The grey numbers are the total number of genes in each category. The names of the genes involved in iron homeostasis have been indicated. The colour code for gene names is at the bottom of the right panel. HTL is a *his3-trp1-leu2- C. glabrata* strain background. BG2 derives from a clinical isolate and has no auxotrophy. Right panel: the Yap5 and CBC shared regulon in *C. glabrata*, based on the Yap5 and Hap5 transcriptomic and ChIP-seq data from Merhej *et al.* and Thiebaut *et al.* [113, 170].

adhesin encoding gene *EPA1* is induced by iron excess under the dependency of the CgHog1 MAP kinase. Accordingly, iron overload renders the *C. glabrata* cells more adherent to epithelial cells [157].

ChIP-seq and transcriptome analyses indicated that the role and targets of CgYap5 are very similar to ScYap5 (Fig. 3). A strain deleted for *CgYAP5* is more sensitive to iron overload than the wild-type strain [113]. CgYap5 binds and controls the expression of several genes encoding iron-containing proteins (*CgRLI1*, *CgACO1*, *CgGLT1*, *CgSDH2*, *CgTYW1*), the vacuolar iron transporter CgCcc1, the Fe-S cluster assembly factor CgIsa1, the rate-limiting enzyme in haem biosynthesis CgHem3 and the glutaredoxin CgGrx4 [113, 171]. The most enriched motif in CgYap5 ChIP peaks is a perfect YRE [113]. As described in *S. cerevisiae*, CgYap5 binds to its target promoters in an iron-independent way [113]. The post-translational regulation of CgYap5 has not yet been investigated, but the CRDs are highly conserved in CgYap5 and it is very likely that its activity is regulated by Fe-S cluster binding, as described in *S. cerevisiae*.

CgYap5 is a regulatory subunit of the CCAAT binding complex

More surprisingly, it was shown that CgYap5 requires the CCAAT binding complex (CBC) for regulating its targets [170]. The CBC is a highly conserved heterotrimeric transcription factor which binds to the CCAAT DNA motif. In fungi, its three core subunits, named Hap2, Hap3 and Hap5, are sufficient for DNA binding but it requires a fourth regulatory subunit for transcription regulation. In *S. cerevisiae*, this regulatory subunit is Hap4 which interacts with Hap2/3/5 though a 16-aa domain called the Hap4L domain [172–174]. In this species, the CBC is mostly known as an activator of the respiratory genes and has no obvious role in iron homeostasis [155, 172, 175]. ChIP-seq and transcriptome analyses have shown that CBC in *C. glabrata* actually plays a dual role as a constitutive activator of the respiratory genes and as an activator of the CgYap5 regulon upon iron overload [170]. In contrast to the respiratory function, the iron excess response does not require CgHap4 [170]. A careful inspection of the CgYap5 protein sequence revealed that it actually has a degenerated Hap4L domain close to its bZIP motif [171]. Mutagenesis and co-immunoprecipitation experiments suggest that this Hap4L domain is required for CgYap5-CBC interaction and subsequently for Yap5 DNA binding and the activation of its target genes [170]. The CgYap5-CBC interaction is further supported by DNA sequence analyses, which revealed a close proximity of the CCAAT and YRE DNA motifs in the promoter of the CgYap5-CBC common targets. More precisely, in the promoter of these genes, the

CCAAT and YRE motifs are spaced by 10–14 bp, which is very unusual in the *C. glabrata* genome [170]. Moreover, experiments using LacZ as a reporter gene under the control of wild-type or mutated versions of the *GRX4* promoter have shown that both the CCAAT and the YRE motifs are necessary for proper regulation of the CgYap5 targets (A. Thiebaut, unpublished results). This regulation is likely to be conserved in *S. cerevisiae*. First, CBC binding has been reported in the promoters of *ScGRX4*, *ScISA1*, *ScCCC1* and *ScGLT1* [176]. Second, the close proximity of the CCAAT and YRE motifs is conserved in the *S. cerevisiae* orthologues of the CgYap5 targets (A. Thiebaut, unpublished results). However, the role of ScCBC in the iron excess response has not been directly addressed.

Is CgYap5 orthologous to the HapX regulator of iron starvation in other fungal species?

