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Iron acquisition in fungal pathogens of humans

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The devastating infections that fungal pathogens cause in humans are underappreciated relative to viral, bacterial and parasitic diseases. In recent years, the contributions to virulence of reductive iron uptake, siderophore-mediated uptake and heme acquisition have been identified in the best studied and most life-threatening fungal pathogens: *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. In particular, exciting new work illustrates the importance of iron acquisition from heme and hemoglobin in the virulence of pathogenic yeasts. However, the challenge of establishing how these fungi gain access to hemoglobin in blood and to other sources of heme remains to be fully addressed. Recent studies are also expanding our knowledge of iron uptake in less-well studied fungal pathogens, including dimorphic fungi where new information reveals an integration of iron acquisition with morphogenesis and cell-surface properties for adhesion to host cells. Overall, the accumulating information provides opportunities to exploit iron acquisition for antifungal therapy, and new work highlights the development of specific inhibitors of siderophore biosynthesis and metal chelators for therapeutic use alone or in conjunction with existing antifungal drugs. It is clear that iron-related therapies will need to be customized for specific diseases because the emerging view is that fungal pathogens use different combinations of strategies for iron acquisition in the varied niches of vertebrate hosts.

1. Introduction

Despite being an abundant metal on Earth, the bioavailability of iron in the environment is extremely limited due to its existence as insoluble ferric oxide. Microbial pathogens face an additional challenge to acquire iron and other key metals, such as zinc, manganese and copper, inside vertebrate hosts.

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Won Hee Jung

Dr Won Hee Jung is currently an associate professor in the Department of Systems Biotechnology at Chung-Ang University, Korea. His current research focuses on molecular and genetic analysis of regulatory mechanisms of iron and zinc uptake systems in the human fungal pathogen Cryptococcus neoformans. He has also recently started to work on the human cutaneous fungal pathogen Malassezia, which causes various skin diseases including seborrheic dermatitis, dandruff and atopic dermatitis. Dr Jung obtained his PhD degree from University of Manchester (UMIST), U.K., where he worked on the cAMP signaling pathway in another human fungal pathogen, Candida albicans.

The mammalian immune system recognizes the importance of metal ions for the proliferation of pathogens and therefore has evolved elegant mechanisms for controlling the blood, tissue and intracellular levels of metals. For example, the process of host-mediated sequestration of iron has been termed as 'nutritional immunity' and its role in the immune response to bacterial pathogens is well established.[1] In fact, much is known about the competition for iron between vertebrate hosts and bacterial pathogens or parasites, and an appreciation of this evolutionary host-adaptation process is also starting to emerge for several fungal pathogens of humans.

Fungal pathogens cause devastating diseases in humans, but have received far less attention than viral, bacterial and parasitic infections.[2–5] This lack of appreciation is surprising given the high risk of fungal infections in people with HIV/AIDS or cancer, or those receiving organ transplants or immunomodulatory therapy. The most common fungal pathogen of humans is *Candida albicans* and this fungus, along with other *Candida* species, causes superficial mucosal infections as well as life-threatening systemic disease.[6] Additionally, the fungus *Cryptococcus neoformans* is a devastating agent of meningitis in HIV/AIDS patients with an estimated 1 million cases per year and over 600 000 deaths.[5,7] Similarly, *Aspergillus fumigatus* and other *Aspergillus* species cause a range of diseases (aspergilloses) that include allergic bronchopulmonary, chronic pulmonary and invasive aspergillosis.[8] Given the serious threats posed by fungal pathogens, there is a pressing need for new approaches to treat infections, especially in the face of increasing resistance to an already limited number of antifungal drugs.[9] In this context, iron acquisition by fungi during host colonization presents a compelling target for new diagnostic and therapeutic approaches.

In this mini-review, we consider the major systems of iron acquisition in pathogenic fungi including reductive iron uptake, siderophore synthesis and transport, and mechanisms to use heme and hemoglobin, and we highlight recent advances that add depth to our understanding of iron acquisition. With reference to information from the model yeast *Saccharomyces cerevisiae*, we focus on the specific fungal pathogens *C. albicans*, *Candida glabrata*, *C. neoformans* and *A. fumigatus*, as illustrated in Fig. 1. In particular, we emphasize the uptake functions for the pathogens that contribute to disease. Additionally, we include new studies for other species of pathogenic fungi where relevant information is available. We also address the iron sources that fungi exploit in vertebrate hosts, and much of this consideration involves exciting new work on fungal exploitation of heme and hemoglobin as abundant sources of iron. The key functions for iron acquisition that we discuss are listed in Table 1. The information on known uptake strategies sets the stage for a consideration of approaches to exploit fungal iron uptake for diagnostic and therapeutic purposes. We refer readers to several recent reviews for additional considerations of iron homeostasis in fungi, including mechanisms of regulation and opportunities for therapy.[10–16]

James W. Kronstad

Dr James Kronstad is a Professor in the Department of Microbiology and Immunology, and Director of the Michael Smith Laboratories at the University of British Columbia in Vancouver. Research in the Kronstad group focuses on interactions between fungal pathogens and their animal or plant hosts, with an emphasis on the characterization of host factors that influence pathogen gene expression and proliferation. One of the goals of his team is to develop antifungal strategies that target mechanisms of iron uptake in fungal pathogens.

