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Candida albicans specializations for iron homeostasis: from commensalism to virulence

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Candida albicans is a fungal commensal-pathogen that persistently associates with its mammalian hosts. Between the commensal and pathogenic lifestyles, this microorganism inhabits host niches that differ markedly in the levels of bioavailable iron. A number of recent studies have exposed *C. albicans* specializations for acquiring iron from specific host molecules in regions where iron is scarce, while also defending against iron-related toxicity in regions where iron occurs in surfeit. Together, these results point to a central role for iron homeostasis in the evolution of this important human pathogen.

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Introduction

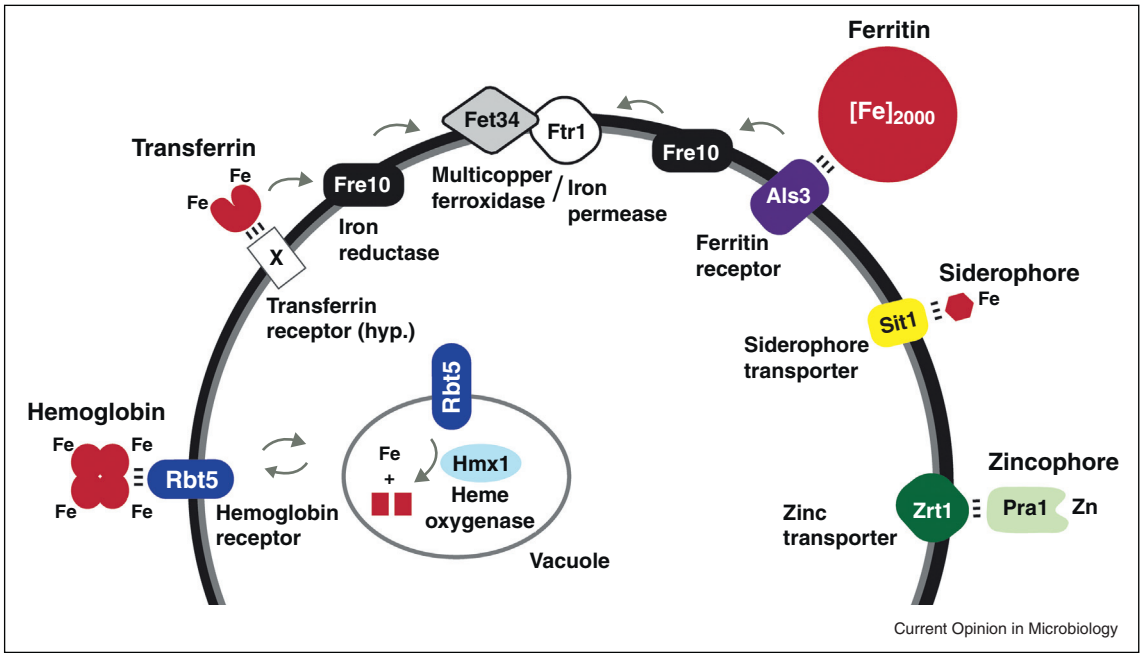
Unlike the majority of pathogenic fungi, *C. albicans* remains perpetually associated with its mammalian hosts [1]. It typically exists as a commensal of the mammalian microbiome, occupying mucocutaneous surfaces such as skin, the genitourinary tract, and particularly the gastrointestinal tract. *C. albicans* also functions as an opportunistic pathogen and can disseminate to virtually any internal organ. This ability to adapt to host microenvironments differing markedly in the levels of key micronutrients is a hallmark of *C. albicans* biology. For example, *C. albicans* is among the most common pathogens recovered from the human bloodstream [2], a region characterized by extremely low levels of iron ($\sim 10^{-24}$ M Fe^{3+}) because of low aqueous solubility at neutral pH, combined with active sequestration by the host [3]. In contrast, dietary iron remains abundant throughout its commensal niche in the gastrointestinal tract, and iron may even approach toxic levels in regions where local acidity or hypoxia increases its bioavailability [4]. Recent investigations of *C. albicans* mechanisms for acquiring necessary iron in the bloodstream and tissues while also defending against iron toxicity in the gut provide insights into the importance of iron as an evolutionary force in the most common human fungal pathogen.

