



Review

Fungal iron homeostasis with a focus on *Aspergillus fumigatus*[☆]Matthias Misslinger^a, Peter Hortschansky^b, Axel A. Brakhage^{b,c}, Hubertus Haas^{a,*}^a Institute of Molecular Biology - Biocenter, Medical University of Innsbruck, Innsbruck, Austria^b Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology-Hans Knöll Institute (HKI), Jena, Germany^c Department Microbiology and Molecular Biology, Institute of Microbiology, Friedrich Schiller University Jena, Jena, Germany

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ABSTRACT

To maintain iron homeostasis, fungi have to balance iron acquisition, storage, and utilization to ensure sufficient supply and to avoid toxic excess of this essential trace element. As pathogens usually encounter iron limitation in the host niche, this metal plays a particular role during virulence. Siderophores are iron-chelators synthesized by most, but not all fungal species to sequester iron extra- and intracellularly. In recent years, the facultative human pathogen *Aspergillus fumigatus* has become a model for fungal iron homeostasis of siderophore-producing fungal species. This article summarizes the knowledge on fungal iron homeostasis and its links to virulence with a focus on *A. fumigatus*. It covers mechanisms for iron acquisition, storage, and detoxification, as well as the modes of transcriptional iron regulation and iron sensing in *A. fumigatus* in comparison to other fungal species. Moreover, potential translational applications of the peculiarities of fungal iron metabolism for treatment and diagnosis of fungal infections is addressed.

1. Introduction

Pathogenic fungi have a tremendous impact on humans, animals, plants, and ecosystem [1,2]. Plant pathogenic fungi impact global economy, e.g., a third of all cultivated food crops is destroyed by plant pathogenic fungi annually [1]. The majority of fungal infections in humans affect skin or mucosa, which is consequently not life-threatening; however, a considerable portion of these are invasive or chronic with high mortality [3]. The latter are often difficult to diagnose and to treat as limited antifungal drugs are available and resistance against these drugs is emerging. Therefore, still over one million people die due to a fungal infection each year, which are more deaths than caused by malaria or tuberculosis [4].

To cope with this fungal threat in agriculture and clinics, it is of importance to understand the mechanisms, which are used by pathogenic fungi to infect a host. These include evasion of the immune system and supply of nutrients, particular of those with limited availability in the different host niches [5].

Iron is an essential trace element, which is usually limited for pathogens during infection and consequently adaptation to iron starvation

is crucial for virulence [6–9]. On the other hand, excessive iron uptake is toxic as free iron causes the production of highly reactive hydroxyl radicals via the Haber Weiss/Fenton reaction [10,11]. Indisputably, characterization of iron homeostasis and metabolism in fungi is most advanced in *Saccharomyces cerevisiae* [12]. However, this yeast species lacks siderophore biosynthesis, which enables high-affinity iron acquisition and iron storage in most filamentous fungal species (see below). Moreover, baker's yeast employs rather unique iron regulatory mechanisms (see below).

This review focuses mainly on iron homeostasis maintaining mechanisms of *Aspergillus fumigatus*, as this mold became a model system for iron metabolism of siderophore-producing fungal species.

2. *A. fumigatus* - pathogen and valuable model organism

The ascomycete *A. fumigatus* is a saprobic fungus ubiquitously dwelling on dead organic-matter. However, as an opportunistic pathogen it can cause life-threatening invasive diseases such as invasive pulmonary aspergillosis, mainly in immunocompromised patients [13]. The produced spores, also termed conidia, are plentifully distributed by

Abbreviations: CIA, cytosolic Fe-S cluster assembly machinery; CBC, CCAAT-binding complex; CRR, cysteine-rich region; Fe-S, iron-sulfur; GRX, non-mitochondrial monothiol glutaredoxins; α -IPM, α -isopropylmalate; ISC, mitochondrial Fe-S cluster assembly machinery; NRPS, non-ribosomal peptide synthetase; RIA, reductive iron assimilation; ROS, reactive oxygen species; SIA, siderophore-mediated iron acquisition; SIT, siderophore iron transporter; SREBP, sterol regulatory element binding protein; TAFC, triacetylfulvarinone C; WGD, whole genome duplication

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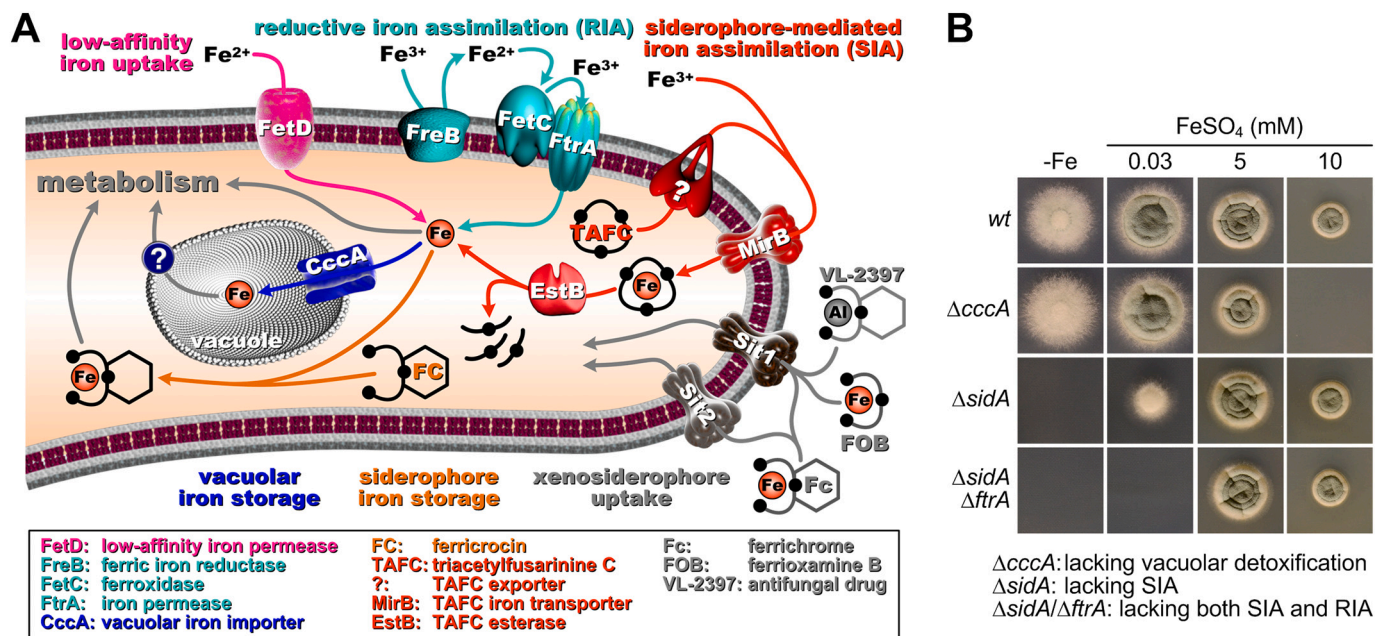


Fig. 1. Summary of iron acquisition and storage mechanisms of *A. fumigatus*. (A) For optimal adaptation to iron starvation, *A. fumigatus* employs several iron uptake systems. High-affinity iron uptake includes RIA (reduction of hardly soluble iron complexes to increase iron solubility) and SIA (siderophore-mediated iron acquisition); SIA involves uptake of endogenous siderophores such as ferric TAFc, which is hydrolyzed by EstB for intracellular iron release after uptake, and xenosiderophores such as FOB. Moreover, the antifungal drug VL-2397 is taken up by the siderophore transporter Sit1. For intracellular transport and storage, iron is bound to the siderophore ferricrocin. Excess of cellular iron is mainly detoxified by its translocation into the vacuole via CccA. (B) Growth-phenotypes of *A. fumigatus* mutants under different iron-availability. Lack of SIA results in a growth defect during iron starvation (-); lack of both SIA and RIA blocks growth also during iron sufficiency (0.03 mM); lack of vacuolar iron detoxification impairs growth under high iron (10 mM) conditions.

air and easily inhaled due to their small size (2.0–3.0 μ m). The average adult inhales roughly more than hundred spores daily [14]. *A. fumigatus* is a thermotolerant fungus, which can grow at temperatures up to 52 °C [15], and with a fast growth rate at 37 °C it is perfectly adapted to grow inside a warm-blooded host. This and other characteristics discriminate *A. fumigatus* from its less pathogenic relatives [16] and make *A. fumigatus* a common airborne fungal pathogen. *A. fumigatus* infections have mortality rates between 15% and > 90% and it is estimated that *A. fumigatus* is responsible for over half a million deaths per year worldwide [17]. *A. fumigatus* reproduces asexually and sexually and the haploid genomes of several isolates are sequenced [18,19]. It is a fast-growing organism, which can easily be cultivated in liquid and on solid medium (Fig. 1B). Meanwhile, an extensive toolbox of molecular methods has made *A. fumigatus* accessible to sophisticated molecular analyses [20,21].

3. Iron - an essential trace element

The ability of iron to easily accept and release electrons to switch between two oxidation states (ferrous iron, Fe^{2+} , and ferric iron, Fe^{3+}) has made it a convenient cofactor in redox biochemistry. Numerous enzymes require iron or iron-containing cofactors to fulfil their specific function. These cofactors include mononuclear or dinuclear non-haem-iron centers, iron-sulfur (Fe-S) clusters, heme, and siroheme [22–25]. With few exceptions [26,27], iron is crucial for all forms of life as it is involved in vital pathways such as oxidative phosphorylation, the TCA cycle, oxidative stress detoxification, P450 enzymes, DNA repair and replication as well as the biosynthesis of amino acids, nucleotides, and sterols. Iron is also crucially involved in less conserved pathways such as secondary metabolism, e.g., biosynthesis of β -lactam antibiotics, penicillin, and cephalosporin, and several metabolites requiring P450 oxidoreductases [28,29].

4. Fungal iron acquisition strategies

Iron is one of the most abundant elements on earth. However, its bioavailability is very low in most biological niches as it is easily oxidized by atmospheric oxygen into hardly soluble ferric complexes with the most common form of ferric iron in natural waters being ferric hydroxides [30]. Hence, fungi have developed sophisticated mechanisms to facilitate iron acquisition, which can be divided into low-affinity and high-affinity iron uptake systems. Low-affinity iron uptake, which will not be further discussed in this review, includes direct uptake of ferrous iron ions, in *S. cerevisiae* involving the transporters Fet4 and Smf1 [31]. *A. fumigatus* mutants lacking high-affinity iron uptake systems are able to grow in the presence of high amounts of ferrous iron demonstrating the presence of low-affinity uptake [32]. In line, *A. fumigatus* encodes uncharacterized homologs of Fet4 and Smf1. Moreover, some fungal species have the ability to use heme as iron source, e.g., *Candida albicans* and *Cryptococcus neoformans* [33,34]. *A. fumigatus* appears to lack efficient use of heme as iron source [32]. High-affinity uptake systems include siderophore-mediated iron acquisition (SIA) and reductive iron assimilation (RIA). It is believed that control of iron uptake and storage is the major mechanism to maintain iron homeostasis in fungi as no excretory mechanism for iron have been identified so far. In contrast, certain mammalian cells, enterocytes and macrophages [35], and possibly bacteria [36] are able to export iron. The strategies for iron uptake and storage of *A. fumigatus* are summarized in Fig. 1 including growth phenotypes of mutants with respective defects that are described in detail below.

4.1. Reductive iron assimilation (RIA)

As mentioned above, ferric iron forms hardly soluble complexes under aerobic conditions. A highly conserved strategy to cope with the low bioavailability of iron is extracellular reduction of ferric to ferrous

iron. The reduction of ferric iron decreases ferric-iron complex formation and thereby increases the solubility and consequently bioavailability of iron. RIA has been mainly characterized in baker's yeast [31,37], but *A. fumigatus* was also shown to employ this strategy [32,38]. In this pathway, plasma membrane-localized metallo-reductases, in *A. fumigatus* mainly FreB (Fig. 1A; [38]), reduce ferric to ferrous iron which is thereby mobilized. Subsequently, the ferrous iron is re-oxidized to ferric iron by a multicopper-oxidoreductase, termed FetC in *A. fumigatus*, to enable its uptake via a ferric-iron transporter, termed FtrA in *A. fumigatus*. These opposing reduction and oxidation steps appear to be counterintuitive at first sight and the reason is actually not completely understood. The combined action of the ferroxidase and the permease might be required to impart specificity to the high-affinity iron transport system and/or protect the organism from toxic effects of ferrous iron [39,40]. RIA is highly conserved in the fungal kingdom. A rare exception is *Aspergillus nidulans*, which lacks homologs of FtrA and FetC (Fig. 1A; [41]).

4.2. Siderophore-mediated iron assimilation (SIA)

In addition to RIA many fungal species employ SIA. In this system low-molecular mass, iron-chelating molecules, termed siderophores (from Greek: "sidero", iron, and "phorein", 'carrier'), are excreted to bind ferric iron (detailed mechanisms are described in [42]). Iron-bound siderophores are then taken up to supply the fungus with iron. The first siderophore, coprogen, was described in 1952 as an essential growth factor of coprophilic (strictly dung inhabiting) fungi such as *Pilobolus* spp. [43]. In the same year, the iron-containing compound ferrichrome isolated from *Ustilago sphaerogena* was shown to have similar growth-promoting activity [44]. Later, it was shown that coprogen and ferrichrome act as iron-transporting agents in microbial metabolism.