Hence, Yap5 needs both the interaction with the YRE by its bZip domain and the interaction with the CBC through its Hap4L domain to control gene expression. In other words, Yap5 belongs to the family of bipartite Hap4L-bZIP proteins, which makes it a probable orthologue of the HapX transcription factor found in many other fungal species [171, 177]. Interestingly, these HapX proteins have a key role in the repression of iron-consuming genes upon iron starvation (see next section). CgYap5 has probably largely lost this role, as the deletion of *CgYAP5* only results in a modest increase in the expression of its targets in iron-limited conditions and has no impact on cell growth in iron-deficient media [113, 170]. Yet, this may explain an apparent paradox: while CgYap5 is required for high-iron stress responses, its expression is inversely correlated with the iron concentration and is much higher in iron-limited conditions [113]. Actually, the expression of *CgYAP5* is directly regulated by CgAft1 and, albeit to a lesser extent, by CgAft2 [101, 106], making *CgYAP5* a member of the *C. glabrata* iron regulon per se. Hence, this surprising expression profile would be a relic from ancient times, when the Yap5 ancestor was a negative regulator required in iron-limited conditions. These findings bring new light on the evolution of iron homeostasis regulation in fungi.

C. GLABRATA REGULATION OF IRON HOMEOSTASIS IN AN EVOLUTIONARY PERSPECTIVE

The Aft/Cth system described above for *S. cerevisiae* and *C. glabrata* is actually restricted to the *Saccharomycetaceae* (i.e. the yeast species between *S. cerevisiae* and *Kluyveromyces lactis*) [159, 160] and not representative of other fungal species. *C. albicans* does have orthologues for Aft2 and Cth2 but the first one has a minor role in iron homeostasis and the latter is involved in biofilm formation [161, 178–180]. In general, fungi control iron homeostasis using a transcriptional regulatory network based on two antagonist repressors (Fig. 4).

Repression of iron uptake by GATA transcription factors

In most fungi, the iron uptake genes are negatively regulated under iron-replete conditions by a GATA transcription factor. Its name differs depending on the species. It is called Sfu1 in *C. albicans*, SreA in *Aspergillus fumigatus* and *A. nidulans*, Sre1 in *Histoplasma capsulatum*, Fep1 in *Schizosaccaromyces pombe*, Cir1 in *Cryptococcus neoformans* and Urbs1 in *Ustilago maydis* [181–188]. Fe-S clusters seem to play a role in the regulation of their activity. In *S. pombe*, Fep1 is an iron-binding protein, whose repressor activity and DNA binding is stimulated by metal chelation. Fep1 interacts constitutively with the glutaredoxin Grx4 [189]. Upon iron starvation, the Fep1-bound iron is transferred to the apo-Grx4 to form an Fe-S cluster, which triggers interaction of Grx4 with the DNA binding domain of Fep1 and loss of Fep1 binding to DNA [189–191]. Consequently, the repression of iron uptake genes is relieved and their expression increases. As described for Aft1/2 regulation in *S. cerevisiae*, this process requires interaction of Grx4 and Fep1 with the *S. pombe* Fra2 orthologue [190, 192]. In the human pathogen *C. neoformans*, Grx4 was shown to bind to the GATA factor Cir1 and to impact on the iron starvation response, although the underlying molecular mechanisms have not yet been elucidated [193]. However, the situation is probably more complex in *C. neoformans* than in *S. pombe* because Cir1 acts both as a repressor of intracellular iron transport and as an activator of extracellular iron uptake [185, 194, 195]. Similarly to Fep1, the Sre1 and SreA GATA transcription factors of *Histoplasma capsulatum* and *Aspergillus* species, respectively, were proposed to bind iron through conserved cysteines, but the potential role of glutaredoxins in the regulation of iron homeostasis has not been examined in these species [184, 188].

Repression of iron-consuming genes by the CBC and an iron-regulated CBC regulatory subunit

Conversely, upon iron starvation the iron-consuming genes are transcriptionally repressed by the CBC and a regulatory subunit which is called Php4 in *S. pombe*, Hap43 in *C. albicans* and HapX in most other fungal species [177, 194, 196–201]. Inactivation of HapX, Hap43 or Php4 leads to dramatic up-regulation of iron-consuming genes upon iron deficiency and these proteins are required for normal growth in iron-limited conditions [161, 177, 197, 198, 200–205].