2. Reductive iron uptake: connections with virulence and dimorphism

The mechanisms of iron acquisition and homeostasis have been well studied in the model yeast *S. cerevisiae*, and this information provides a foundation for many of the studies in fungal pathogens.[17–19] *S. cerevisiae* primarily employs two different mechanisms for iron uptake that involve reductive and non-reductive systems.[17] Reductive iron uptake involves two sequential steps: (1) reduction of ferric iron to soluble ferrous iron by ferric reductases encoded by the *FRE* genes, *FRE1* and *FRE2*, and; (2) re-oxidation to the ferric form by a multicopper ferroxidase (Fet3) coupled with transport into the cell by a permease (Ftr1). Nonreductive iron uptake involves the use of siderophore-bound iron *via* specific transporters, although *S. cerevisiae* does not synthesize its own siderophores.[18]

The fungal pathogens *C. albicans*, *C. neoformans*, and *A. fumigatus* also use a reductive iron uptake system involving conserved orthologs of the cell-surface ferric reductases, ferroxidases and iron permeases identified in *S. cerevisiae*.[19–23] The *FRE* reductases are integral membrane proteins that require heme, FAD and NADPH for activity.[24] At least 17 genes encoding putative ferric reductases have been identified in the *C. albicans* genome. Of these genes, the *FRE10* and *FRE7* genes encode highly active cell-surface ferric reductases and deletion of these genes results in decreased ferric iron uptake by the fungus.[21,22,25] More recent work on the Cfl1 ferric reductase from *C. albicans* expands our appreciation of the roles of these enzymes in fungal pathogens. Specifically, transcription of *CFL1* is regulated in response to iron and copper, and deletion of the gene results in a remarkably large number of phenotypes.[26–28] These phenotypes include increased accumulation of cellular iron, altered cell wall architecture, loss of mitochondrial function, impaired filamentous growth, sensitivity to oxidative stress, and attenuated virulence in mouse model of systemic candidiasis.[26–28] Interestingly, a mutant lacking Cfl1 is not defective for iron acquisition, perhaps because of the observed compensatory upregulation of other ferric reductase genes in a *cfl1* deletion mutant.[27] Cfl1 therefore

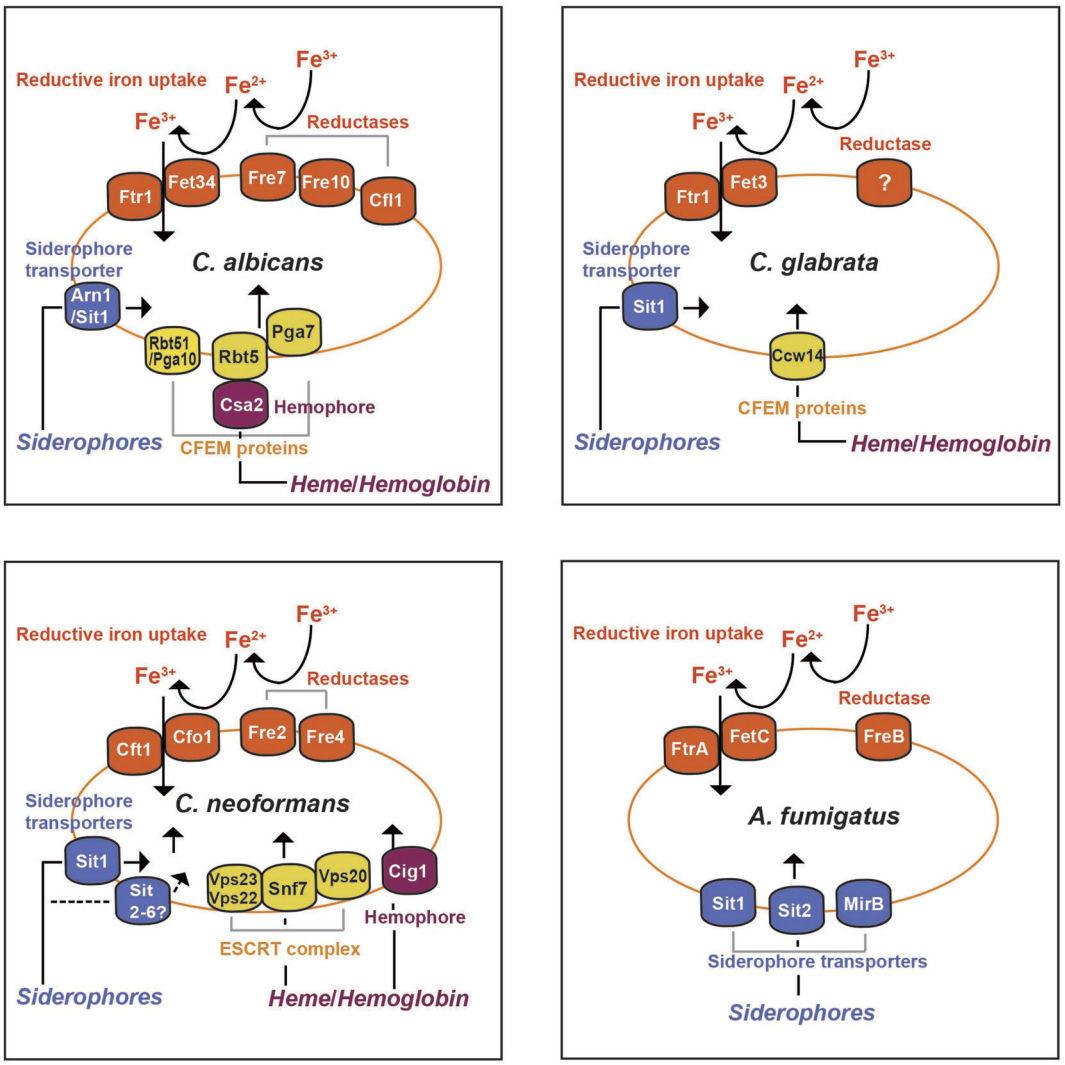

Fig. 1 Iron acquisition systems for four of the best-studied fungal pathogens of humans including *Aspergillus fumigatus*, *Candida albicans*, *Candida glabrata* and *Cryptococcus neoformans*.