4.2.1. Siderophore biosynthesis

Most fungal siderophores are of the hydroxamate-type, which can be grouped into four structural families: fusarinines, coprogens, ferrichromes, and rhodotorulic acid; representatives of each family are shown in Fig. 2A. Detailed chemistry of siderophores has been reviewed previously [45,46]. The iron-chelating hydroxamate groups of these siderophores are synthesized from the same building blocks, namely the non-proteinogenic amino acid *N*⁵-hydroxyornithine and an acyl-CoA ester such as acetyl-CoA (e.g., ferricrocin, rhodotorulic acid), hydroxymethylglutaryl-CoA (e.g., ferrichrome A), malonyl-CoA (e.g., malonichrome) or anhydromevalonyl-CoA (e.g. fusarinines), via transacylation of ornithine that is beforehand *N*⁵-hydroxylated by an enzyme termed SidA. The siderophore biosynthetic pathway of *A. fumigatus*, shown in Fig. 2B, is hence largely conserved in most siderophore-producing fungi; e.g., *Talaromyces marneffeii*, *Fusarium oxysporum*, *Cochliobolus heterostrophus*, and *Ustilago maydis* [47–50]. SIA of *A. fumigatus* has been extensively reviewed [51]. Hydroxamate groups are excellent ligands in coordination chemistry [46]. In siderophores, these groups strongly coordinate ferric iron via a bidentate structure [46]. Most fungal siderophores have three hydroxamate groups linked by ester- or peptide bonds to form hexadentate structures, which enormously increase the affinity for ferric iron. Both peptide and ester bond formation is catalyzed by non-ribosomal peptide synthetases (NRPSs), which are best known from biosynthetic pathways involved in secondary metabolism [52]. Fusarinine- and coprogen-type siderophores are examples for products of NRPSs catalyzing the formation of ester bonds [51].

A. fumigatus produces four siderophores, extracellular fusarinine C and its derivative triacetylfusarinine C (TAFC) for iron acquisition as well as the ferrichrome-type siderophores ferricrocin and hydroxyferricrocin for intracellular iron storage and handling (see below and Fig. 2A). Fusarinine- and coprogen-type siderophores are typically excreted, ferrichrome-type siderophores are often found strictly

intracellularly as in *A. fumigatus*, but some fungi also secrete them, e.g., *U. maydis*, *Aspergillus niger*, and *A. terreus* [53–55]. Siderophores produced by selected fungal species are summarized in Table 1. The types of siderophores produced can vary within the same genus, e.g., *Aspergillus* spp.; on the other hand, *A. nidulans* and *A. fumigatus*, which are only distantly related, produce the same extracellular and hyphal siderophores.

Most *Ascomycota* and *Basidiomycota* species produce hydroxamate-type siderophores [56,57]. However, there are famous exceptions of economic and/or medical relevance such as the entire *Saccharomycotina* clade, including *S. cerevisiae* and *C. albicans*, as well as the basidiomycete genus *Cryptococcus* spp. Interestingly, the majority of these exceptions are yeast morphotypes. On the other hand, there are yeast species that produce siderophores such as *Schizosaccharomyces pombe*, *Rhodotorula* spp., and *Aureobasidium melanogenum* [58–60]. Moreover, *Mucoromycota* lack the ability to produce hydroxamate-type siderophores but produce a carboxylate-type siderophore, termed rhizoferrin, originally isolated from *Rhizopus microsporus* [61,62]. Compared to most hydroxamate-type siderophores, rhizoferrin displays a significantly lower affinity to iron. Recently, rhizoferrin biosynthesis has been shown to depend on an NRPS-independent siderophore (NIS) enzyme in *Rhizopus delemar* [63].

Several *Saccharomycotina* species produce and/or are able to take up the iron-chelating compound pulcherrimin, which is composed of two cyclized and modified leucine molecules [64]. In contrast to classical siderophores, its production is not confined to iron starvation conditions. Pulcherrimin has been shown to mediate antagonistic interactions toward nonproducing yeast species. Consequently, pulcherrimin's role appears to be iron monopolizing rather than iron scavenging for iron supply. Also *A. fumigatus* produces an iron chelating hexapeptide, hexadecahydroastechrome, which lacks classical siderophore activity; its artificial overexpression even causes iron starvation via cellular iron deprivation [65]. Taken together, not all low molecular-mass iron chelating metabolites serve as classical siderophores.

Notably, most bacteria employ SIA, involving in addition to hydroxamate-type siderophores also catecholate-, carboxylate-, and mixed-types [46,66]. In contrast, some plants utilize rather simple iron chelators including nicotianamine, mugineic acid family phytosiderophores, and citrate for iron mobilization [67]. Remarkably, biosynthesis of the hydroxamate groups of bacterial siderophores involves homologs of the fungal *N*⁵-ornithine hydroxylase SidA (Fig. 2B), e.g., for biosynthesis of pyoverdine by *Pseudomonas* spp., aerobactin by *Escherichia coli*, mycobactin by *Mycobacterium* spp., or feroxamines produced by *Streptomyces* and distinct *Enterobacteriaceae* [68], which underlines the overlap of biosynthetic modules used in bacterial and fungal SIA.

4.2.2. Siderophore uptake and intracellular release of iron

Uptake of siderophore-iron chelates in fungi is mediated by members of the siderophore iron transporter (SIT) family, which is a subfamily of the major facilitator superfamily. SIT proteins are exclusively found in the fungal kingdom and here in most species [57]. Notably, three SIT proteins appear to have siderophore-independent functions; *S. cerevisiae* Gex1 was found to act as a glutathione exchanger [69], *S. pombe* Str3 is required for low-affinity heme uptake [70], and the gene encoding *A. fumigatus* CrmC is localized in a secondary metabolite gene cluster and was shown to be regulated by copper and not by iron [71].

Despite the fact that there are fungal species lacking siderophore biosynthesis, the ability to utilize siderophore-chelated iron appears to be highly conserved in the fungal kingdom, i.e. most species from all fungal phyla (*Ascomycota*, *Basidiomycota*, *Mucoromycota*, and *Chytridiomycota*) possess SIT proteins [57]. However, there are exceptions such as the *Chytridiomycota* species *Batrachochytrium dendrobatidis*. In line, *Mucoromycota* are able to take up hydroxamate siderophores despite the fact that they do not synthesize this siderophore type [72,73]. As most fungal species appear to produce siderophores, it is likely that the non-siderophore-producing species have

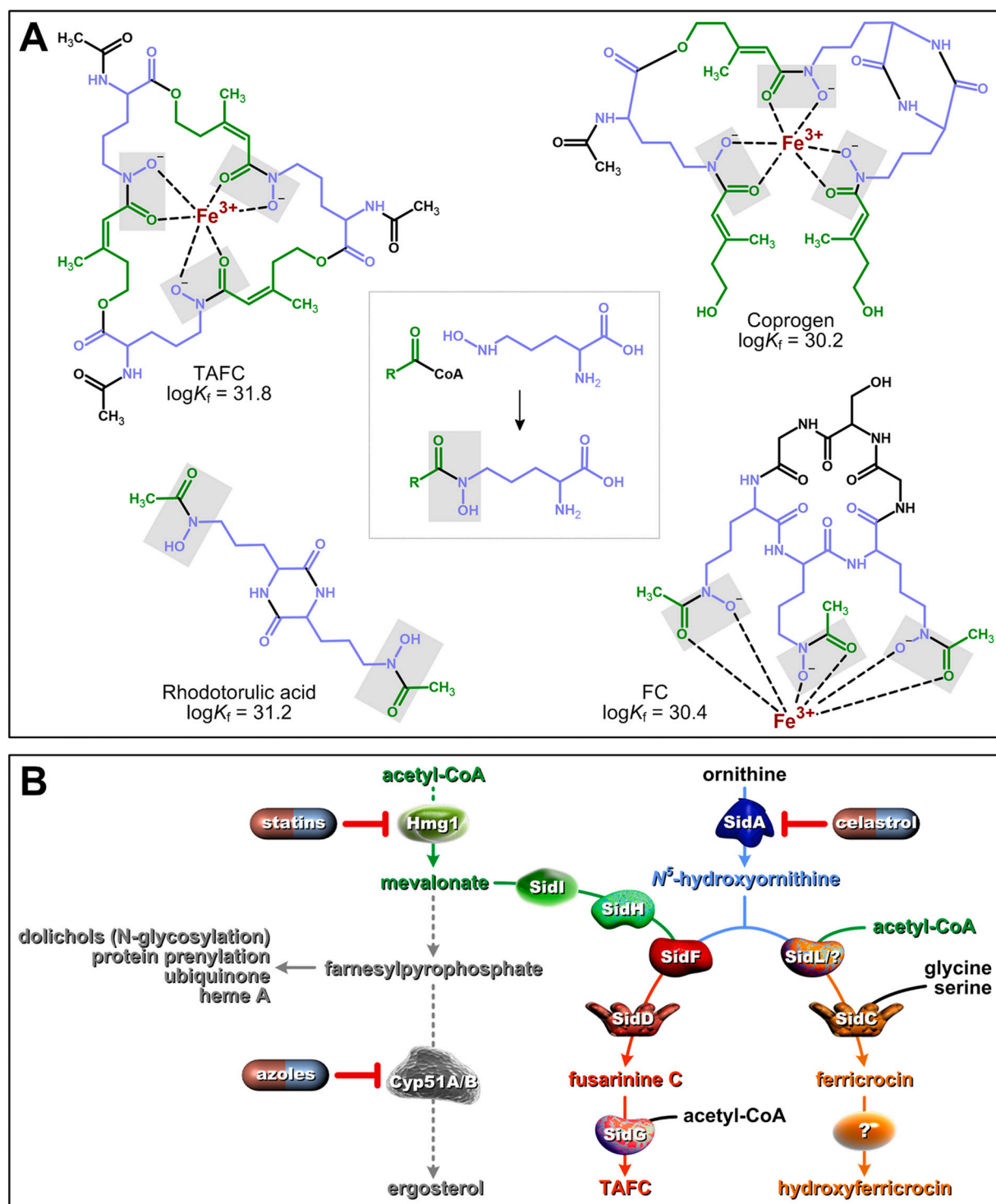


Fig. 2. Structures of hydroxamate siderophore representatives and siderophore biosynthetic pathway in *A. fumigatus*. (A) All shown siderophores share common building blocks, N^5 -hydroxyornithine (in blue) and acyl/acetyl groups (in green), which constitute the hydroxamate-groups (boxed in gray; middle panel). Hydroxamate groups are shaded in gray. In fusicarbinol C, T AFC and coprogen, the acyl group is anhydromevalonyl; in rhodotorulic acid and ferricrocin it is acetyl. The acetyl groups lacking in fusicarbinol C compared to T AFC are shown in black. Siderophore- Fe^{3+} formation constants ($\log K_f$) were taken from references [276–278]. (B) The first committed step in the biosynthesis of *A. fumigatus* siderophores is the N^5 -hydroxylation of ornithine catalyzed by SidA. Subsequently, the pathways for biosynthesis of extra- and intracellular siderophores split. For intracellular siderophores, acetyl is transferred to N^5 -hydroxyornithine by SidL and an unidentified transacetylase. For extracellular siderophores, the transacetylase SidF transfers anhydromevalonyl to N^5 -hydroxyornithine. The required anhydromevalonyl-CoA moiety is derived from mevalonate by CoA-ligation and dehydration catalyzed by the enzymes SidI and SidH, respectively, thereby linking isoprenoid and siderophore biosynthetic pathways. Fusicarbinol and ferricrocin are then assembled by the non-ribosomal peptide synthetases SidD and SidC, respectively. T AFC is derived by triple N^2 -acetylation of fusicarbinol C catalyzed by SidG. For ferricrocin biosynthesis, two glycine residues and one serine residue are incorporated in addition to the three hydroxamate groups. Hydroxyferricrocin is derived from ferricrocin by a single hydroxylation step catalyzed by an unknown enzyme. SidI, SidF and SidH are localized in peroxisomes, while the other dedicated siderophore biosynthetic enzymes are believed to operate in the cytosol [279]. The targets of statins (HMG-CoA reductase, Hmg1), azoles (Cyp51A), and celastrol (SidA) are indicated.

Table 1

Siderophores of selected species. Ferrichrome-type, coprogen-type, and fusarinine-type siderophores are shown in blue, green, and red, respectively; “?” marks species in which the conidial siderophore has not been analyzed; species that do not produce conidia or hydroxamate siderophores are marked by “na” (not applicable); * Epichloënin A is an atypical ferrichrome-type siderophore but with a cyclic structure consisting of eight amino acid residues. Detailed siderophore structures have been excellently reviewed by Hider and Kong [46]. Some references are exclusively found in this Table [261,262,264–267].