HapX and Hap43 are bZip-Hap4L bipartite proteins which share many similarities with Yap5. First, their expression is induced in iron-limited conditions and repressed when iron concentration increases [181, 206]. Second, HapX and Yap5 interact with Fe-S clusters through similar conserved CRDs [168, 206]. Third, most of the orthologues of the CgYap5 regulon are also targets of HapX in *Aspergillus* species and of Hap43 in *C. albicans* (i.e. *ACO1*, *CCC1*, *GLT1*, *ISA1*, *HEM3*, *SDH2*, *RLI1*) [161, 205]. Fourth, HapX constitutively binds to its promoter targets, independently of the iron concentration [206]. Fifth, HapX and Hap43 require both interaction with the CBC and interaction with DNA for their activity. Consequently, mutations in the Hap43 bZIP domain severely impair

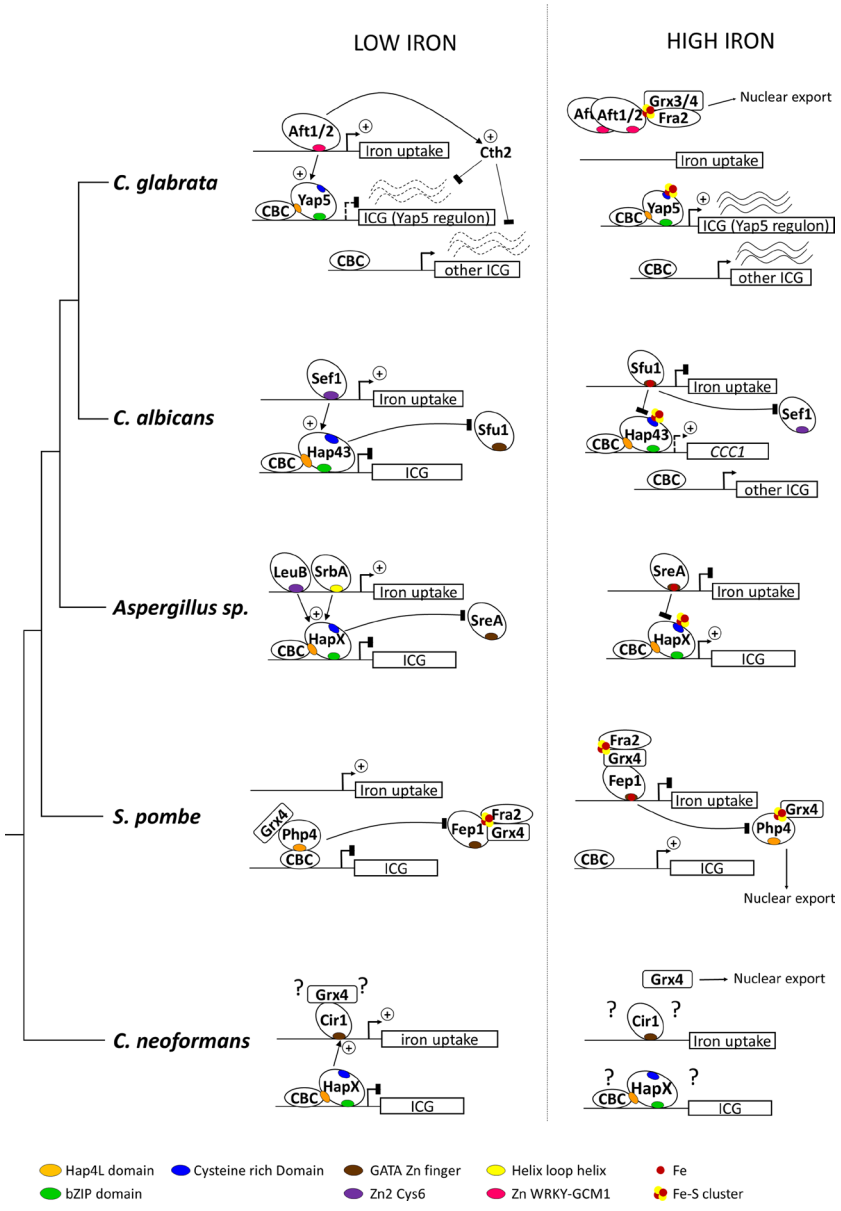

Fig. 4. The evolution of iron-responsive regulatory networks in fungi. ICG, 'ion-consuming genes'. In the archeascomycete *Schizosaccaromyces pombe*, a system with two antagonist repressors is used. Iron-consuming genes are repressed by the Php4 subunit of the CBC, while iron uptake is repressed by the Fep1 GATA factor. Upon iron excess, Fep1 binds iron and is active, while Php4 is exported from the nucleus due to its interaction with Grx4 and an Fe-S cluster. Upon iron starvation, Fep1 transfers its iron to a Grx4-Fra2 complex and his therefore inactivated, while Php4 relocates to the nucleus and represses its targets. Additionally, Fep1 and Php4 negatively cross-regulate their expression. In *Aspergillus* species, as in many euascomycetes (e.g. *Histoplasma capsulatum*, *Fusarium oxysporum*, *Verticillium dalhiae*), this general scheme is conserved, except that HapX, the functional homologue of Php4, binds DNA directly through its bZip domain and is also able to positively control ICG expression in iron overload conditions upon binding of an Fe-S cluster. Also, additional regulators (e.g. LeuB and SrbA) positively control the expression of *HAPX* and of some iron uptake genes upon iron deficiency. The role of Grx4 in these regulations has not been examined in these species. In the hemiascomycete *C. albicans*, the situation is very similar to euascomycetes, except that Hap43, the equivalent of HapX, only has a minor role in iron excess conditions, by activating the vacuolar iron importer-encoding gene *CCC1*. In *C. glabrata*, the GATA repressor has been lost. Iron uptake genes are activated upon iron starvation by Aft1 and Aft2 and repressed in high iron conditions because of the release of Aft1/2 from DNA following its interaction with a Grx3/4-Fra2-Fe-S complex. Yap5, the orthologue of HapX and Hap43, has conserved a role in the activation of some ICGs upon iron excess. However, it has almost completely lost its repressor properties. This role has been undertaken by the negative post-transcriptional regulation of ICG by Cth2. Yet, the expression of *YAP5* is positively controlled by Aft1/2. Note that the post-translational regulation of Aft1/2 and of Yap5 represented here have not been formally established in *C. glabrata* and