plays a variety of roles and the recent work illustrates the pleiotropy of iron-related functions with regard to fungal pathogenesis. Transcriptional analysis of several other *C. albicans* genes encoding ferric reductases indicates that this fungus can regulate the expression of its iron-uptake machinery in different host environments.[25] This finding illustrates the need to consider the host niche in determining the relevance of an iron uptake system for disease. In *C. neoformans*, eight genes encoding putative ferric reductases have been identified (*FRE1–7*, *FRE201*).[29] Notably, the transcription of *FRE2* and *FRE4* is regulated by hemin or $FeCl_3$ suggesting a crucial role of these genes in maintaining iron homeostasis during iron-limited conditions. Furthermore, *FRE2* is required for robust growth in the presence of heme and transferrin, and for full virulence in a mouse inhalation model of cryptococcosis.[29] Similar to the *S. cerevisiae FRE* genes, the *FRE* genes in both *C. albicans* and *C. neoformans* also show differential transcriptional regulation in the presence of copper.[29,30]

In *S. cerevisiae*, the second step of reductive iron uptake involves transport of the reduced ferrous iron *via* a high-affinity heterodimeric transport complex consisting of the multicopper ferroxidase Fet3 and the permease Ftr1, as mentioned above. Ferrous iron is oxidized to ferric iron by Fet3 and is subsequently transported into the cell by Ftr1. The transport of ferric iron involves a classic metabolite channeling mechanism where amino acid residues in both Fet3 and Ftr1 interact with each other to drive the iron into the cell.[31] The corresponding components of the multicopper ferroxidase-iron permease system have been identified and characterized in *C. albicans*, *C. neoformans*, *A. fumigatus*, *C. glabrata*, and other species (see below).[32–37] For example, *C. albicans* harbors five genes, *FET3*, *FET31*, *FET33*, *FET34* and *FET99* that are orthologs of the *S. cerevisiae FET3* gene.[32,38,39] Transcriptional analyses demonstrated that *FET3* and *FET34* are regulated by iron starvation.[22,40] The characterization of *FET34* revealed that this gene encodes a functional ferroxidase which localizes to the plasma membrane and contributes to iron accumulation, hyphal development and virulence in a mouse model of systemic candidiasis.[39,40] Transcription of the *C. albicans* ortholog of the *S. cerevisiae* iron permease *FTR1* is also induced

Table 1 Iron acquisition functions in *Saccharomyces cerevisiae* and selected fungal pathogens of humans

Type of transport system	Species	Protein	Functions	Virulence of mutants	Ref.
Reductive iron uptake					
Ferric reductases					
	S. cerevisiae	Fre1	Ferric iron reduction at the cell surface	NA[a]	17
	S. cerevisiae	Fre2	Ferric iron reduction at the cell surface	NA	17
	C. albicans	Fre7	Ferric iron reduction at the cell surface	ND[b]	21, 22 and 25
	C. albicans	Fre10	Ferric iron reduction at the cell surface	ND	21, 22 and 25
	C. albicans	Cfl1	Ferric iron reduction at the cell surface	Attenuation in a mouse model of systemic candidiasis	27
	C. neoformans	Fre2	Ferric iron reduction at the cell surface	Attenuation in a mouse model of cryptococcosis	29
	A. fumigatus	FreB	Ferric iron reduction at the cell surface	ND	41
Multicopper ferroxidase					
	S. cerevisiae	Fet3	Multicopper oxidase. Ferrous iron oxidation and high-affinity iron uptake, coupled with Ftr1	NA	17
	C. albicans	Fet3	Putative multicopper oxidase	ND	32, 38 and 39
		Fet31	Ferrous iron oxidation and high-affinity iron uptake	Not required	32 and 38
		Fet33	Putative multicopper oxidase	Not required	39
		Fet34	Ferrous iron oxidation and high-affinity iron uptake, coupled with Ftr1	Attenuation in a mouse model of systemic candidiasis	38 and 39
		Fet99	Putative multicopper oxidase	ND	32 and 38
	C. glabrata	Fet3	Ferrous iron oxidation and high-affinity iron uptake	Reduced kidney colonization	37
	C. neoformans	Cfo1	Ferrous iron oxidation and high-affinity iron uptake	Attenuation in a mouse model of cryptococcosis	44
	C. neoformans	Cfo2	Putative multicopper oxidase	Not required	44
	A. fumigatus	fetC	Ferrous iron oxidation and high-affinity iron uptake	ND	36
	T. marneffei	fetC	Ferrous iron oxidation and high-affinity iron uptake	ND	49
Iron permease					
	S. cerevisiae	Ftr1	High-affinity iron uptake, coupled with Fet3	NA	17
	C. albicans	Ftr1	High-affinity iron uptake, coupled with Fet3	Avirulent in a mouse model of systemic candidiasis	23
	C. albicans	Ftr2	Putative iron permease	Not required	23
	C. glabrata	Ftr1	High-affinity iron permease	Reduced kidney colonization	37 and 46
	C. neoformans	Cft1	High-affinity iron permease	Attenuation in a mouse model of cryptococcosis	35
	C. neoformans	Cft2	Putative iron permease	Not required	35
	C. neoformans	Cft3	Putative iron permease	Not required	43
	A. fumigatus	FtrA	High-affinity iron permease	Not required	36
	T. marneffei	Ftr1	High-affinity iron permease	No growth defect in macrophages	49
	R. oryzae	Ftr1	High-affinity iron permease	Reduced virulence in a mouse model of mucormycosis	63 and 64
Siderophore transport[c]					
	S. cerevisiae	Arn1	Ferrichrome and ferrichrome A transport	NA	17, 24 and 53
		Arn2/Taf1	Triacetylfusarinine C transport	NA	17, 24 and 53
		Arn3/Sit1	Ferrichrome and ferrichrome A transport	NA	17, 24 and 53
		Arn4/Enb1	Enterobactin transport	NA	17, 24 and 53
	C. albicans	Arn1/Sit1	Ferrichrome-type xenosiderophore transport	Required for epithelial invasion	58–60
	C. glabrata	Sit1	Ferrichrome transport	Reduced survival within macrophages	37 and 61
	C. neoformans	Sit1	Ferrioxamine transport	Not required	62
	A. fumigatus	Sit1	Ferrichrome and ferrioxamine B transport	Not required	67
		Sit2	Ferrichrome transport	Not required	67
		MirB	Triacetylfusarinine C transport	ND	69
	H. capsulatum	Mfs1	Putative siderophore transporter	ND	70
		Abc1	Putative siderophore transporter	ND	70
	R. oryzae	Fob1, Fob2	Ferrioxamine binding at the cell surface	Required for virulence in a mouse model of mucormycosis	63