Species	Extracellular siderophore	Hyphal/yeast siderophore	Conidial siderophore	Reference
Ascomycota				
<i>Schizosaccharomyces pombe</i>	Ferrichrome	Ferrichrome	na	[59]
<i>Saccharomyces cerevisiae</i>	-	-	na	[57]
<i>Candida glabrata</i>	-	-	na	[57]
<i>Candida albicans</i>	-	-	na	[57]
<i>Aspergillus fumigatus</i>	Fusarinine C T AFC	Ferricrocin	Hydroxyferricrocin	[100]
<i>Aspergillus nidulans</i>	Fusarinine C T AFC	Ferricrocin	Ferricrocin	[261]
<i>Aspergillus niger</i>	Coprogen B Ferrichrome	Ferrichrome	?	[54]
<i>Aspergillus terreus</i>	Coprogen Ferrichrysin	Ferrichrysin	?	[55]
<i>Penicillium chrysogenum</i>	Coprogen	Ferrichrome	Ferrichrome	[261]
<i>Neurospora crassa</i>	Coprogen	Ferricrocin Ferrichrome C	Ferricrocin	[262]
<i>Fusarium oxysporum</i>	Fusarinine C Malonychrome	Ferricrocin Ferrichrome C Malonychrome	?	[49]
<i>Fusarium graminearum</i>	T AFC Malonychrome	Ferricrocin	?	[228,263]
<i>Cochliobolus heterostrophus</i>	Coprogen Neocoprogen I Neocoprogen II	Ferricrocin	?	[103,228]
<i>Alternaria brassicicola</i>	Dimethylcoprogen			[228]
<i>Acremonium chrysogenum</i>	Coprogen B, Methylcoprogen B, Dimethylcoprogen	Ferricrocin	?	[264]
<i>Magnaporthe grisea</i>	Coprogen, coprogen B Methylcoprogen Methylcoprogen B	Ferricrocin	?	[265]
<i>Colletotrichum graminicola</i>	Coprogen Coprogen B Methylcoprogen B	Ferricrocin	?	[230]
<i>Aureobasidium melanogenum</i>	Fusarinine C	Ferricrocin Hydroxyferricrocin	?	[58]
<i>Metarhizium robertsii</i>	Metachelins	Ferricrocin	?	[266]
<i>Epichloë festucae</i>	Epichloënin A*	Epichloënin A* Ferricrocin	?	[243,267]
Basidiomycota				
<i>Cryptococcus neoformans</i>	-	-	na	[57]
<i>Microbotryum violaceum</i>	Rhodotorulic acid	Rhodotorulic acid	na	[235]
<i>Rhodotorula glutinins</i>	Rhodotorulic acid	Rhodotorulic acid	na	[60]
<i>Ustilago maydis</i>	Ferrichrome Ferrichrome A	Ferrichrome Ferrichrome A	na	[53]
Mucoromycota				
<i>Rhizopus delemar</i>	Rhizzoferrin	rhizzoferrin	na	[63]
<i>Mucor mucedo</i>	Rhizzoferrin	rhizzoferrin	na	[61]

lost the ability to synthesize siderophores due to the lack of selection pressure in their niche, while they have preserved the ability to utilize siderophores. The ability to utilize xenosiderophores (siderophores of non-self origin) can be explained by the fact that siderophore-chelated iron is highly valuable due to its solubility. Moreover, the utilization of xenosiderophores most likely plays a role in microbial competition and can thus be observed also in siderophore-producing fungi (see below). Uptake of xenosiderophores via SIT members has been demonstrated for example for *S. cerevisiae*, *C. albicans*, *C. glabrata*, *C. neoformans*, *A. fumigatus*, and *A. nidulans* [74–80], whereby also bacterial siderophores have been shown to be utilized by fungi including the hydroxamate siderophore ferroxamine B and the catecholate siderophore enterobactin. *A. fumigatus* possesses five potential siderophore transporters, which are induced by iron starvation [81]. MirB transports the endogenous siderophore T AFC [82], Sit1 and Sit2 transport ferrichrome, whereby Sit1 transports additionally ferroxamine B (Fig. 1A;

[75]). The substrate specificity of MirC and MirD remains elusive whereby a function of MirC in ferricrocin biosynthesis was suggested [83].

The high affinity of siderophores to iron requires mechanisms to release the chelated iron from siderophores to make it available for metabolic processes after uptake. In *Aspergillus* spp., three esterases have been identified, which intracellularly hydrolyze siderophores with strict substrate specificity: *A. fumigatus* EstB for T AFC (Fig. 1A), *A. fumigatus* SidJ for fusarinine C, and *A. nidulans* EstA for enterobactin [84–86]. Recently the X-ray crystal structures of all three enzymes have been resolved providing new insights on the mode of action and substrate specificity [86]. Noteworthy, EstA and EstB are homologs of bacterial esterases hydrolyzing enterobactin and salmochelins [87], which again underlines the overlap of modules used in bacterial and fungal SIA.

5. Iron storage and detoxification

Two different mechanisms for iron storage have been described in fungi, namely vacuolar and siderophore-mediated iron storage. Moreover, ferritin-like iron-containing proteins have been detected in *Mucoromycota* [88].

5.1. Vacuolar iron storage and detoxification

The role of the vacuole in iron homeostasis has been extensively investigated in *S. cerevisiae*. In this yeast, iron is imported into the vacuole by the transporter Ccc1 for iron storage, reuse, and detoxification as ferric polyphosphate [89]. Reuse of vacuolar-stored iron is mediated by paralogs of RIA components localized in the vacuolar membrane, *i.e.*, by the metalloredutase Fre6 and a complex consisting of the ferroxidase Fet5 and the iron permease Fth1 [90,91]. Moreover, the NRAMP transporter Smf3 appears to play a role in vacuolar iron export in *S. cerevisiae* [92]. As shown in *S. cerevisiae* and *C. albicans*, vacuolar iron management is linked to mitochondrial iron metabolism [93,94]. Similar to *S. cerevisiae*, inactivation of the Ccc1 homolog CccA highly decreases resistance to iron toxicity in *A. fumigatus* demonstrating the conservation of vacuolar iron detoxification *via* the Ccc1 homolog CccA (Fig. 1; [95]). However, it is not clear if vacuolar stored iron can be reused in *A. fumigatus*. In *S. pombe*, the Ccc1 homolog Pcl1 mediates vacuolar iron import and the ABC transporter Abc3 is involved in reuse of vacuolar stored iron [96,97].

5.2. Siderophore-mediated iron storage

Siderophore-producing fungi use siderophores also for intracellular iron handling. The rationale for this strategy might be that siderophore-bound iron does not participate in Fenton chemistry. Intracellular siderophores might lower the labile iron pool to prevent formation of deleterious hydroxyl radicals [98,99].

A. fumigatus employs the ferrichrome-type siderophore ferricrocin for hyphal- and its hydroxylated version hydroxyferricrocin for conidial iron handling [100]. Conidial siderophore-mediated iron storage was shown to be important for germination of conidia in *A. fumigatus*, *A. nidulans*, *Neurospora crassa*, and *Penicillium chrysogenum* [57,101]. Moreover, several lines of evidence indicate that siderophores are also involved in iron trafficking within hyphae, *i.e.*, (i) in *A. fumigatus*, *A. nidulans*, *C. heterostrophus*, and *Giberella zeae* lack of ferricrocin reduces sporulation and/or blocks sexual development, both of which depend on transport of nutrients from substrate to aerial hyphae [98,102,103], (ii) conidia produced by ferricrocin lacking mutants display an iron starvation signature at transcriptional and protein levels under iron replete conditions [102], (iii) lack of ferricrocin increases the hyphal iron content about 2-fold in *A. fumigatus* [100], and (iv) lack of ferricrocin decreases the growth rate during iron starvation in *A. fumigatus* and *A. nidulans* [98,100].

6. Fungal iron regulatory systems to maintain iron homeostasis

As described above, different fungal species evolved diverse, partially overlapping, mechanisms for iron acquisition and storage. To avoid both iron shortage and toxicity, these mechanisms require finetuned regulation based on cellular iron-sensing. The common conserved outcome of iron regulation in all species is upregulation of high-affinity iron acquisition and downregulation of iron consumption to spare iron during iron starvation as well induction of iron detoxification during iron excess. Dependent on the species, tightly regulated high-affinity iron acquisition systems include SIA, RIA and/or heme acquisition. Regulated iron-consuming pathways include respiration, TCA cycle, biosynthesis of heme, amino acids, and sterols. The major iron detoxifying mechanism conserved in most species is vacuolar iron deposition. Notably, different fungal species evolved diverse iron-regulatory

mechanisms. Examples of fungal iron-regulatory factors are summarized in Table 2. Remarkably, the model eukaryote *S. cerevisiae* displays a rather unique system as one of its iron-sensing transcription factors, Aft1/2, apart from *Saccharomycetes*, is not widespread. In contrast, the regulators employed by *A. fumigatus*, SreA and HapX, are largely conserved in the fungal kingdom. The differences are probably best understood in the context of phylogeny and evolutionary events as discussed below (Fig. 3). HapX homologs are found in *Ascomycota* and *Basidiomycota*. SreA appears to be the iron-regulatory protein with the highest phylogenetic conservation as it is found in *Ascomycota*, *Basidiomycota*, and *Mucoromycota*. Despite the species-specific differences, certain iron-regulatory/sensing modules within transcription factors are highly conserved in all fungal species analyzed so far. Evolutionary conservation of the DNA-binding and iron-sensing domains of SreA homologs is displayed in Fig. 4; domain architecture of HapX homologs is illustrated in Fig. 5A and evolutionary conservation of the DNA-binding domain of HapX homologs is shown in Fig. 5B. Species-specific details are discussed below.

6.1. Iron regulation in *A. fumigatus*

6.1.1. Iron regulation via iron-sensing transcription factors

A. fumigatus employs two iron-sensing transcription factors, termed SreA and HapX [51]. SreA harbors two Cys₂Cys₂ GATA-type zinc fingers, which recognize the consensus DNA sequence ATCWGATAA, separated by a cysteine-rich region (CRR), which mediates iron sensing (Fig. 4). HapX comprises several phylogenetically conserved domains (Fig. 5): (i) a bZIP-type DNA-binding domain, (ii) a Hap4-like domain (Hap4L) for physical interaction with the CCAAT-binding complex (CBC, termed Hap complex in *S. cerevisiae*; see below), and (iii) four CRR, whereby two CRR (CRR-A and CRR-B) have been shown to be involved in iron sensing, most likely *via* [2Fe-2S] cluster binding similar to SreA, while the other two (CRR-C and CRR-D) lack assigned functions.

During iron sufficiency, SreA represses high-affinity iron uptake, including RIA and SIA, to avoid iron toxicity. During iron starvation, HapX represses iron-consuming pathways such as respiration, heme biosynthesis, TCA cycle, and vacuolar iron deposition to spare iron. Furthermore, HapX transcriptionally activates SIA but also iron-independent functions such as ribotoxin AspF1 [104]. Remarkably, iron excess converts HapX into an activator of iron-dependent pathways, particularly vacuolar iron deposition, which appears to be the major mechanism of iron detoxification [105]. The functions of HapX during iron starvation and iron excess can be confined to specific protein domains, *i.e.*, mutation of the CRR-B impairs adaptation of *A. fumigatus* to iron excess but not iron starvation; inversely, truncation of the C-terminus impairs adaptation to iron starvation but not iron excess. HapX executes all functions *via* physical interaction with the CBC [106]. The CBC has HapX-independent functions and is speculated to affect expression of about 30% of all genes; *i.e.*, the CBC-HapX regulon is a subset of the CBC regulon. Taken together, both SreA and HapX are important for adaptation to iron sufficiency, while only HapX is also crucial for adaptation to iron starvation. Inactivation of both HapX and SreA is synthetically lethal, underlining the critical role of iron homeostasis in cellular survival [104,107].

Most fungal species possess homologs to SreA and HapX. *Saccharomycotina*, *Taphrinomycotina*, *Basidiomycota*, and *Mucoromycota* are discussed below. Apart from these, SreA-like functions have been confirmed for homologs of the ascomycete species *A. nidulans*, *A. terreus*, *A. niger*, *N. crassa*, *Histoplasma capsulatum*, *C. heterostrophus*, *A. melanogenum*, *Alternaria alternata*, *Epichloë festucae*, *Fusarium fujikuroi*, and *Blastomyces dermatitidis* [54,55,108–116]; HapX-like functions have been confirmed for homologs of *A. nidulans*, *A. terreus*, *Fusarium graminearum*, *F. oxysporum*, *Verticillium dahliae*, and *B. dermatitidis* [49,55,107,114,117]. The mechanism of iron sensing by fungal transcription factors is discussed below.

Table 2
Examples of conserved fungal regulators securing iron homeostasis. Proteins shaded in the same color are homologs or share at least conserved domains.

Species	Repression of iron acquisition during iron starvation	Repression of iron-consuming processes during iron starvation	Activation of iron acquisition during iron starvation	Activation of iron detoxification during iron excess
<i>S. cerevisiae</i>		Cth1/2 ¹	Aft1/2	Yap5
<i>C. glabrata</i>		Cth2 ¹	Aft1	Yap5
<i>S. pombe</i>	Fep1	Php4		
<i>C. albicans</i>	Sfu1	Hap43		
<i>C. neoformans</i>	Cir1 ²	HapX		
<i>A. fumigatus</i>	SreA	HapX		

¹, In contrast to the other regulators, Cth1/2 do not act transcriptionally but posttranscriptionally.
², Cir1 has also activating functions, e.g., for siderophore uptake and virulence determinants.

6.1.2. Iron homeostasis is tightly interwoven with other metabolic pathways

Due to the central metabolic role of iron, it is not surprising that a variety of regulatory circuits affect cellular iron handling. Most obvious, balance of iron, oxygen, and redox is intimately linked. On the one hand, oxidation of iron is the reason for the low iron availability. On the other hand, most redox reactions are catalyzed by proteins taking advantage of the electron carrying function of iron, often with oxygen as the final electron acceptor such as in oxidative phosphorylation. As byproduct of these reactions iron can generate reactive oxygen species (ROS) leading to oxidative stress. However, iron is required to detoxify ROS as catalases and other peroxidases often contain heme-iron as a cofactor. Moreover, upregulation of iron dependent pathways requires coordinated iron acquisition and siderophore biosynthesis requires coordinated precursor supply. Noteworthy, RIA and SIA are both oxygen-dependent pathways due to oxygen consumption of the reactions mediated by SidA and FtrA.

Regarding redox balance, it is fascinating that the CBC, the essential interaction partner of iron-sensing HapX, was found to sense ROS through oxidative modification of cysteine residues in order to mediate oxidative stress response [118], i.e., the CBC:HapX complex combines regulation of iron and redox balance. Recently, oxidative stress was

found to be more deleterious during iron starvation and to overrule CBC:HapX complex-mediated repression during iron starvation [119].