Mechanisms of iron acquisition in the bloodstream

Roughly two-thirds of mammalian total body iron occurs in the bloodstream, mostly in the form of hemoglobin in red blood cells [5]. In addition, the minute amount of extracellular transferrin-bound iron serves as the major mechanism for iron distribution [5]. Such fastidious sequestration of iron protects healthy hosts against iron-catalyzed toxic free radical generation (via Fenton chemistry [6]) and provides 'nutritional immunity' against infection with iron-dependent pathogens [7]. Moreover, in the presence of pathogens such as *C. albicans*, host inflammatory responses further reduce serum iron levels through mechanisms such as decreased intestinal absorption and retention within reticuloendothelial cells [8,9]. People lacking these defenses, such patients with hemochromatosis and other iron overload syndromes, have an excess risk for bloodstream infections with *C. albicans* and other pathogens [10].

Modern medical practices have nevertheless contributed to an increased incidence of candidemia, worldwide [11–13]. Medical therapies such as intravenous catheters [12,14,15], surgery [12,14,15], antibiotics [15,16], and immunosuppressants [15] promote *C. albicans* bloodstream infections through disruption of host immune and epithelial barriers and by altering the microbiome. Once introduced into the bloodstream, *C. albicans* can acquire iron from the very molecules that are used by the host to sequester it [17–19,20•] (Figure 1). For example, several groups have identified *C. albicans* hemolytic activity capable of releasing hemoglobin from host erythrocytes [21–23]. Free hemoglobin or its heme/hemin metal-porphyrin ring is bound by a hemoglobin receptor, Rbt5, on the fungal cell surface [24•], followed by endocytosis of Rbt5-hemoglobin complexes [25] and release of ferrous iron by the heme oxidase, Hmx1 [19,26]. Notably, *C. albicans* encodes four additional homologs of Rbt5, of which one (Rbt51) has also been demonstrated to bind to hemin and to confer hemin utilization capability when expressed in the nonpathogenic model yeast, *Saccharomyces cerevisiae* [24•].

Figure 1



C. albicans mechanisms for acquiring iron and zinc from the host. *C. albicans* has evolved systems targeting host hemoglobin, host transferrin, host ferritin, and siderophores as sources of iron, and the Pra1/Zrt1 system for scavenging zinc. The depicted transferrin receptor ('X') is hypothetical (hyp.). The depicted variants of the hemoglobin receptor, iron reductase, multicopper ferroxidase, and iron permease are members of larger gene families with demonstrated *in vitro* activity.

C. albicans can also utilize host transferrin *in vitro* as a sole source of iron [20•]. It is uncertain whether *C. albicans* expresses a transferrin receptor, similar to certain bacterial pathogens [27], but the observation that it requires direct contact with transferrin in order to utilize it [20•] suggests that this may be the case. Ferric iron derived from transferrin is taken up by a reductive iron uptake system that is conserved with the well-described high affinity iron uptake system of *S. cerevisiae* (reviewed in [27,28]). Fe^{3+} is first reduced to soluble Fe^{2+} by a cell surface-associated ferric reductase [29,30]. In coupled reactions, Fe^{2+} is then oxidized and imported into the fungal cytoplasm by a multicopper ferroxidase/iron permease complex [30,31••]. *C. albicans* encodes 17 putative ferric reductases, five putative multicopper ferroxidases, and four putative ferric permeases [32] with potential functions in reductive iron uptake, and different subsets of these enzymes are expressed under different *in vitro* conditions (e.g. [20•,33,34]). Of the two ferric permeases, only Ftr1 is expressed when iron is scarce, and *FTR1* is essential in a murine bloodstream infection model of virulence [34].

Strategies for iron acquisition in tissues

Approximately one-third of mammalian total body iron occurs bound to ferritin in tissues and macrophages [5]. A single ferritin heteropolymer binds as many as 4500 iron atoms, and cytoplasmic iron-ferritin complexes are generally extremely stable [35]. Only a few bacterial pathogens such as *Neisseria meningitidis* have been shown to use ferritin as an iron source [36]. Among fungi, *C. albicans* can also utilize ferritin when provided under standard *in vitro* conditions or directly from host epithelial cells in culture [37••]. When co-cultured with a human oral epithelial cell line, invading *C. albicans* hyphae aggregate host ferritin onto their surfaces using a hypha-specific cell surface protein, Als3. *In vitro*, fungal-mediated acidification of the laboratory culture media is required to dissociate Fe^{3+} from ferritin [37••]. Fe^{3+} is transported into the fungal cytoplasm via the same reductive iron uptake system [37••] described above for transferrin. Intriguingly, Als3 also plays important roles in *C. albicans* biofilm formation [38,39], adhesion to host epithelial and endothelial cells [40], and induced endocytosis of hyphae [41]. Further, deletion of *ALS3* abrogates *C. albicans* virulence in oral epithelial infection models [37••,40] but not in a bloodstream infection model [42]. Thus, Als3 integrates iron uptake and virulence functions, a characteristic displayed by several other key players in *C. albicans* iron homeostasis, as discussed below.