are inferred from studies in *S. cerevisiae*. The basidiomycete *C. neoformans* has an orthologue of HapX which negatively regulates the ICG upon iron starvation. However, the GATA factor Cir1 and HapX exert a positive effect on the expression of many iron uptake genes, which is independent of iron concentration. Of note, *C. neoformans* may not be representative of all basidiomycetes: in *U. maydis*, a GATA repressor acting similarly to SreA in *Aspergillus* species has been described.

its repression potential [177, 207]. The DNA binding properties of HapX have been examined in detail in *Aspergillus nidulans*. The CBC-HapX DNA binding domain is composed of the CCAAT box and a TGA(T/C)TCA motif spaced by 11–12 bp [206, 208], which is reminiscent of the CCAAT-YRE bipartite DNA binding motif described for CBC-CgYap5 [170]. Sixth, HapX has been shown to play an important role in the iron excess response of *Aspergillus* species and several other euascomycetes by activating the gene encoding the orthologue of the Ccc1 vacuolar iron transporter and of some iron-consuming genes (e.g. *ACO1*, *CYC1* and *LEU1*) [206]. Consequently, like Yap5 in *C. glabrata*, HapX is required for optimal growth upon iron overload in these species [199, 201, 206]. As described for Yap5, the binding of Fe-S clusters to the HapX CRD is probably the key signal to turn it from a repressor to an activator in high iron conditions [168, 206]. In contrast, Hap43 has only a minor role in the iron excess response of *C. albicans*. *Hap43Δ* strains only show a modest decrease of *CaCCC1* induction and have no growth defect upon iron overload [209].

In *S. pombe*, the situation is slightly different. Php4 does contain a conserved Hap4L domain and binds to the CBC but it has neither a bZIP domain nor conserved CRDs [200, 206]. Hence, Php4 does not bind DNA directly [204]. Also, Php4 does not bind Fe-S clusters on its own and requires Grx4 for its regulation [210]. In iron replete cells, Grx4 forms a complex with Fe-S clusters and Php4, hence triggering the dissociation of Php4 from CBC and its nuclear export [210–212]. Upon iron limitation, the Grx4-Php4 complex loses its Fe-S cluster, Php4 binds the CBC and represses the transcription of its targets. The potential role of Php4 in the iron excess response of *S. pombe* has not been extensively addressed, but because Php4 is actively exported from the nucleus in iron-replete conditions, it is very unlikely to have one.

Cross talks between the GATA factor, the CBC and other iron-responsive transcriptional regulators

In many fungal species, a negative transcriptional cross-regulation exists between the GATA factor and the CBC. In iron starvation conditions, HapX, Hap43 and Php4 directly repress the expression of *SreA*, *SFU1* and *FEP1*, respectively. Reciprocally, in iron-repleted cells, SreA, Sfu1 and Fep1 repress the expression of *HAPX*, *HAP43* and *PHP4*, respectively [161, 181, 182, 200–204, 213]. A notable exception is *C. neoformans* in which HapX positively regulates the expression of Cir1 upon iron deficiency [194].