Adaptation to oxygen limitation represents another link between oxygen and iron homeostasis. Hypoxic adaptation requires upregulation of glycolysis, the TCA-cycle, respiration, and ergosterol biosynthesis, and as a consequence increased cellular iron and heme contents, which is coordinated by the transcription factor SrbA, a member of the sterol regulatory element binding protein (SREBP) family [120,121]. SrbA is proteolytically activated upon depletion of the cellular sterol pool, which appears to serve as “oxygen sensor” because ergosterol biosynthesis is highly oxygen consuming [122]. However, ergosterol biosynthesis is also highly dependent on iron-dependent enzymes (e.g., P450 enzymes such as Cyp51A). SrbA was found to be crucial not only for sterol-feedback regulation and consequently resistance against triazole drugs and hypoxic growth, but also for activation of high-affinity iron acquisition including RIA and SIA [123]. This indicates that SrbA coordinates cellular oxygen and iron handling. Inversely, the iron-sensing CBC:HapX complex was recently identified as transcriptional repressor of the iron-dependent ergosterol biosynthetic enzyme Cyp51A and the cytochrome b₅ CybE, which is required to fuel P450 enzymes including Cyp51A with electrons (Fig. 6A; [29,124]). Via this link, the

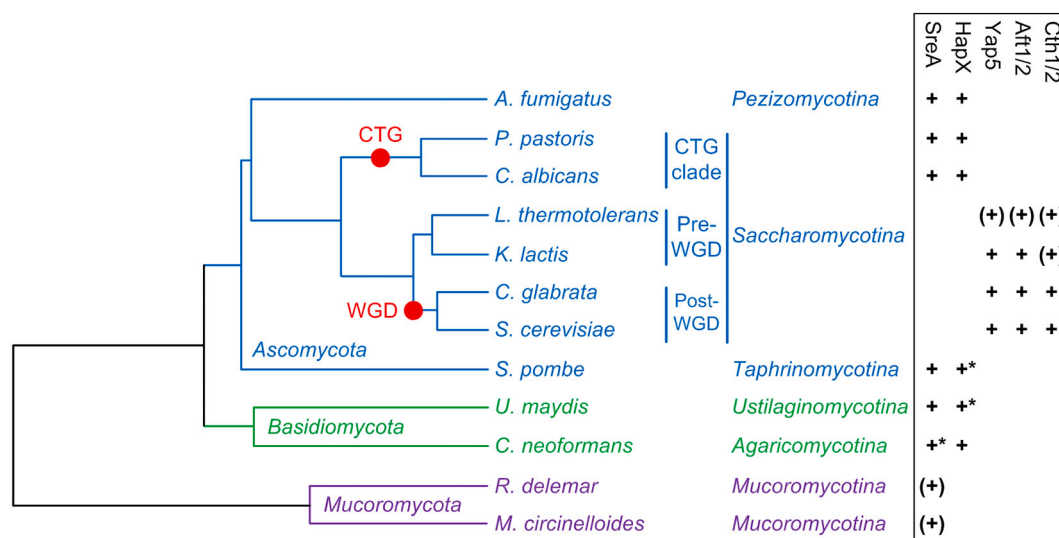


Fig. 3. Phylogeny of selected fungal species including their key iron regulators. The phylogenetic tree is schematically drawn according to Gabaldón et al. [147]. (+) marks regulators based solely on protein similarity (E-value < e-50). +* marks homologous regulators with functional differences in comparison to *A. fumigatus* HapX or SreA.

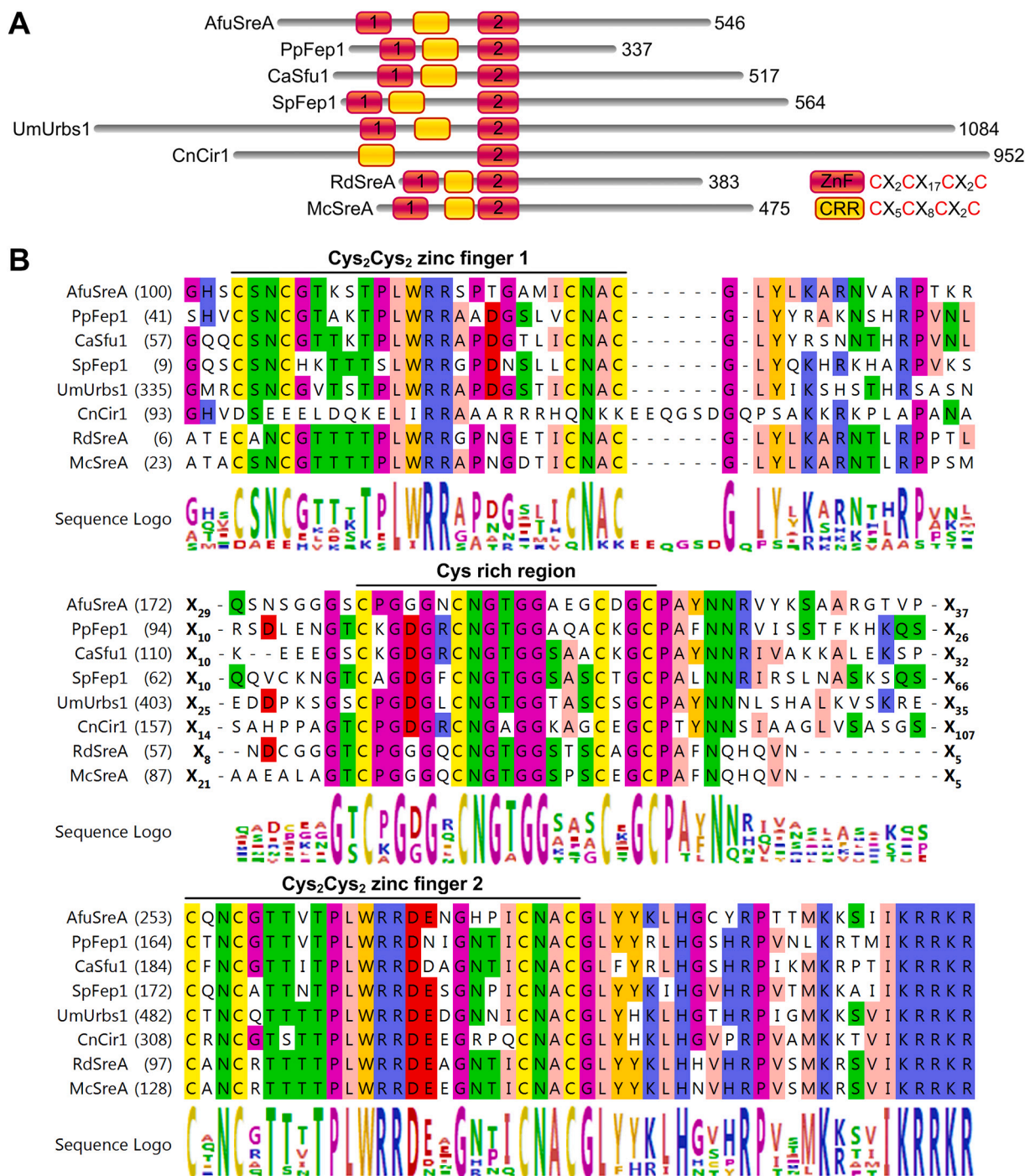


Fig. 4. Evolutionary conservation of the DNA-binding and iron-sensing domains of SreA homologs from *A. fumigatus* (Afu), *P. pastoris* (Pp), *C. albicans* (Ca), *S. pombe* (Sp), *U. maydis* (Um), *C. neoformans* (Cn), *R. delemar* (Rd), and *M. circinelloides* (Mc). (B) Amino acid sequence alignment of the Cys₂Cys₂ GATA-type zinc finger (ZnF) and cysteine-rich regions (CRR) of fungal SreA orthologs. Residues that match the consensus sequence are colored according to their physico-chemical properties. Alignments were generated using MegAlign Pro 17 (DNASTAR, Inc.).

CBC:HapX complex also modulates azole resistance. Moreover, the dominating class of pan-azole resistance, characterized by the occurrence of a tandem repeat of at least 34 bases within the promoter of the Cyp51A encoding gene, is linked to iron because the repeat increases transcriptional activation by SrbA and decreases transcriptional repression by the CBC:HapX complex [124]. Increased azole resistance caused by a mutation in one of the subunits of the CBC, HapE^{P88L}, underlines CBC:HapX-mediated regulation of Cyp51A [125]. Notably, there is not only a SrbA-mediated regulatory link between sterol/isoprenoid metabolism and SIA but also a direct enzymatic link because

the sterol/isoprenoid biosynthetic intermediate mevalonic acid is a precursor of extracellular siderophore biosynthesis (Fig. 2B; [51]). The SREBP family is conserved in most eukaryotes but *S. cerevisiae* lacks SREBP-mediated adaptation to oxygen limitation, which underlines fundamental regulatory rewiring in this yeast as also shown for iron regulation as discussed below.

Another example for the integration of an iron-dependent metabolic pathway into the regulation of iron acquisition is biosynthesis of the branched-chain amino acids isoleucine, leucine, and valine [126,127]. This biosynthetic pathway comprises the two Fe-S-dependent enzymes

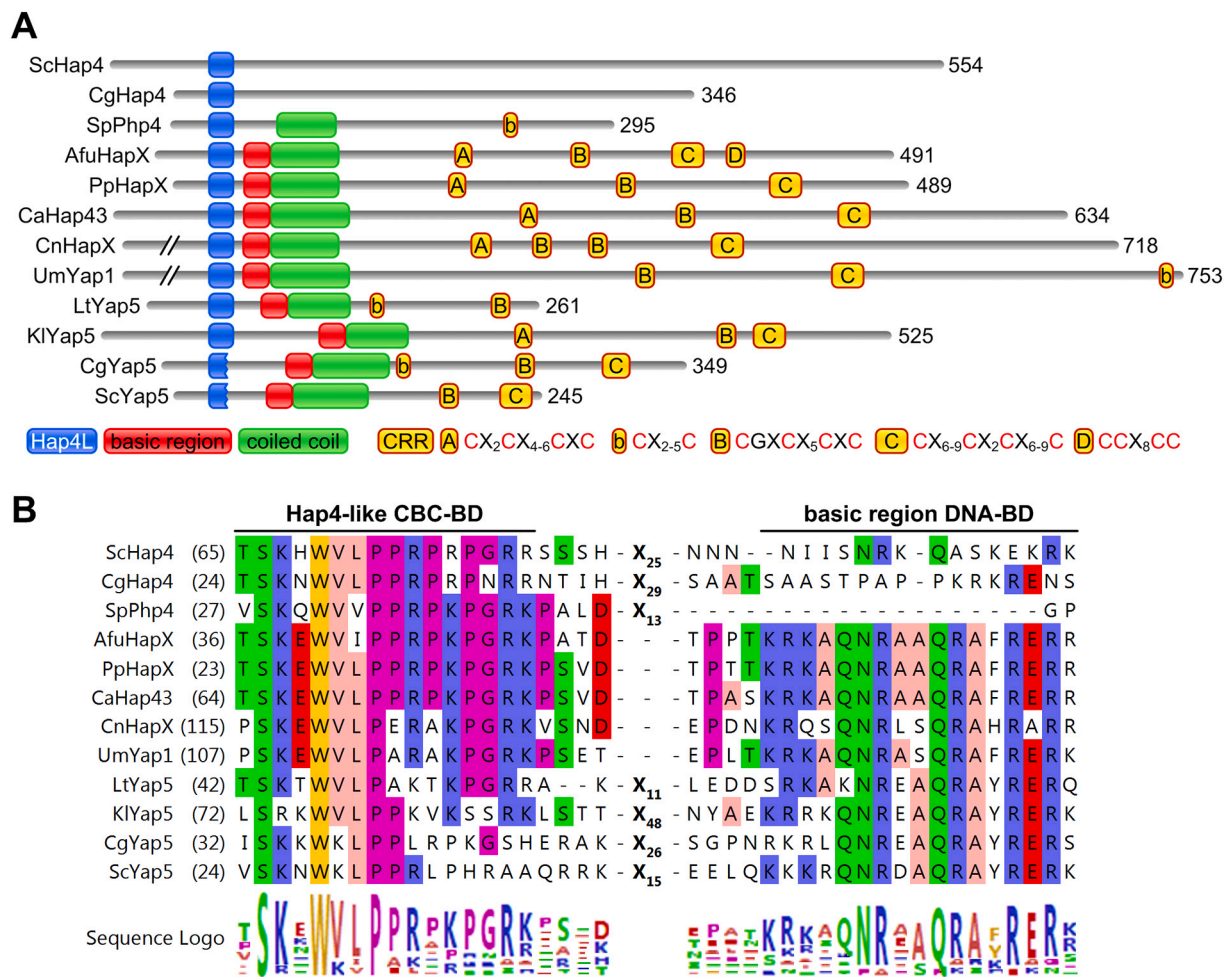


Fig. 5. Domain architecture of fungal Hap4-type and HapX/Yap5-type iron regulators that require the CBC as DNA-binding scaffold for their function. (A) The Hap4 activators of respiratory gene expression in *S. cerevisiae* (Sc) and *C. glabrata* (Cg) contain the canonical 16 aa Hap4-like CBC-BD, but lack a bZIP domain. The same applies to Php4 in *S. pombe* (Sp). *A. fumigatus* (Afu) HapX and its homologs in *P. pastoris* (Pp), *C. neoformans* (Cn), and Hap43 in *C. albicans* (Ca) as well Yap1 in *U. maydis*, Yap5 in *Lachancea thermotolerans* (Lt), and *K. lactis* (Kl) are bZIP transcription factors that share the full 16 aa Hap4-like CBC-BD. In contrast, Yap5 bZIP-type proteins in *C. glabrata* and *S. cerevisiae* contain a degenerated Hap4-like domain. Full and rudimentary Hap4-like domains required for CBC-binding are shown in blue. Basic regions, coiled coil domains, and cysteine-rich regions are depicted in red, green and yellow, respectively. (B) Amino acid sequence alignment of the Hap4-like CBC-BD and basic region DNA-BD spanning regions in the N-Termini of regulatory CBC subunits shown in (A). Residues that match the consensus sequence are colored according to their physico-chemical properties. Alignments were generated using MegAlign Pro 17 (DNASTAR, Inc.).

dihydroxyacid dehydratase (Ilv3A) and α -IPM isomerase (LeuA) providing the rationale for downregulation of the encoding genes during iron starvation by the CBC:HapX complex [104]. Leucine shortage is sensed by the Zn₂Cys₆-type transcription factor LeuB, which is post-translationally activated by accumulation of the leucine pathway intermediate α -IPM [127,128]. Recently, LeuB was shown to serve not only as transcriptional activator of leucine biosynthesis but also of HapX and several genes involved in high affinity iron acquisition; *i.e.*, it cross-regulates leucine biosynthesis and iron acquisition [126]. In agreement with α -IPM as posttranslational activator of LeuB for this process, inactivation of LeuC, which blocks α -IPM synthesis, but not inactivation of LeuA, which causes accumulation of α -IPM, was shown to block adaptation to iron starvation [127]. A scheme of these regulatory links is shown in Fig. 6B.