Table 1 (*continued*)

Type of transport system	Species	Protein	Functions	Virulence of mutants	Ref.
Heme assimilation CFEM proteins					
	C. albicans	Rbt5	Heme uptake, putative GPI-anchored hemoglobin receptor	Required for biofilm formation, not required for virulence	75 and 78
	C. albicans	Rbt51	Heme uptake	ND	75
	C. albicans	Pga7	Hemoglobin and heme utilization	Attenuation in a mouse model of systemic candidiasis	80
	C. glabrata	Ccw14	Unknown	Reduced survival in the liver and the spleen in a mouse model of systemic candidiasis	37
	P. brasiliensis	Rbt5	Putative hemoglobin receptor	Impaired survival within macrophages	84 and 85
	P. lutzii	Rbt5	Putative hemoglobin receptor	Reduced survival in the spleen	84 and 85
ESCRT complex					
	C. neoformans	Vps23	ESCRT-I complex, heme uptake	Avirulent in a mouse model of cryptococcosis	88
	C. neoformans	Vps22	ESCRT-II complex, heme uptake	Avirulent in a mouse model of cryptococcosis	89
	C. neoformans	Snf7	ESCRT-III complex, heme uptake	Avirulent in a mouse model of cryptococcosis	89
	C. neoformans	Vps20	ESCRT-III complex, heme uptake	ND	89
Hemophore					
	C. albicans	Csa2	Putative hemophore	ND	77
	C. neoformans	Cig1	Putative hemophore	Not required, a *cig1* and *cfo1* double mutant shows attenuated virulence in a mouse model of cryptococcosis	90
Haemolysin					
	C. glabrata	Mam3	Putative haemolysin	Reduced fungal burden in the kidney, liver and brain	37

[a] NA: not applicable. [b] ND: data not available. [c] Mutants defective in siderophore biosynthesis are attenuated in mouse models of virulence for *A. fumigatus* and *H. capsulatum*.[15,16,69]

upon iron deprivation.[33] Importantly, *FTR1* in *C. albicans*, along with the reductase encoded by *FRE10*, is implicated in iron acquisition from transferrin and ferritin, and Ftr1 is important for virulence in a mouse model of systemic disease.[22,23,33]

A. fumigatus also possesses the components of the reductive iron uptake system including cell-surface ferric reductases and a ferroxidase (FetC) and iron permease (FtrA).[36,41] The *ftrA* gene of *A. fumigatus* is highly homologous to *C. albicans FTR1*, and its expression is induced upon iron depletion. However, the mutant lacking *ftrA* grew normally in iron-restricted medium and was as virulent as the wild-type strain, thus suggesting that the iron permease is dispensable for virulence in *A. fumigatus*.[36] The *fetC* gene in *A. fumigatus* encodes an ortholog of *FET3* from *S. cerevisiae*. Iron-responsive transcriptional regulation of *fetC* was observed, but the contribution of the gene to virulence has not been determined.[36] Notably, a low-affinity iron utilization system, dependent on the putative vacuole iron importer Ccc1, has also been identified in *A. fumigatus*.[42] Ccc1 contributed to the ability of the fungus to grow in the presence of high iron levels when both the high-affinity and siderophore-mediated iron uptake systems were absent.[42]

Three paralogous genes in *C. neoformans* (*CFT1-3*) are orthologs of *S. cerevisiae FTR1* and, among them, *CFT1* encodes the high-affinity iron permease in the reductive iron uptake system.[35,43] *CFT1* is required for ferric iron uptake and acquisition of iron from holo-transferrin *in vitro*, and the *cft1* mutant is attenuated for virulence in both inhalation and tail vein injection models of cryptococcosis in mice.[35] Moreover, fungal colonization in the brain of the infected mice was dramatically reduced for the *cft1* mutant implying that transferrin may be an important iron source for *C. neoformans* during disseminated disease and meningitis.[35] The ferroxidase of the reductive uptake system is encoded by *CFO1* in *C. neoformans*, and the *cfo1* mutant shows reduced growth in iron-restricted medium.[44] Moreover, the *cfo1* mutant was unable to utilize holo-transferrin as a sole iron source, while heme and the siderophore feroxamine supported the growth of the mutant (as was found with the *cft1* mutant). Deletion of *CFO1* also attenuated virulence in a mouse model of cryptococcosis.[44] Together, the results with Cft1 and Cfo1 suggest that transferrin may be an important iron source for *C. neoformans* during infection, and that high-affinity reductive iron uptake is important during cryptococcosis.