In some species, additional positive transcriptional controls have been shown to interfere with the CBC-HapX core network. For instance, in *C. albicans*, Sef1 positively controls *HAP43* and genes involved in iron uptake upon iron starvation. The expression of *SEF1* is required for growth in iron-limited conditions and is repressed by Sfu1 [161]. Similarly, in *A. fumigatus*, the SrbA and LeuB transcription factors, which are involved in the regulation of sterol and haem biosynthesis in response to hypoxia and in the biosynthesis of branched-chain amino acids respectively, also contributes to the induction of *HAPX* and of some iron uptake genes upon iron deficiency [214, 215].

An evolutionary scenario towards the *C. glabrata* regulation of iron homeostasis

Hence, the global regulatory logic of iron homeostasis has been conserved despite a significant rewiring of the underlying regulatory networks. Of note, other similar cases have been described in the literature and the molecular mechanisms allowing dramatic changes of regulators while conserving the gene expression patterns have been nicely described in the particular case of sexual determination in hemiascomycete yeasts [216].

Compared to this well-studied case, our knowledge of iron homeostasis regulation in different fungal species clearly lacks completion. For instance, the role of glutaredoxins has not been investigated in *C. albicans* or *Aspergillus* species. Still, it is tempting to propose an evolutionary scenario that could have led to the *C. glabrata* situation. The ancestral state is very probably the GATA/CBC-HapX model, because it is the most widespread in ascomycetes and it is also found in basidyomycetes (e.g. *Ustilago maydis*) [183]. The acquisition of a role for an Aft transcription factor in the iron starvation response probably appeared in a common ancestor of *C. albicans* and *C. glabrata*, because *C. albicans* does have an Aft2 protein with minor role in iron regulation. In the *C. albicans* lineage, the ancestral state was preserved and is still predominant today. In the *S. cerevisiae* and *C. glabrata* lineage, the positive regulation of iron uptake by Aft totally replaced the negative regulation by GATA factors. Meanwhile, the repression of iron-consuming genes shifted from a transcriptional repression by CBC to a post-transcriptional repression by Cth RNA-binding proteins. Consequently, the modern Yap5 proteins have almost totally lost their transcriptional repression properties. Interestingly, Yap7, the ohnologue of Yap5 in *S. cerevisiae* (i.e. the Yap5 paralogue which arose from the whole genome duplication) is a CBC-dependent transcriptional repressor [171], suggesting that Yap5 lost its inhibitory properties after the whole genome duplication. Also, the fact that *CgYAP5* is still induced by iron starvation under the control of Aft transcription factors in modern species suggests that the Aft-positive regulation appeared before the replacement of Yap5-CBC repression by the post-transcriptional regulation mediated by Cth proteins.

It is impossible to say if the role in the iron excess response described for HapX in *Aspergillus* and for Yap5 in *Saccharomycetacea* is an ancestral or a derived state, because it has not been studied in *S. pombe* and in basidiomycetes. It certainly followed different evolutionary paths in hemiascomycetes: this role was almost totally lost in *C. albicans* but conserved in *Saccharomycetacea* (e.g. *C. glabrata*, *S. cerevisiae* and *K. lactis*) [171].

In conclusion of this part, while the global logic of iron regulation is remarkably conserved among fungi, the transcriptional and post-transcriptional underlying networks have been considerably rewired. Still, the central roles of Fe-S clusters and glutaredoxins in iron sensing seem to be widely

conserved, together with the involvement of CBC-interacting proteins such as Yap5 and HapX.

GENERAL CONCLUSION

The analyses of *C. glabrata* iron homeostasis are at their beginning and our knowledge is much less advanced than in *S. cerevisiae*. Still, the published studies revealed intriguing, and then potentially very interesting, particularities in *C. glabrata*. For instance, elucidation of the roles of Ccw14 and Mam3 in iron homeostasis, the basis of the requirements for ribosome recycling factors in iron-limited media, the physiological rationale of the opposite regulatory patterns of *CgGRX3* and *CgGRX4*, clarification of the role of CgSef1 in the iron starvation response or the impact of mitophagy on iron metabolism represent fascinating ways of investigation for the near future. Considering the importance of iron homeostasis for *C. glabrata* virulence, these studies may result in new therapeutic approaches to efficiently cure candidemia.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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