Iron-starvation induces siderophore production and the amount of produced siderophores can reach up to 10% of the fungal biomass [51]. This accomplishment is achieved by coordinated upregulation of the siderophore biosynthetic pathway and precursor supply. A major precursor of siderophores is the non-proteinogenic amino acid ornithine, which can be produced within the mitochondria, as intermediate of

arginine biosynthesis, or within the cytosol *via* arginase-mediated hydrolysis of arginine [129]; during iron starvation, both pathways are upregulated [51]. With glutamine as nitrogen source, ornithine is produced mainly *via* the mitochondrial pathway followed by export into the cytosol, where siderophore biosynthesis starts. In contrast, with arginine as nitrogen source, ornithine is produced mainly in the cytosol from arginine [129,130]. The first enzyme involved in siderophore biosynthesis, the ornithine monooxygenase SidA, has been demonstrated to be activated allosterically by arginine [131]. This regulation prevents excessive depletion of the cellular arginine pool, which is essential also for other purposes such as protein biosynthesis, for the sake of siderophore biosynthesis.

Moreover, the gluconeogenesis activating transcription factors AcuK and AcuM [132,133], nitrogen metabolite repression-mediating transcription factor AreA [134], unfolded protein response-mediating proteins IreA and HacA [135], pH response regulator PacC [136], cross-pathway control regulator CpcA [137], protease activating factor PrtT [138], and the mitogen-activated protein kinase (MAPK) MpkA [139], which is involved in maintaining cell wall integrity and protection against ROS, have been implicated in the regulation of iron uptake.

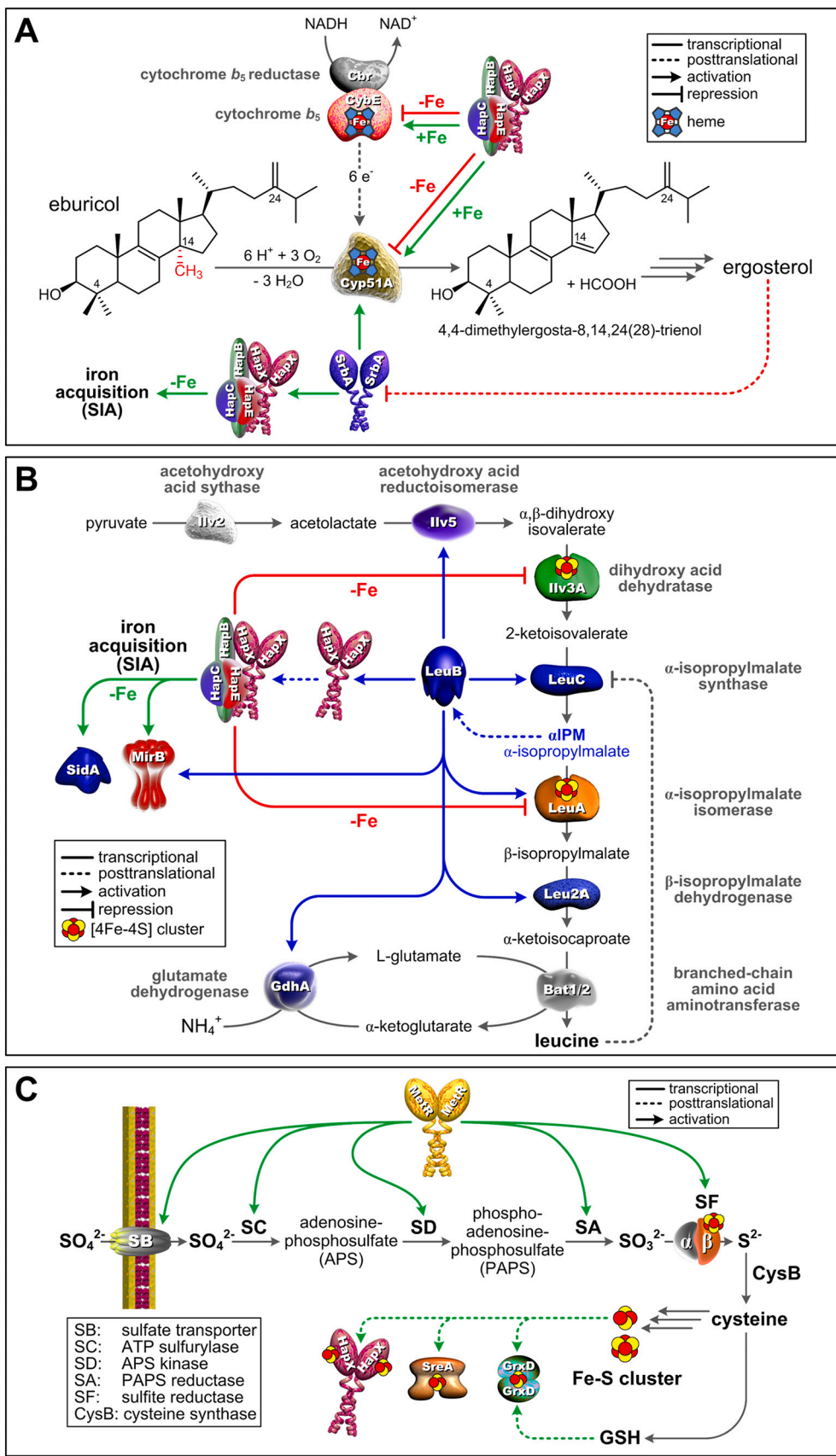


Fig. 6. Iron-regulatory links of selected metabolic pathways. (A) The target of azoles, heme-dependent ergosterol biosynthetic Cyp51A, and the cytochrome *b*₅ CybE, which fuels Cyp51A with electrons, are both transcriptionally repressed by the CBC:HapX complex during iron starvation. Transcriptional activation of Cyp51A and iron acquisition is mediated by SreA, which is activated by ergosterol depletion caused for example by iron starvation due to iron dependence of ergosterol biosynthesis. With respect to SreA-mediated regulation of iron acquisition, ergosterol depletion might serve as an indirect signal for iron starvation. (B) Iron availability is indirectly sensed via the iron-dependent activity of leucine biosynthesis. LeuB transcriptionally activates leucine biosynthesis and iron acquisition directly and indirectly via HapX. LeuB is activated by α-IPM, which accumulates during iron starvation due to the Fe-S dependency of Ilv3A and LeuA. In a feedback loop, Ilv3A and LeuA are transcriptionally repressed by the CBC:HapX complex during iron starvation. (C) MetR is a transcriptional activator of sulfate assimilation, which is required for biosynthesis of sulfur containing amino acids, Fe-S and GSH. Consequently, MetR is required for iron sensing by SreA and HapX.

6.2. Iron regulation in *C. albicans* and *Pichia pastoris*

CTG clade species including *P. pastoris* and *C. albicans* employ SreA and HapX homologs (Fig. 3). In *C. albicans*, these are called Sfu1 and Hap43, respectively. Sfu1 functions similarly to *A. fumigatus* SreA [140]. Despite high sequence similarity of Hap43 and *A. fumigatus* HapX, Hap43 is dispensable for growth in high iron conditions, i.e., for iron detoxification [141]. In other words, only its function during iron starvation is conserved in comparison to *A. fumigatus*. The regulatory feedback between Sfu1 and Hap43 includes a third regulator, Sef1, which is subject to posttranslational regulation via phosphorylation [142,143]. Sef1, is activated during iron starvation, and in turn, transcriptionally activates iron acquisition and Hap43. Sfu1 and Hap43 possess domains for sensing of [2Fe-2S] availability and consequently the cellular iron status (Fig. 5). What might be the rationale for integration of a third transcription factor? In this respect it is interesting to note that Sef1 displays considerable sequence similarity with *S. cerevisiae* Leu3, *A. nidulans* LeuB, and *A. fumigatus* LeuB, which all activate leucine biosynthesis in response to shortage of leucine, which is an iron-dependent metabolite [126,144]. *A. fumigatus* LeuB has been shown to transcriptionally activate HapX and several components of high-affinity iron acquisition (see above). Consequently, it is tempting to speculate that Sef1 senses iron starvation via shortage in iron-dependent leucine pathway intermediates similar to LeuB/Leu3 transcription factors. In *P. pastoris*, only the SreA homolog was functionally characterized and shown to function similar to *A. fumigatus* SreA [145]. Regulation of iron detoxification is not clarified in these species but lack of Sef1 was found to increase susceptibility to iron toxicity in *C. albicans* [141].

6.3. Iron regulation in *S. cerevisiae* and *C. glabrata*

As shown in Fig. 3, *Saccharomycetales* split into the CTG clade (including *C. albicans* and *Pichia* spp), which exhibits non-canonical codon usage [146], and *Saccharomycetaceae* [147]. The latter can be divided into two clades, one of which conducted a whole genome duplication (WGD) followed by gene loss (Post-WGD, including *S. cerevisiae* and *C. glabrata*) and the other without this genome re-arrangement (Pre-WGD species including *Kluyveromyces lactis* and *Ashbya gossypii*). As discussed above, the CTG clade species employ homologs of *A. fumigatus* SreA and HapX. In contrast, the *Saccharomycetaceae* lost SreA, conserved only the iron detoxification function of HapX leading to Yap5 proteins, and evolved novel iron regulators, Aft1/2 and Cth1/2. As Pre-WGD and Post-WGD species employ largely the same iron regulators (Fig. 3), this transcriptional rewiring most likely occurred before the WGD. Consequently, *S. cerevisiae*, represents a rather unique system for iron regulation despite the fact that it is the fungal system that has been characterized in most detail. In response to iron starvation, the two paralogous transcription factors Aft1 and Aft2 activate high-affinity iron acquisition, RIA and siderophore uptake, as well as the two paralogous proteins Cth1 and Cth2 [148]. Cth1/2 initiate degradation of mRNAs encoding iron consuming pathways and iron detoxification (e.g., Ccc1) by binding to AU-rich elements in their 3'-UTRs [149]. Moreover, Cth2 also represses the translation of these genes [150]. Taken together, Cth1/2 repress iron use posttranscriptionally, a function that is carried out transcriptionally by species employing HapX. Iron detoxification via vacuolar iron deposition by Ccc1 is activated by Yap5, which shares several domains with *A. fumigatus* HapX (Fig. 5), but apparently lacks any function during iron starvation [148]. Notably, neofunctionalization of the duplicated version of Yap5 upon WGD led to Yap7 proteins, which act as repressors of nitric oxide detoxification [151].

C. glabrata uses a similar system as *S. cerevisiae* but employs only a single homolog of Aft1/2 and Cth1/2 [152,153]. Moreover, it includes a third regulator, Sef1, which is transcriptionally repressed by Cth2 and which activates TCA cycle enzymes and ISC assembly factors during

iron starvation [153].

Interestingly, both *C. albicans* and *P. pastoris* possess a protein that contains a zinc finger DNA-binding domain that is homologous to that of *S. cerevisiae* Aft1/2 but lack the typical Aft1/2 iron-responsive motif (Cys-Asp-Cys); in these organisms lack of this transcription factor has only a very minor or no impact on iron metabolism [154,155]. Possibly, this protein was the ancestor of Aft1/2-mediated iron regulation in *Saccharomycetaceae*.

6.4. Iron regulation in *S. pombe*

For iron regulation *S. pombe* that belongs to the *Taphrinomycotina*, which split early from the other *Ascomycota* clades (Fig. 3), employs a classical SreA homolog, termed Fep1 (Fig. 4), and a protein termed Php4 [156]. Php4 only shares the Hap4-like domain with HapX homologs (Fig. 5), which is essential for interaction with the CBC. Similar to HapX homologs, in concert with the CBC, Php4 transcriptionally represses iron-consuming pathways, vacuolar iron detoxification, and Fep1. In contrast to HapX homologs, Php4 is not involved in activation of iron detoxification, i.e., it lacks a function during iron excess similar to *C. albicans* Hap43.