More recent studies extended the analysis of reductive iron uptake to another clinically important *Candida* species, *C. glabrata*. This pathogen, which is actually more closely related to *S. cerevisiae* than to *C. albicans*, has emerged as the second leading cause of

candidiasis after *C. albicans*.[45] An ortholog of the *FTR1*-encoded iron permease was identified in *C. glabrata* and found to be required for growth in iron-restricted medium.[37,46] Additionally, deletion of *FTR1* caused reduced colonization of the kidneys of infected mice suggesting that the iron permease is essential for the virulence of *C. glabrata*.[37] The same study also identified the *C. glabrata* ortholog of the Fet3 ferroxidase of *S. cerevisiae*. Similar to the *ftr1* mutant, the *C. glabrata* strain lacking the *FET3* ortholog also showed reduced colonization of the kidneys of mice, supporting the essentiality of high-affinity reductive iron uptake for virulence.[37] Interestingly, plasma membrane-localized Ftr1 moves to the vacuole in a Vps34-dependent manner upon exposure of *C. glabrata* to excess iron.[46] *VPS34* encodes a phosphoinositide 3-kinase, and the *C. glabrata* mutant lacking the gene shows impaired iron homeostasis and a significant reduction of organ colonization in mice. The inability of a *vps34* mutant to properly traffic Ftr1 as well as other virulence-related phenotypes (*e.g.*, impaired biofilm formation) may contribute to the observed colonization defect.

Reductive iron uptake is also associated with morphogenesis in dimorphic or pleiomorphic fungal pathogens that can switch between yeast-like growth and hyphal or pseudohyphal growth.[47–49] The yeast to hyphal morphological transition has been well studied in *C. albicans* where the ability to switch contributes to virulence.[6] Emerging evidence indicates that iron acquisition is intertwined with morphological switching in some dimorphic fungi. For example, the reductive iron uptake system in *C. albicans* is coordinated with the function of the adhesin-like receptor protein, Als3, to facilitate iron acquisition from ferritin specifically during hyphal but not yeast growth.[47] A strong link between the expression of other adhesion proteins (*e.g.*, *MP65* and *PGA62*) and iron was also established in a study of the influence of iron on chromatin remodeling in *C. albicans*.[50] The connection between surface protein expression and iron may be a general feature of pathogenic yeasts because the *EPA1* gene encoding the major adhesin in *C. glabrata* is also regulated by iron.[51] Very recently, Pasricha *et al.* reported a thorough characterization of the connections between cell morphology and both reductive iron uptake and siderophore-mediated uptake in the dimorphic pathogen *Talaromyces marneffei*.[49,52] The pathogenic yeast phase of this fungus proliferates intracellularly in macrophages and causes disease in immunocompromised people, particularly in endemic areas such as Southeast Asia. Initial transcriptional profiling experiments revealed co-incident regulation of iron uptake functions with the temperature-mediated switch from hyphal (25 °C) to yeast (37 °C) growth.[52] Subsequent detailed experiments demonstrated that the *T. marneffei fetC* (ferroxidase) and *ftrA* (iron permease) genes have higher transcript levels both in response to iron limitation and in yeast cells compared to hyphae. In agreement, a deletion mutant lacking *ftrA* displayed reduced growth on low iron medium at 37 °C but not at 25 °C.[49] These results suggest that an additional iron uptake system may be active at 25 °C or that the yeast cells at 37 °C may have a higher demand for iron. Interestingly, the *ftrA* mutant did not display a defect in intracellular proliferation in macrophages suggesting that another uptake pathway is used in this context. As discussed below, the siderophore biosynthetic genes in *T. marneffei* were also characterized, and these genes also show interesting cell-type regulation and contributions to growth.[49]

3. Siderophore iron uptake: a potential drug target for some but not all fungal pathogens

Along with reductive iron uptake, siderophore-mediated non-reductive iron uptake also contributes to iron acquisition in fungi.[14,16] *S. cerevisiae, C. neoformans* and *C. albicans* do not synthesize their own siderophores, but can use exogenous siderophores (xenosiderophores) produced by other organisms. Early studies in *S. cerevisiae* again provide a model for uptake mechanisms for xenosiderophore-iron chelates, in this case *via* Arn/Sit transporters of the major facilitator superfamily.[24] These transporters, termed Arn1, Arn2/Taf1, Arn3/Sit1, and Arn4/Enb1, individually show specificity for different bacterial and fungal xenosiderophores such as enterobactin, ferrichrome, ferrichrome A, triacetylfusarine C, and ferrioxmaine B.[17,24,53] The siderophore bound Arn transporters are internalized and transported to late-endosomal compartments for vacuolar degradation to release the iron, as revealed by the trafficking of ferrioxamine B and ferrichrome-bound Arn3/Sit1 and Arn1 transporters, respectively.[53,54] The trafficking of Sit1 to the vacuolar compartment depends on Rsp5-mediated ubiquitylation and the clathrin adaptor protein Gga2, and is regulated by the presence of the substrate ferrichrome B.[55,56] A recent study by Kang *et al.* added further depth to the connection between intracellular trafficking and iron.[57] Specifically, it was found that the transcriptional activator Aft1 in *S. cerevisiae*, which responds to iron availability and regulates the expression of the *FET3*, *FTR1* and *ARN* genes, interacts with the Arn3/Sit1 transporter and influences its ubiquitination and vacuole-dependent protein degradation.[56,57] The Aft1-Arn3/Sit1 interaction illustrates the potential for additional sensing mechanisms that fine-tune the deployment of iron uptake strategies.

As indicated above, some pathogenic yeast exploit xenosiderophores produced by other microbes. *C. albicans*, for example, employs a Sit1/Arn1 transporter to use xenosiderophores such as ferricrocin, ferrichrysin, ferrirubin, coprogen and triacetylfusarine C.[58–60] Given that *C. albicans* is a human commensal, the fungus may encounter xenosiderophores from bacteria that co-inhabit mucosal or gastrointestinal niches. The Sit1/Arn1 transporter in *C. albicans* is required for efficient invasion of reconstituted epithelium as a model of the human oral mucosa, but does not contribute to virulence in a mouse model of systemic candidiasis.[60] Thus, siderophore-mediated iron uptake may not be important during bloodstream infection by *C. albicans*.[60] *C. glabrata* also possesses a Sit1 siderophore transporter, and Nevitt and Thiele investigated the influence of Sit1 on virulence by monitoring the survival of a *sit1* mutant in a mouse macrophage cell line.[61] They observed reduced survival of the *sit1* mutant within phagolysosomes of the infected macrophages compared to the wild-type strain. However, reduced survival of the mutant was