6.5. Iron regulation in basidiomycota: *U. maydis* and *C. neoformans*

The prototype of iron regulatory GATA factors homologous to SreA was identified in *U. maydis*, termed Urbs1 (Fig. 4; [157]). Moreover, *U. maydis* possesses a protein showing significant similarity with HapX/Yap5 proteins, termed UmYap1, which was found to regulate the oxidative stress response in this plant pathogen. Interestingly, UmYap1 and HapX/Hap43/Php4 proteins share the full Hap4-like domain and two cysteine-rich regions, CRR-B and CRR-C (Fig. 5). Mutation of cysteine residues within the CRR-B abolished the function of UmYap1 during oxidative stress response. The presence of the CGFC motif containing CRR-B indicates that UmYap1 recognizes [2Fe-2S] clusters and might function also in iron regulation as putative HapX/Yap5 homolog.

C. neoformans employs a SreA homolog displaying structural differences compared to other SreA homologs by lacking the N-terminal GATA-type zinc finger. Similar to the other SreA homologs, Cir1 represses RIA [158]. However, in contrast to SreA homologs, it functions not only as repressor but also as activator for several virulence determinants (e.g., for capsule formation) and uptake of siderophore-chelated iron, which is usually co-regulated with RIA. *C. neoformans* also possesses a classical HapX homolog, which represses Cir1 and iron consuming pathways but activates uptake of siderophore-chelated iron [159].

6.6. Iron regulation in *Mucoromycota*

Iron regulation has not been investigated in detail in *Mucoromycota* so far. However, it is interesting to note that *Mucoromycota*, including *Mucor circinelloides* (EPB84775.1) and *R. delemar* (EIE82954.1) encode proteins containing two GATA-type zinc fingers and an intervening CRR, which is typical for SreA homologs (Fig. 4). These data underline the wide conservation of SreA homologous regulators. In contrast, these species seem to lack typical HapX/Yap5 homologs.

7. Iron-sulfur (Fe-S) cluster biosynthesis and iron sensing

Fe-S clusters are essential cofactors in numerous proteins. Moreover, eukaryotes have been shown to sense iron mainly via intracellular Fe-S cluster availability rather than iron availability *per se*. Iron-sulfur cluster biogenesis has been extensively reviewed [22] and is also covered in detail by other articles in this issue. However, since Fe-S cluster biogenesis is inextricably connected to regulation of iron homeostasis, a brief overview about this pathway is provided here.

Although the composition of Fe-S clusters is usually rather

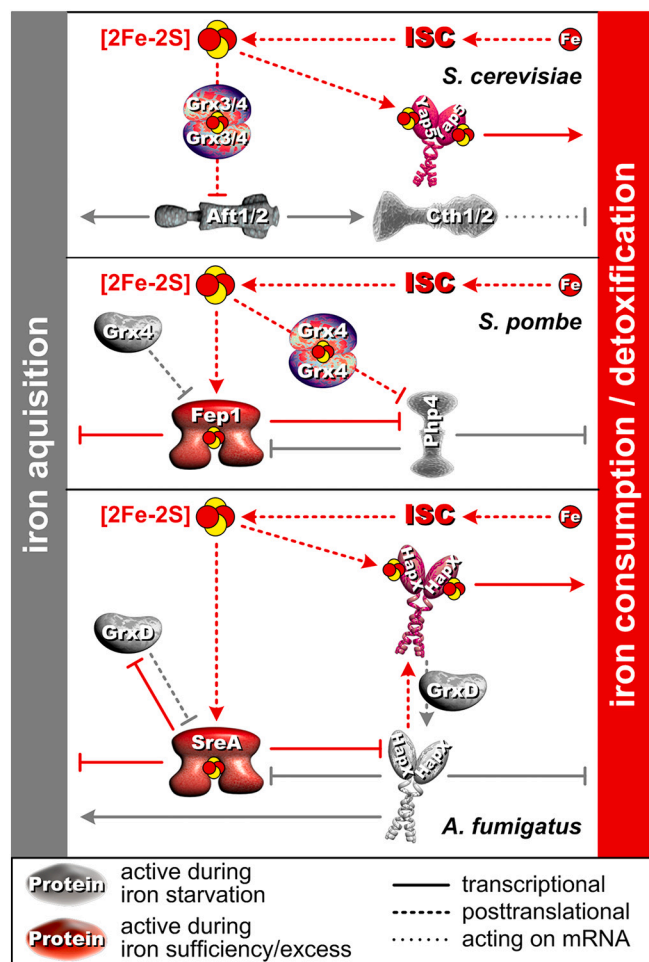


Fig. 7. Schematic overview of iron sensing and regulatory mechanisms in *S. cerevisiae*, *S. pombe*, and *A. fumigatus* with a focus on the role of GRX. In all three species, GRX is essential for iron sensing, however with different roles. For *S. cerevisiae* Aft1/2 and *S. pombe* Php4 GRX it is important for [2Fe-2S] cluster transfer (sensing iron availability), while *S. cerevisiae* Yap5, *S. pombe* Fep1, *A. fumigatus* SreA, and *A. fumigatus* HapX appear to recognize [2Fe-2S] clusters independent of GRX. For *S. pombe* Fep1, *A. fumigatus* SreA, and *A. fumigatus* HapX, and potentially *S. cerevisiae* Yap5, GRX is crucial for sensing iron starvation (potentially [2Fe-2S] cluster removal from transcription factors).

unspectacular, their biosynthesis is, and the functions enabled by Fe-S clusters are plentiful. The production of Fe-S clusters can be roughly subdivided into two parts, the mitochondrial iron-sulfur cluster machinery (ISC), consisting of [2Fe-2S] cluster biosynthesis (core ISC) and build on [4Fe-4S] cluster biosynthesis (late ISC) machinery, and the [4Fe-4S] cluster producing cytosolic iron-sulfur cluster assembly (CIA), which depends on an exported product of the core ISC.

Fe-S cluster biosynthesis starts with the import of iron into mitochondria, in *S. cerevisiae* by the paralogous transporters Mrs3/4; *A. fumigatus* employs a single homolog, termed MrsA [160,161]. Subsequently, iron and sulfur, which is provided by desulfuration of cysteine, are complexed to a [2Fe-2S] cluster via a complex cascade of enzymes and chaperones [162]. This pathway provides the precursor for mitochondrial [4Fe-4S] cluster generation, but also for CIA via a compound that is exported into the cytosol by the mitochondrial ABC transporter Atm1 and that contains iron and sulfur [163]. Whether this compound is already a [2Fe-2S] cluster or a precursor thereof is a matter of debate [162,164,165]. The central role of Fe-S cluster biosynthesis for cell homeostasis is reflected by the fact that almost all genes involved in this pathway are essential. The only exceptions in *S. cerevisiae* are Yfh1 (frataxin), Grx5, and Atm1 [166,167]. The non-

essentiality of Atm1 is explained by partial substrate redundancy with the mitochondrial ABC transporter Mdl1 [168]. The non-essentiality of Atm1 has also been reported in *S. pombe*, *F. graminearum*, and *C. neoformans* [134,169,170]; the non-essentiality of frataxin has been confirmed for *S. pombe*, *C. albicans* and *C. neoformans* [171–173]. The essentiality of the core ISC component Nfs1 and the CIA component Nbp35 was recently confirmed in *A. fumigatus* [174].

In mammalian cells, the iron regulatory protein IRP1 senses [4Fe-4S] cluster availability, which originates from CIA [175], while IRP2 was recently discovered to sense indirectly not only iron availability but also [2Fe-2S] cluster availability [176], which most likely originates from core ISC. In fungi, the crucial role of the core ISC for iron sensing has been demonstrated for *S. cerevisiae*, *C. albicans*, *S. pombe*, *C. neoformans*, *A. fumigatus*, and *F. graminearum* [134,171,172,174,177,178], as deficiency for Mrs3/4, Nfs1, Yfh1, Grx5 or Atm1 caused an iron starvation response in the presence of iron. However, dispensability of CIA for iron sensing has only been demonstrated in *S. cerevisiae* and *A. fumigatus* [174,178], which emphasizes the conserved importance of mitochondrially produced [2Fe-2S]- rather than cytosolically produced [4Fe-4S] clusters for iron sensing in fungal species, particularly as there is no indication for de-novo cytosolic [2Fe-2S] cluster biosynthesis [22].

One of the few [2Fe-2S] cluster coordinating proteins in the cytosol of eukaryotes are monothiol glutaredoxins (GRX, non-mitochondrial monothiol glutaredoxins), which are homologs of the mitochondrial Grx5 that is involved in [2Fe-2S] biosynthesis [179]. *S. cerevisiae* possesses two paralogs of GRX, namely Grx3 and Grx4. The [2Fe-2S] cluster is coordinated by four sulfur moieties originating from two glutathiones (GSH) and two monothiol glutaredoxins [180]. The sulfur group of GRX is the cysteine residue within the highly conserved CGFS motif. Grx3/4 has been shown to be crucial as [2Fe-2S] cluster chaperone for the assembly of cytosolic Fe-S proteins [181–183]. Recently, several studies indicated that GRX from plants, mammals, and yeast are important for cytosolic [4Fe-4S] cluster biosynthesis, i.e., they link core ISC with CIA [162,184–186]. In line, the single GRX of *A. fumigatus*, termed GrxD, was found to physically interact with components of the CIA machinery [187]. In *S. cerevisiae*, lack of both GRX homologs is lethal depending on the genetic background [182]. Similarly, lack of GRX is lethal in *A. fumigatus* and *C. neoformans* [187,188], while *S. pombe* mutants lacking the GrxD homolog are viable only under microaerophilic conditions [189]. In contrast, GRXs are not essential in *F. graminearum* and *C. albicans* [134,190]. In all fungal species analyzed, GRXs are additionally involved in iron sensing (see below). As partner of both Grx5 and GRX, GSH is required for coordination of [2Fe-2S] clusters [191]. Therefore, GSH is required for ISC and CIA and consequently also iron sensing.

Taken together, iron sensing in *A. fumigatus* has been linked to MrsA-mediated mitochondrial iron import, core ISC, GSH, and GrxD [174,187]. Moreover, extreme sulfur starvation via inactivation of the transcriptional activator of sulfate assimilation, MetR, renders *A. fumigatus* iron-blind due to derepression of high-affinity iron acquisition even in the presence of iron [192]. The most likely explanation is defective iron sensing due to the requirement of sulfur for [2Fe-2S] cluster biosynthesis and/or glutathione (Fig. 6C).

7.1. Iron sensing by transcription factors

A common theme in fungal iron homeostasis is the sensing of the cellular iron status via [2Fe-2S] cluster abundance by iron-regulatory transcription factors in concert with GRX. Besides GRX, a second [2Fe-2S] cluster chaperone, termed Bol2 (also termed Fra2) is involved in iron sensing by some transcription factors. However, there are significant mechanistic differences in iron sensing between species despite the use of similar components. Fungal iron sensing has recently been comprehensively reviewed by Gupta and Outten [193]. Iron sensing is best understood in *S. cerevisiae*, *S. pombe*, and *A. fumigatus*, which will

be compared in the following section (Fig. 7).

During iron starvation (low [2Fe-2S] cluster abundance), *S. cerevisiae* Aft1/2 binds to DNA to activate iron acquisition and iron sparing via Cth1/2. During iron availability (high [2Fe-2S] cluster abundance), a heterocomplex consisting of one GRX, one Bol2, and one glutathione transport [2Fe-2S] clusters to Aft1/2, which promotes DNA dissociation, dimerization, and nuclear export [181,194–196]. [2Fe-2S] cluster recognition by Aft1/2 depends on a conserved Cys-Asp-Cys (CDC) motif located C-terminal of the DNA-binding domain [181,197]; replacement of one or both cysteine residues renders Aft1/2 constitutively active [198]. Importantly, Aft1/2 coordinates a [2Fe-2S] in cooperation with Grx3/4 and Bol2. Also the activity of Yap5, which orchestrates the response to iron excess is regulated by [2Fe-2S] recognition. However, in contrast to Aft1/2, [2Fe-2S] cluster binding activates Yap5 by inducing a conformational change. Yap5 dimers bind [2Fe-2S] clusters *in vitro* via two CRR [199]. One of these CRR, CGFCX₅CXC, is well conserved among fungal Yap5 and HapX orthologs and activity of Yap5 depends mainly on this CRR (Fig. 5). Interestingly, the N-terminal CGFC motif of this CRR is reminiscent of the Grx3/4 [2Fe-2S] cluster binding site (CGFS) in Grx3/4 homologs. In contrast to Aft1/2, [2Fe-2S] cluster transfer to Yap5 appears to be independent of Grx3/4 and [2Fe-2S] cluster coordination by Yap5 appears to be independent of other proteins. Taken together, lack of Grx3/4 causes constitutive activation of Aft1/2, *i.e.*, iron acquisition independent of iron availability (Fig. 7). The role of Grx3/4 in activity of Yap5 has not been studied, but due to the homology to *A. fumigatus* HapX (see below) it appears likely that lack of Grx3/4 results in constitutive activation of Yap5 (Fig. 7). As a result, apart from its function in Fe-S cluster biosynthesis, lack of Grx3/4 causes iron-regulatory defects during iron sufficiency.

In *S. pombe*, Php4 represses iron-consuming pathways unless the availability of iron is signaled. Iron availability is sensed by GRX, which inactivates Php4 function through bridging a [2Fe-2S] cluster with Php4 involving two cysteine residues within Php4 [200]. Repression of iron acquisition during iron availability by Fep1 depends on [2Fe-2S] cluster coordination via a conserved CRR [201–203] which appears to be independent of other proteins including Grx4 [204]. However, the inactivation of Fep1 under iron starvation depends on GRX (Grx4) and Bol2; the simplest explanation would be that these two proteins remove the [2Fe-2S] cluster from Fep1 to inactivate this transcription factor [189]. In summary, lack of Grx4 causes constitutive activation of both Php4 and Fep1 (Fig. 7), *i.e.*, constitutive repression of iron consumption [205] with constitutive repression of iron acquisition [206]. Consequently, with respect to iron homeostasis, lack of Grx4 causes iron-regulatory defects during both iron starvation and sufficiency.

In *A. fumigatus*, HapX represses iron consuming pathways. During iron excess, HapX is assumed to coordinate [2Fe-2S] clusters, most likely independent of other proteins including GRX (GrxD), which transforms HapX into an activator of iron consuming pathways and iron detoxification [187]. *In vivo*, the latter function has been shown to depend on two CRR, whereby the major role is played by CGFCX₅CXC motif that is conserved in *S. cerevisiae* Yap5 [105]. Repression of iron acquisition during iron availability by SreA is assumed to depend on [2Fe-2S] cluster coordination via a conserved CRR (Fig. 4) in a GrxD independent manner similar to the homologous *S. pombe* Fep1. During iron starvation, GrxD appears to mediate [2Fe-2S] cluster removal of both SreA and HapX to inactivate SreA and to convert HapX from the “iron excess-” into the “iron starvation function” [187]. In contrast to *S. pombe*, [2Fe-2S] cluster removal from transcription factors appears to be independent of BolA as both BolA-like proteins of *A. fumigatus* possess mitochondrial targeting sequences; however, a dual localization cannot be excluded [187]. Taken together, lack of GrxD locks HapX and SreA in the “iron form” (Fig. 7), *i.e.*, constitutive derepression of iron consumption paired with constitutive repression of iron acquisition. Consequently, lack of Grx4 causes iron-regulatory defects exclusively during iron starvation.

In summary, GRXs are involved in iron sensing by all discussed

transcription factors. However, for Aft1/2 and Php4 GRX is involved in [2Fe-2S] cluster trafficking and coordination together with the transcription factors. In contrast, for SreA/Fep1- and Yap5/HapX-type transcription factors, GRX appears to be required for [2Fe-2S] cluster removal. Alternative to [2Fe-2S] cluster removal, GRX might modify the coordination environment of [2Fe-2S] clusters to signal iron starvation. BolA proteins have been shown to be involved in iron sensing by Aft1/2 and Fep1, while Yap5 and Php4 act independently of this Fe-S chaperone [193].

8. DNA recognition mode of HapX and homologous transcription factors

Heterotrimeric CBCs (transcriptional regulators of the HAP/NF-Y family) consist of three subunits, initially identified as Hap2/Hap3/Hap5 in yeast and as NF-YA/B/C in mammals and later as HapB/HapC/HapE in *A. nidulans* and *A. fumigatus*. While heterotrimeric CBCs are found in all eukaryotes, an additional fourth regulatory subunit of this core complex is present only in the fungal kingdom of life [118]. The prototype of this subunit, Hap4, was found first in *S. cerevisiae* as the transcriptional activator and global regulator of respiratory gene expression [207]. Recently, Hap4 was shown to be posttranslationally regulated by heme [208]. In line with the observation that fungal Hap5 homologs feature a conserved domain that is important for recruitment of Hap4 into the Hap2/3/5 complex, Hap4-like proteins were identified subsequently in most fungal species, however with diverging functions. According to the current state of knowledge, Hap4 requires the presence of CCAAT-containing DNA for formation of a stable Hap2/3/5/4 tetrameric complex, but the question of why the interaction of Hap4 with the Hap2/3/5 complex requires DNA-binding as a prerequisite remains unaddressed [209].

Hap4-like iron-sensing transcription factors of the HapX-type share only a small 16 amino acid (aa) stretch with Hap4 (termed Hap4-like domain), an indispensable part of Hap4 for association with the core Hap2/3/5 complex (Fig. 5). The incorporation of a Yeast activator protein 1 (Yap1) like basic region leucine zipper (bZIP)-type DNA-binding domain, which is C-terminal relative to its Hap4-like CBC-binding domain, represents a structural hallmark of all HapX-type regulators. This leads to the question of how HapX recognizes its target DNA sites. Initial studies in *A. nidulans* indicated that a HapX homodimer binds promoter sites cooperatively with the CBC. The conserved core CBC covers and bends a DNA double strand of 23 base pairs (bp) in length [210] and escorts HapX to the 3'-end of CBC covered DNA comprising the respective CBC consensus motif 5'-CCAAT-3' (Fig. 8A; [211]). This multi-protein recognition mode is mediated via protein-protein interaction between the Hap4-like domain of HapX and the HapX recruitment domain present in the CBC subunit HapE [107].

A recent study in *A. fumigatus* revealed the mode of discrimination between the around 100 genome-wide promoter sites recognized cooperatively by the CBC:HapX complex and the about 2500 target sites bound solely by the CBC. Intriguingly, HapX and the CBC exert an extremely flexible, but evolutionary conserved promoter-specific DNA recognition [106]. The minimal common bipartite CBC:HapX consensus DNA-binding motif 5'-CSAAT-N₁₂-RWT-3' comprises the pentameric 5'-C(C/G)AAT-3' core sequence (recognized by the CBC) and a small 3 bp A/T-rich sequence motif (bound by HapX) with a fixed distance of 12 bp between both submotifs. In addition, a second HapX binding site consisting of single, or less frequently, overlapping 5'-TKAN-3' motifs is located downstream of the 5'-CSAAT-3' sequence, however with high variability regarding its position (11 to 23 bp downstream), but with clear orientation and spacing preferences (Fig. 8B, C). The 5'-TKAN-3' motifs represent half-sites which, as overlapping half sites, are generally preferred targets of Yap1-type (Yap1, Gcn4) bZIP homodimers. Thus, the astonishing plasticity of HapX DNA recognition in cooperation with the CBC cannot be explained with the well-defined bZIP core DNA binding sites (5'-TTACTAA-3' and 5'-TGACTCA-3'). However,

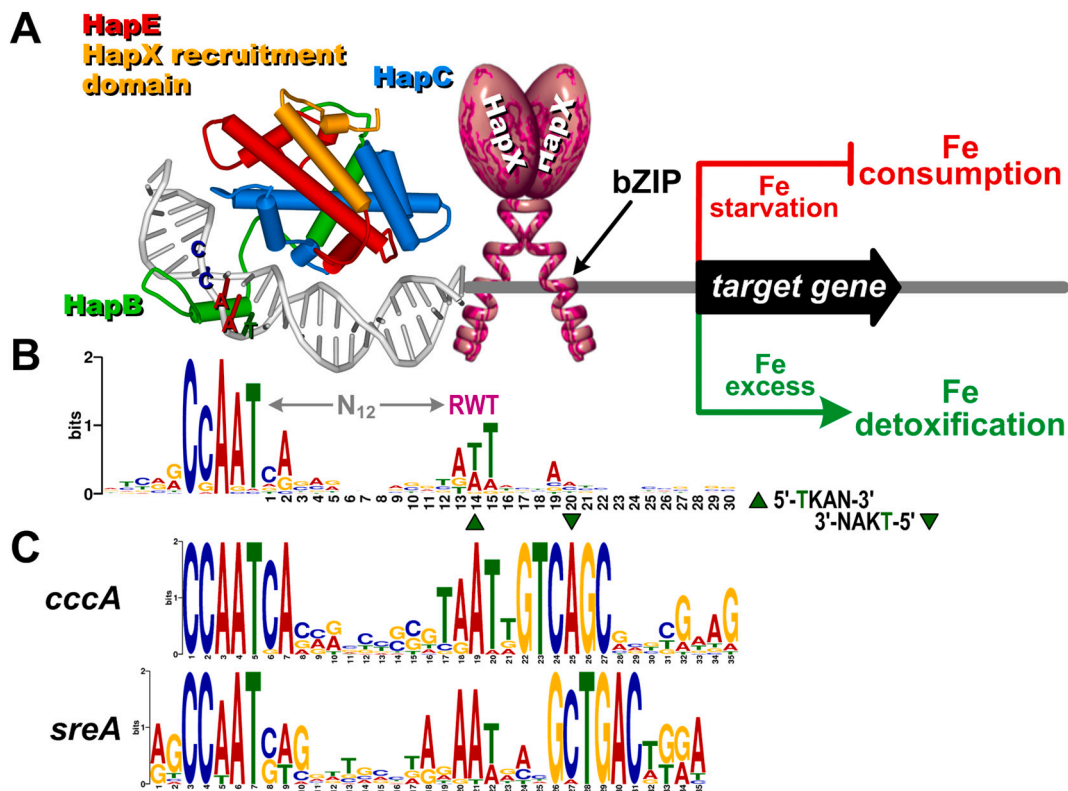


Fig. 8. Proposed model for cooperative CBC:HapX DNA recognition based on the binary CBC:DNA structure and genome-wide ChIP-seq analyses. (A) The CBC with its two histone-like subunits HapC and HapE as well as the sequence-specific HapB subunit recognizes the CCAAT pentanucleotide by inserting the C-terminal anchor helix of HapB into the minor groove and DNA-bending in a nucleosome-like manner. (B) The minimal common CBC:HapX consensus DNA-binding motif has the bipartite structure 5'-CSAAT-N₁₂-RWT-3' but is not sufficient for tight and specific interaction. HapX high-affinity binding requires additional bZIP half-sites (5'-TKAN-3', indicated by green triangles), which are located preferably 14 or 20 bp downstream of the CSAAT consensus motif either on the same or the opposite strand relative to the CCAAT box. How the bZIP domains of a HapX dimer target the DNA in this region and how the Hap4-like CBC-binding domain of HapX (not shown in the figure) interacts with the HapX recruitment domain of the CBC subunit HapE has to be clarified. (C) Phylogenetically conserved CBC:HapX target motifs in the *cccA* and *sreA* promoter regions from 20 *Aspergillus* spp. reflecting the promoter specificity of CBC:HapX complex binding and lack of HapX regulon-wide similarity in the region downstream of the 5'-RWT-3' motif.

transcriptional regulation often involves the assembly of multi-protein complexes on DNA and it has been reviewed that such complexes can exhibit novel DNA-binding specificities not predictable from those of the single transcription factors [212].

As mentioned above, *S. cerevisiae* and *C. glabrata* Yap5 and HapX have the same function in protecting the cell from toxic iron effects. Moreover, they share conserved domains: the bZIP domain and a rudimentary Hap4-like domain [151] and it was shown that CgYap5 requires the CBC for regulating its targets [213]. However, the DNA recognition modes of HapX and CgYap5/ScYap5 are obviously different. In contrast to Hap4, Php4 and HapX-type regulators, CgYap5/ScYap5 lack the C-terminal pentapeptide of the canonical Hap4-like domain (⁴⁷KPGRK⁵¹ in HapX, Fig. 5B). Nevertheless, CBC interaction of the truncated 11 aa Hap4-like domain of CgYap5 (³²ISKWKLPPLR⁴²) and cooperative binding to CCAAT and \underline{Y} ap response element (YRE) motifs (5'-TTACTAA-3') spaced by 10–14 bp is sufficient to establish CBC:Yap5:DNA interaction [213,214]. Similar to Hap4 from *S. cerevisiae* and *C. glabrata*, *S. pombe* Php4 lacks the bZIP basic region for DNA binding, again displaying a different mode of DNA recognition.

Recent advances in elucidation of the HapX and Yap5 DNA binding modes shed new light on their DNA recognition modes and may suggest that the canonical 16 aa spanning Hap4-like domains of HapX- and Hap4-type transcription factors not only mediate CBC protein-protein interaction, but also have an intrinsic DNA-binding propensity for DNA target site discrimination. Several lines of evidence are in line with this scenario. First, HapX cooperates with the CBC to bind two different DNA motifs (a least one bZIP half-site and a short A/T-rich sequence).

Second, *A. fumigatus* HapX bZIP-domain loss of function mutants retained a residual regulatory potential for activation of target genes under conditions of iron starvation (*sidG*) and overload (*cccA*). Third, the degenerated Hap4-like domain of CgYap5 was unable to functionally replace the canonical one of HapX. Fourth, the CBC in *C. glabrata* has a dual role. It is involved in iron detoxification (cooperatively with Yap5) as well as in activation of respiratory genes in cooperation with Hap4. An intrinsic DNA-binding propensity of CgHap4 would be a logical explanation for the discrimination of CBC:Yap5 and CBC:Hap4 target promoter sites. And finally, direct DNA contact of Hap4, which means cooperative promoter recognition with the CBC, would explain that Hap4 requires the presence of CCAAT-containing DNA for formation of a stable complex with Hap2/3/5 in *S. cerevisiae*.