only observed for fungal cells pre-treated with the xenosiderophore ferrichrome, a substrate of Sit1, and no difference was observed between the *sit1* mutant and the wild-type strain without ferrichrome pre-treatment. *C. neoformans* also has a highly conserved transporter, Sit1, for the uptake of ferrioxamine B, and xenosiderophores may be important during proliferation of the fungus in the environment (*i.e.*, in soil, bird excreta and in association with trees).[62] However, Sit1 in *C. neoformans* does not contribute to virulence in a mouse model of cryptococcosis.[62] Xenosiderophores are also important in the disease mucormycosis caused by *Rhizopus oryzae* and other fungi in the order Mucorales.[63] Mucormycosis is a threat to patients who are experiencing iron overload as a result of kidney dialysis and are receiving chelation therapy with the bacterial siderophore deferoxamine. The ability of *R. oryzae* to exploit deferoxamine to grow in the host, and the ability to use the siderophore, depends on cell surface binding proteins (Fob1 and Fob2) as well as the reductive uptake system.[63] Consistent with the latter requirement, defects in iron permease activity attenuate virulence in a deferoxamine-treated mouse model of mucormycosis.[63,64] In contrast, the reductive uptake system encoded by *CFT1* and *CFO1* is not required for iron acquisition *via* the siderophore ferrioxamine B in *C. neoformans*.[33,44]

Siderophore-mediated iron uptake has been extensively studied in *Aspergillus* species including *A. nidulans* and *A. fumigatus*, and also in the dimorphic pathogen *Histoplasma capsulatum*.[15,16,36,65,66] Unlike *C. albicans* and *C. neoformans*, both the *Aspergillus* species and *H. capsulatum* synthesize different hydroxamate-type siderophores including ferricrocin, hydroxyferricrocin, fusarinine C, coprogen B and triacetylfusarinine C.[36,65,66] Extensive studies have characterized the genetics and regulation of siderophore production, and defined the role of siderophores in the virulence of *A. fumigatus*.[15,16] In this pathogen, the *sidA* gene encoding L-ornithine-N^5-monooxygenase is essential for siderophore biosynthesis and virulence.[36] Putative siderophore transporters have also been identified in both *A. fumigatus* and *A. nidulans*.[67–69] In a recent study, Park *et al.* described the roles of highly conserved orthologs of the *S. cerevisiae* Sit1 and Sit2 transporters in *A. fumigatus* and demonstrated their participation in the uptake of ferrichrome and ferrioxamine B.[67] However, these siderophore transporters did not contribute to the virulence of *A. fumigatus* in immunosuppressed mice. Another siderophore transporter, MirB, has also been characterized for *A. fumigatus*, but its contribution to virulence remains to be determined.[69] For *H. capsulatum*, a microarray analysis identified genes with increased expression under iron-poor conditions.[70] These genes encoded functions for siderophore synthesis (*e.g.*, *SID1* the ortholog of sidA) or transport (*MFS1* and *ABC1*). Importantly, deletion of *SID1* in *H. capsulatum* abolished siderophore biosynthesis as well as growth in bone marrow-derived macrophages and in mice, indicating that siderophore production is essential for the virulence of this fungus.[70]

As introduced earlier, work in *T. marneffei* has provided interesting insights into the integration of iron acquisition with virulence-associated dimorphism. With regard to siderophore production, a characterization of the genetics and expression of siderophore biosynthesis revealed a suite of genes orthologous to most of those defined in *A. fumigatus* for the production of extracellular and intracellular siderophores. Interestingly, two orthologs of the *A. fumigatus sidA* gene (*sidA* and *sidX*) were found, and these genes make distinct contributions to growth in response to iron.[49] In particular, the growth of the *sidA* deletion mutant was not impaired but instead enhanced by iron limitation relative to the wild-type strain at 25 °C or 37 °C.[49] In contrast, the *sidX* deletion mutant grew poorly in low iron conditions at 25 °C or 37 °C, but behaved like wild type upon iron repletion. The *sidA* and *sidX* deletion mutants were not impaired for growth in macrophages. Finally, it was observed that the *fetC* gene for the multicopper ferroxidase was upregulated in the *sidA* and *sidX* deletion mutants indicating cross regulation between uptake pathways.[49] Taken together, these findings strengthen the view that iron acquisition is integrated with morphogenesis in some fungal pathogens, and they provide insights into the relative contributions of siderophore *versus* reductive uptake mechanisms for growth in phagocytic cells and for yeast *versus* hyphal cell types.

4. Heme and hemoglobin are important iron sources for pathogenic yeasts

Approximately 80% of the iron inside a mammalian host is bound to heme in hemoglobin (Hb) and other heme-containing proteins. In addition to hemoglobin, other host iron-containing proteins include lactoferrin (Lf), transferrin (Tf), haptoglobin, haemopexin, lipocalin-1 and lipocalin-2 (Lcn1/Lcn2).[71] Additionally, intracellular iron availability to microbes is also limited due to the sequestration of iron in transferrin and ferritin.[71,72] Consequently, the ability of fungal pathogens of humans to obtain iron from different host iron-binding proteins to sustain growth and proliferation is a key determinant of the outcome of infection. In this regard, some fungal pathogens target heme and hemoglobin as key iron sources. For example, *C. albicans* has robust machinery to obtain iron from heme/hemoglobin.[73–75] This system is independent of the reductive and siderophore uptake mechanisms and depends on a conserved family of proteins (Rbt5, Rbt51/Pga10, Pga7 and Csa2) which contain the cysteine-rich Common in Fungal Extracellular Membrane (CFEM) domain.[74–79] Among these proteins, Rbt5 is a mannosylated plasma membrane-anchored protein that mediates heme uptake.[75] The paralog of *RBT5*, *RBT51*/*PGA10*, also confers the ability to use heme as a sole iron source in *S. cerevisiae*, and this finding provided a fruitful avenue to investigate heme and hemoglobin uptake. Specifically, a large-scale screen of the deletion library in *S. cerevisiae* expressing *C. albicans RBT5* suggested that the Rbt5 protein is the GPI-anchored hemoglobin receptor that facilitates the endocytosis of hemoglobin into the vacuole. The functions identified in the screen implicated the involvement of acidification of the lumen of the late secretory pathway, a type I myosin and the activity of the ESCRT pathway in hemoglobin utilization in *C. albicans*.[76] A recent study also identified a role for another CFEM protein Pga7 in heme uptake