9. The role of iron and siderophores in biotic interactions

Iron is an essential trace element for both pathogens and their hosts. Consequently, hosts contain iron. Nevertheless, pathogens typically encounter iron limitation during infection because the host iron is typically tightly sequestered by proteins. In mammalian hosts, the majority of iron is found as heme in hemoglobin, as ferric iron in the intracellular iron storage protein ferritin, as ferric iron in the iron-transporting serum protein transferrin and in enzymes as Fe-S clusters and heme. Furthermore, innate immunity effectively increases restriction of iron availability to fight infections, termed “nutritional immunity”, leading to anemia of inflammation [215,216]. Consequently, pathogens evolved strategies to “steal” the iron from their hosts. Thus,

competition for iron is a critical battleground that determines the outcome of the host-pathogen relationship. Therefore, it is not surprising that iron overload due to frequent blood transfusions, ineffective erythropoiesis and/or defects in iron metabolism decreases resistance against infection including invasive aspergillosis [217]. For example, microhemorrhage-associated tissue iron enhances the risk for *A. fumigatus* invasion in a murine model of airway transplantation [218]. In healthy individuals, plasma iron accessible for cells is bound to the iron transporting protein transferrin, whereby transferrin is typically only about 30% iron-saturated, i.e., serum transferrin has a high capacity to scavenge free iron. However, pathologic iron excess conditions can exceed the binding capacity of transferrin leading to non-transferrin bound iron (NTBI), also termed enhanced labile plasma iron (eLPI). Recently, eLPI was found to stimulate the *in vitro* growth of *A. fumigatus* in serum from hematopoietic stem cell transplanted patients [219]. The growth stimulation by eLPI could be neutralized by apo-transferrin (iron-free transferrin) and the therapeutically used iron chelator deferasirox but not by the therapeutically used iron chelator deferiprone. These results underline the importance of iron sequestration by the host and might open treatment options. In line, by independent studies deferasirox has been shown to have an activity against *A. fumigatus* *in vitro* and *in vivo* models [217,220]. However, application of deferasirox for mucormycosis in mice and humans yielded inconsistent outcomes with adverse events in humans, so that these data must be interpreted with caution [221].

The importance of siderophores in virulence of bacteria has been recognized early, e.g., in 1979, Williams [222] demonstrated the crucial role of the ferrichrome-type siderophore aerobactin in virulence of invasive *E. coli* strains using genetic techniques. About 10 years later, an association between the occurrence of infection with *Mucoromycota* species in dialysis patients and the use of deferoxamine treatment against aluminum overload and/or iron excess was reported; a link that could be confirmed in animal models [223]. Later on, deferoxamine was shown to serve as xenosiderophore for these species [224]. Characterization of the genetics of fungal siderophore biosynthesis began in the maize pathogen *U. maydis*, when in 1993 siderophore biosynthesis was shown to be dispensable for virulence of this maize pathogen [225]. This finding generally diminished the interest in fungal siderophore metabolism, which changed significantly, when siderophore biosynthesis was found to be essential for virulence of *A. fumigatus* in murine models for invasive aspergillosis in 2004 [32]. Notably, also intracellular siderophore biosynthesis was found to be crucial for virulence as shown initially in *A. fumigatus* [100], which underlines its role in germination and intracellular iron handling. In contrast, RIA was found to be dispensable for virulence of *A. fumigatus*. Nevertheless, RIA might play a role in virulence as lack of exclusively extracellular or intracellular siderophore caused only attenuation of virulence [100]. Subsequently, siderophore biosynthesis was also found to be crucial for virulence of several plant pathogenic species such as *F. graminearum*, *C. heterostrophus*, *Cochliobolus miyabeanus*, *A. alternata*, and *Alternaria brassicicola*, which employ SIA for full virulence [226–229]. These fungal species are necrotrophs, i.e., they rapidly kill plant tissue after invasion to live saprobically on the dead remains; a virulence style similar to most fungal animal/human pathogens. In contrast, *U. maydis* is a biotrophic pathogen that establishes a long-term feeding relationship with its hosts without causing immediate damage. Hemibiotrophs have a short initial biotrophic phase followed by a necrotrophic phase. In the hemibiotrophic plant pathogen *Colletotrichum graminicola*, the siderophore system was found to be repressed during biotrophic- and activated during necrotrophic growth [230]; in agreement, both SIA and RIA were found to be crucial for virulence of this pathogen. This and other studies indicated that the presence of siderophores modulates the plant immune response and that downregulation of siderophore biosynthesis serves to evade the plant immune response stimulation to maintain biotrophy [231–233]. In agreement, exclusively RIA was shown to be important for virulence of the biotrophic plant pathogens

U. maydis and *Microbotryum violaceum* [234,235]. In pathogenic fungal species lacking endogenous siderophore production such as *C. albicans* and *C. neoformans*, RIA and heme-mediated iron acquisition were found to be crucial for virulence [236–238]. In *C. albicans*, the adhesin and invasin Als3, was shown to mediate iron scavenging from host ferritin via RIA [239]. In *Mucor circinelloides*, ferroxidases representing RIA were shown to be crucial for virulence [240], while the role of the carboxylate-type siderophore rhizoferrin in virulence of *Mucoromycota* remains unresolved. A unique predisposition for mucormycosis is diabetic ketoacidosis, which lowers the ability of host transferrin to chelate iron. The non-transferrin bound iron together with the elevated glucose level enhances fungal growth and attenuates host defense mechanisms [241]. The role of iron acquisition systems in virulence of different fungal species is summarized in Table 3.

The importance of SIA and iron homeostasis for virulence of *A. fumigatus* is underlined by the fact that metabolic pathways, which are not exclusively required for SIA or iron homeostasis, are crucial for virulence of *A. fumigatus* including mitochondrial production of the siderophore precursor ornithine [129], leucine biosynthetic enzymes that control posttranslational activation of LeuB and consequently affect iron regulation (Fig. 6B; [127]), biosynthesis of riboflavin that is essential for the first step of siderophore biosynthesis (SidA; Fig. 3; [242]), and biosynthesis of pantothenic acid that is essential for post-translational activation of the siderophore biosynthetic NRPS (SidD and SidC; Fig. 3; [242]).

Besides iron acquisition, transcriptional adaptation to iron starvation; i.e., activation of iron acquisition and downregulation of iron consumption has been demonstrated to be crucial for virulence of animal and plant pathogenic fungal species as lack of HapX/Hap43 was proven to attenuate virulence of several species including *A. fumigatus* as summarized in Table 3.

Fungal iron homeostasis has also been shown to be crucial for symbiotic interactions, e.g., impairment of extracellular siderophore biosynthesis or SreA resulted in perturbation of the mutualistic interaction of the endophyte *E. festucae* and its perennial ryegrass host [110,243]. Moreover, the SreA homolog Sfu1 has been shown to be dispensable for virulence but required to promote gastrointestinal commensalism [142]. The role of conserved components involved in iron acquisition and regulation of different fungal species is summarized in Table 3.

The battle for iron significantly impacts also the interaction of microorganisms leading to beneficial and antagonistic relationships. The interplay between *A. fumigatus* and the bacterium *Pseudomonas aeruginosa* is of particular interest as these two opportunistic pathogens frequently co-infect the airways of cystic fibrosis patients. Interestingly, *P. aeruginosa* can iron-starve *A. fumigatus* via secretion of the iron-chelating 1-hydroxyphenazine [244] and iron sequestration by its major siderophore pyoverdine, which cannot be utilized as xenosiderophore by *A. fumigatus* [245]. In this competition for iron, siderophore production by *A. fumigatus* plays an important role in protection against *P. aeruginosa* [246]. The importance of the use of xenosiderophores for saving energy or avoiding competition is underlined by the fact that many bacteria possess specific transporters for utilization of ferrichrome- and coprogen-type siderophores, which are produced exclusively by fungal species, e.g., *E. coli* FhuA and FhuE [247,248]. Moreover, some diatoms can use siderophore-bound iron via endocytosis exhibiting a species-specific recognition for siderophore types [249]. Furthermore, there are natural siderophore-auxotrophic fungal species. In addition to the previously mentioned mucoromycete *Pilobolus* spp., that requires coprogen or ferrichrome as growth factor [43], the ascomycete *Debaryomyces mycophilus*, that lives as endosymbiont in the guts of woodlice, and the basidiomycete *Tritirachium egeum*, a mycosymbiont identified to grow in association with *Penicillium rugulosum*, were found to lack independent high-affinity iron acquisition and to depend on xenosiderophore supply [250,251].

Table 3

Examples of the role of iron uptake and regulation in virulence, symbiosis, and commensalism. Requirement for full virulence is denoted by +, in case of siderophore biosynthesis (SB) by T+ for total, E+ for extracellular, or I+ for intracellular siderophore biosynthesis; proven dispensability for virulence is denoted by -; for RIA, (-) denotes that inactivation did not cause a virulence defect but that an assisting role cannot be excluded.

Species	Host	HapX homologs	SreA homologs	RIA	SB	Heme uptake	References
Virulence in animal host							
<i>Candida albicans</i>	Mouse	+		+		+	[236,237,268]
<i>Aspergillus fumigatus</i>	Mouse, insect	+	-	(-)	T+, E+, I+		[32,100,269,270]
<i>Histoplasma capsulatum</i>	Mouse				T+		[271]
<i>Cryptococcus neoformans</i>	Mouse	+	+	+			[159,238]
<i>Metarhizium robertsii</i>	Insect		-		E-, I+		[272]
Virulence in animal host and necrotrophic plant virulence							
<i>Fusarium oxysporum</i>	Tomato, mouse	+					[49]
Necrotrophic plant virulence							
<i>Fusarium graminearum</i>	Wheat			(-)	T+, E+		[226,228]
<i>Cochliobolus heterostrophus</i>	Maize			+	T+, E+, I+		[228,263,273]
<i>Cochliobolus miyabeanus</i>	Rice				E+		[228]
<i>Alternaria brassicicola</i>	<i>Arabidopsis thaliana</i>				E+		[228]
<i>Verticillium dahliae</i>	Smoke tree	+					[117]
<i>Magnaporthe grisea</i>	Rice				I+		[274]
Biotrophic plant virulence							
<i>Ustilago maydis</i>	Maize			+	T-		[225,234]
<i>Microbotyum violaceum</i>					T-		[235]
Hemibiotrophic plant virulence							
<i>Colletotrichum graminicola</i>	Maize			+	T+, E+		[230,275]
Symbiosis							
<i>Epichloë festucae</i>	Perennial ryegrass		+		E+		[110,243]
Commensal fitness							
<i>C. albicans</i>	Mouse		+				[142]

10. Translational aspects of the fungal siderophore system

The use of siderophores distinguishes fungal from plant and mammalian cells, which might enable translational applications. Invasive fungal infections such as aspergillosis are rather rare but nevertheless life-threatening [13]. The diagnosis of fungal infections is difficult, lacking specificity and sensitivity. Recently, TAFC was reported to be an attractive novel biomarker for systemic *A. fumigatus* infection enabling non-invasive diagnosis in urine [252,253]. Furthermore, replacing iron in siderophores such as TAFC by the radionuclides gallium-68 allowed *in vivo* imaging of *A. fumigatus* infection by positron emission tomography (PET) due to specific uptake and accumulation of the siderophore in fungal cells. However, this approach is so far limited to preclinical models of pulmonary infection in mouse and rat [73,254]. Moreover, the conjugation of siderophores and fluorescent dyes enabled the generation of hybrid imaging compounds, allowing the combination of PET and optical imaging in preclinical aspergillosis models [255]. The latter indicated that SITs tolerate substantial derivatization of their substrate. This is particularly interesting as SITs represent one of few protein families that are unique to the fungal kingdom, *i.e.*, they are not present in prokaryotes or other eukaryotes. Consequently, this protein might allow fungal-specific drug delivery by a Trojan horse approach [256], in which toxic compounds are conjugated to siderophores for selective import into fungal cells. Comparatively, there are natural antibacterial siderophore-antibiotic conjugates, termed sideromycins, and recently the first synthetic siderophore-antibiotic conjugate, cefiderocol, was FDA-approved to combat multidrug-resistant gram-negative bacteria [257]. Notably, the novel antifungal drug VL-2397 (previously termed ASP2397), which has a ferrichrome-type structure containing aluminum instead of iron, was recently shown to require the SIT Sit1 for uptake and activity against *A. fumigatus* (Fig. 1; [258]). Due to its crucial role in virulence, the siderophore biosynthetic pathway represents a promising target for selective therapeutic intervention. In this respect noteworthy, celastrol,

a natural quinone methide, was identified as a noncompetitive inhibitor of SidA [259]. Moreover, treatment of fungal keratitis in a murine model by dual topical therapy with the iron chelator deferiprone and statins, which target biosynthesis of isoprenoids and extracellular siderophores (Fig. 2B), showed restriction of fungal growth [260].

11. Conclusion

During the past decades, it has become clear that siderophore metabolism is a central constituent of iron handling and consequently virulence in numerous but not all pathogenic fungi. Recent work in various pathogenic fungi revealed a high plasticity in iron sensing mechanisms by different transcription factors and associated proteins. However, sensing of mitochondrial Fe-S cluster productivity was identified to be a common feature in all investigated species. Moreover, the conserved role of GRX with a conserved CGFS motif has become evident. In the present understanding, GRXs are required for trafficking and/or modulating Fe-S cluster signal to determine the activity of iron-responsive transcription factors. However, the detailed mechanism of how GRXs affect distinct transcription factors can be variable. These mechanistic differences in GRX-mediated iron sensing might reflect unique features of the respective transcription factor, *e.g.*, some species employ repressors where others use activators. In this context, HapX homologs, which have both a repressing function under iron starvation and an activating function under iron excess, are of special interest. The recent characterization of the mode of target promoter recognition of the CBC:HapX complex revealed astonishing complexity.

In summary, knowledge about fungal iron homeostasis in pathogenic fungi might be of importance to improve diagnosis of fungal infections and to develop antifungal strategies in agriculture and clinics. Since pathogens encounter iron-limited conditions during infection, perturbation of these fine-tuned systems could potentially be exploited for novel antifungal treatments due to significant mechanistic differences to the iron homeostasis-maintaining machinery in host cells.

CRedit authorship contribution statement

Matthias Misslinger: Writing - original draft, Writing - review & editing, Visualization. **Peter Hortschansky:** Writing - review & editing, Visualization. **Axel A. Brakhage:** Writing - review & editing. **Hubertus Haas:** Writing - original draft, Writing - review & editing, Visualization, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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