in *C. albicans*. Pga7 was shown to cooperate with Rbt5 within the cell envelope for hemoglobin and heme utilization, and the mutant lacking *PGA7* displayed a more significant growth deficiency than the *rbt5* mutant in the medium with heme or hemoglobin as a sole iron source.[80] Furthermore, the *pga7* mutant was attenuated for virulence in a mouse model of systemic infection suggesting that heme iron uptake contributes to *C. albicans* pathogenesis.[80]

Another CFEM domain protein, Csa2, is required for robust growth on hemoglobin. Csa2 binds hemoglobin and may serve as a hemophore to deliver the protein to other CFEM proteins such as Pga7 and Rbt5.[77,80,81] In this context, a recent study presented the structural basis of heme-iron acquisition by Csa2, in *C. albicans*.[81] It was found that the CFEM domain in Csa2 adopts a novel six α-helical basket form with a flat hydrophobic platform on top to allow the attachment of a planer heme molecule.[81] A conserved aspartic residue in the CFEM helical basket serves as an axial ligand to confer the specificity of Fe^{3+} heme binding.[81] This aspartic-heme-Fe axial ligand coordination in Csa2 partially resembles the tyrosine-heme-Fe axial ligand coordination of the bacterial hemophore, HasA, as described in the Gram-negative bacteria *Serratia marcescens* and *Pseudomonas aeruginosa*.[82,83] The work on Csa2 further strengthens the model for heme-iron acquisition in *C. albicans* that involves removal of heme from hemoglobin by Csa2, transfer to envelope-anchored CFEM proteins Rbt5 and Pga7, and internalization by endocytosis (Fig. 1).[80]

Other fungi also use heme and hemoglobin as iron sources in various capacities.[75] For example, the *Candida* species *C. tropicalis* and *C. parapsilosis* share the ability to use these iron sources with *C. albicans*, perhaps reflecting the fact that all three species belong to the same clade. However, other *Candida* species such as *C. glabrata* and *C. krusei* are unable to grow with heme and hemoglobin as iron sources. In addition, anti-Rbt51 antiserum reacted with cell lysates of *C. tropicalis* and *C. parapsilosis*, but not with lysates of *C. glabrata* and *C. krusei*, implying that the first two may possess an Rbt51 ortholog for heme uptake.[75] Other heme-related functions have been described in *C. glabrata* including a putative cell-surface bound CFEM domain-containing protein (Ccw14), a haemolysin-like protein (Mam3), and an intracellular haem oxygenase (Hmx1).[37] Both Ccw14 and Mam3 are involved in iron homeostasis and virulence for *C. glabrata*, although their role in the use of heme or hemoglobin requires further investigation.[37] CFEM proteins are important for hemoglobin use by other species including the dimorphic pathogens *Paracoccidioides brasiliensis* and *P. lutzii*.[84,85] In these fungi, an ortholog of Rbt5 serves as a hemoglobin receptor and loss of the protein results in mutants with impaired survival in macrophages and in the spleens of infected mice. In contrast, deletion of three genes (*cfmA-C*) encoding CFEM-domain proteins in *A. fumigatus* did not reveal roles in heme uptake or biofilm formation, although cell wall defects were observed.[86] The lack of an involvement of CfmA-C in heme use is interesting given a recent detailed RNA-Seq analysis indicates that *A. fumigatus* expresses functions for iron homeostasis, secondary metabolism and detoxification to survive in blood, but does not grow.[87] This has been termed a ''just wait and see'' resting stage behavior for the fungus.

The use of heme as an iron source has also been described for *C. neoformans*.[88–90] In this fungus, a screen for genes required for growth on heme initially revealed a role for Vps23, a component of the ESCRT-I complex involved in endocytosis or regulation of iron acquisition. Vps23 was also required for virulence in a mouse inhalation model of cryptococcosis.[88] An additional study showed that other ESCRT proteins, specifically Vps22 (ESCRT-II) and Snf7/Vps20 (ESCRT-III), also contribute to heme use in *C. neoformans*.[89] Similar to *C. albicans*, a putative hemophore, Cig1, has also been described in *C. neoformans* that is involved in heme binding, uptake and virulence.[90] The Cig1 protein does not contain a CFEM domain and therefore must interact with heme *via* a different mechanism than the *C. albicans* proteins. The reductive iron uptake system can mask the absence of Cig1 during colonization of mice because a *cig1* mutant is as virulent as the wild-type strain, and a *cig1cfo1* double mutant is more attenuated than a *cfo1* mutant. These results contribute to the general view that multiple mechanisms of iron assimilation contribute to host colonization and that additional systems remain to be discovered in *C. neoformans*.[90] One outstanding issue concerns the access of *C. neoformans* to heme given that hemolytic activity has not been reported. The surface of Cryptococcal cells is unique among fungal pathogens in that the fungus elaborates a large polysaccharide capsule in response to a variety of signals including iron deprivation.[91] Interestingly, deletion of the *VTC4* gene encoding a polyphosphate polymerase in *C. neoformans* resulted in impaired ability of the fungus to trigger blood coagulation *in vitro*. Although virulence was not attenuated for a *vtc4* deletion mutant, cells of this strain did accumulate to a higher level than the wild type in the lungs of infected mice. Polyphosphate may therefore function in lung colonization or dissemination of the fungus from the lungs. A connection between the master iron regulator Cir1 in *C. neoformans* and the expression of functions for phosphate uptake and polyphosphate formation has been established.[92] It is therefore tempting to speculate that polyphosphate on the fungal cell surface might contribute to interactions with erythrocytes, and potentially to iron acquisition.

5. Targeting iron acquisition to augment antifungal therapy

The accumulating knowledge on the iron acquisition systems that contribute to fungal virulence promises to inform new approaches to diagnostics and therapy. In general, current strategies to target pathogen iron acquisition include the use of iron chelation therapy, siderophore-drug conjugates, inhibitors of uptake systems or biosynthetic enzymes, vaccines against surface uptake components, and immunological strategies to influence iron availability to intra and extracellular pathogens.[93–99] The approach of targeting biosynthetic enzymes for inhibitor development shows considerable promise for pathogens that deploy siderophores during host colonization.[97] Success in this area was recently demonstrated in a screen using an assay for the L-ornithine-N^5-monooxygenase

encoded by *sidA* in *A. fumigatus*.[100] The crystal structure of the enzyme has been determined and detailed biochemical studies led to the development of a fluorescence polarization binding assay involving a chromophore linked to ADP as a probe for active site binding of small molecules.[101,102] A screen of 2320 compounds with the assay yielded celastrol as a specific SidA inhibitor, and follow up work showed that this compound at 50 μM inhibits the growth of *A. fumigatus* on defined minimal medium without iron and on blood agar.[100] As expected, the growth inhibition was rescued by siderophores. Interestingly, celastrol was previously found to have antifungal activity against phytopathogenic fungi.[103]

The use of chelators for treatment of fungal infections also shows potential utility and recent studies illustrate the development of new chelators, the use of these molecules in combination with antifungal drugs and their use in treating topical fungal infections.[104–107] In a search for new chelators, Helsel *et al.* screened a set of metal chelating agents for their ability to inhibit the growth of *C. neoformans*.[105] The tested compounds included general chelators, iron chelators including desferrioxamine B, and extracellular and intracellular molecules that bind copper. Interestingly, chelating compounds that bound iron or copper did not limit fungal growth in the assay conditions, but agents that acted to increase intracellular copper levels were inhibitory. The active molecules were ionophores and, importantly, were fungicidal for *C. neoformans*. The ability of combinations of iron chelators and antifungal drugs to inhibit the growth of *C. neoformans*, *Cryptococcus gatti* and *S. cerevisiae* has also been tested.[107] In particular, the chelators ethylenediamine tetraacetic acid, deferiprone, deferasirox, ciclopirox olamine and lactoferrin reduced the minimal inhibitory concentration of amphotericin B (AmB), a front line antifungal for cryptococcosis. The only combination that showed synergy was lactoferrin with AmB but iron supplementation did not ablate growth inhibition suggesting that another mechanism besides iron chelation may explain the lactoferrin contribution. Finally, recent work examined chelation as a topical treatment for corneal infections caused by *A. fumigatus* and another opportunistic fungal pathogen *Fusarium oxysporum*.[104] Iron availability as modulated by Fe-dextran and deferrioxamine treatment, and extracellular siderophore production, were shown to influence infection in a murine corneal infection model. Additionally, sequestration with the human siderophore-binding protein lipocalin-1 also reduced fungal growth during infection. Extracellular siderophore biosynthesis by *A. fumigatus* requires mevalonate as a precursor, and mutants unable to convert mevalonic acid to anhydromevalonyl CoA for incorporation (Δ*sidH* and Δ*sidI*) had reduced growth in infected tissue.[104] Together, these results led to a demonstration that topical treatment with statins that block HMG-CoA reductase activity for mevalonate production and the chelator deferiprone could protect corneal tissue from infection by *A. fumigatus* or *F. oxysporum*.[104] Overall, this study provides compelling support for combination treatment approaches, and the results hold particular promise for topical therapy of fungal infections on mucosal surfaces, skin and nails.

6. Conclusions

Our understanding of the mechanisms of iron acquisition systems and their contributions to virulence has expanded in the past three years for the best studied fungal pathogens *A. fumigatus*, *C. albicans*, *C. glabrata* and *C. neoformans*. Additionally, new insights are emerging for a broader range of pathogens including dimorphic species in the genera *Talaromyces* and *Paracoccidioides*. In particular, we gained insights into the roles of candidate ferric reductases at the cell surface and the complexities of the contributions of different enzymes in the face of redundancy. Redundancy is also a challenge in determining the potential contributions of multiple siderophore transporters in fungi that exploit xenosiderophores. Additional information from *T. marneffei* illustrates the integration of iron acquisition *via* reductive uptake and siderophores with virulence properties such as the temperature-dependent morphological switch that generates the pathogenic yeast cell type. A major recent accomplishment was the molecular characterization of the relay system of CFEM domain proteins for heme and hemoglobin use in *C. albicans*. This system will undoubtedly serve as a paradigm for many fungal pathogens, particularly among pathogenic yeasts, but perhaps not for *A. fumigatus* and other molds. Together, the accumulating information provides the foundation for therapeutic applications and recent studies support the value of targeting siderophore biosynthesis and metal chelation. It will be important to understand the specific strategies of each pathogen so that targeted interventions can be deployed. Many questions remain to be answered, particularly in the context of understanding the relative contributions of uptake systems for specific host iron sources. One challenge will be to fully understand the range of uptake systems when multiple mechanisms support growth in the host, and to develop the experimental models to understand niche-specific access to iron, particularly in the blood.

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