In common with numerous other fungal and bacterial species, *C. albicans* possesses a third system of iron uptake that targets siderophores rather than host molecules [43]. Siderophores are small ferric iron chelators that bind with extremely high affinity (iron formation

constants K_f range from 10^{20} to $10^{50}\,M^{-1}$), some of which can extract iron from transferrin and lactoferrin [27,44–46]. It is unclear whether *C. albicans* synthesizes its own siderophores: siderophore activity has been reported for this species [47,48] but its genome does not encode the known fungal biosynthetic enzymes [44]. Regardless, *C. albicans* has been demonstrated to utilize exogenous ferrichrome-type siderophores via the Sit1 siderophore importer [43], an ortholog of *S. cerevisiae* Arn1 [49]. Similar to *ALS3*, deletion of *SIT1* abrogates *C. albicans* virulence in a reconstituted human epithelial infection model [43] but not in a bloodstream infection model [43,50••]. Future studies will be required to determine whether Sit1 plays a role in mixed infection models that include additional siderophore-producing microorganisms.

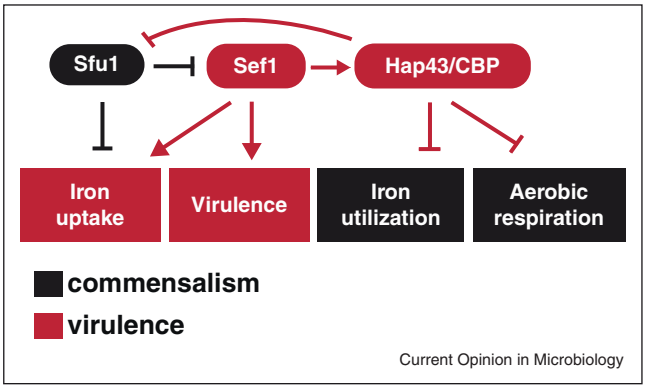
Zinc acquisition via a 'zincophore'

Iron is not the only essential micronutrient, and host sequestration of manganese and zinc also contributes to nutritional immunity [51•]. *C. albicans* was recently reported to scavenge zinc by means of Pra1, a small, secreted protein dubbed a 'zincophore,' by analogy with iron-scavenging siderophores [52••]. Similar to the story with ferritin utilization, during infection of human endothelial cell monolayers, invading *C. albicans* hyphae are able to aggregate host zinc onto their cell surfaces. The aggregation activity requires both Pra1, which exhibits zinc-binding activity *in vitro*, and a predicted cell surface zinc transporter, Zrt1, which binds to Pra1 and is thought to recruit soluble Pra1-zinc complexes to the fungal cell surface [52••]. Pra1 was previously shown to have multiple interactions with components of the host innate immune system, including engagement of a leukocyte receptor that promotes neutrophil migration and fungal killing [53] and interactions with host complement regulators that favor fungal escape [54]. Notably, *PRA1* and *ZRT1* are adjacent on *C. albicans* chromosome 4, and the conservation of synteny among evolutionarily distant fungi (including nonpathogens) suggests that zinc acquisition is the original function of this system [52••].

Defense against iron excess in the gut, a major site of commensalism

Topologically, the mammalian gut exists outside of the body, and dietary iron is not included in estimates of total body iron. A typical Western diet contains approximately 15 mg of daily iron, of which less than 10% is absorbed [4]. Net iron remains relatively high throughout the GI tract, with large boosts in iron bioavailability predicted in regions of acidity, such as the stomach [4], and oxygen-depletion, such as the large intestine [55]. Gastrointestinal commensals thus face the risk of potential iron-associated toxicity (toxic free radicals generated in the Fenton reaction [56]), at least in some regions of the gut.

Figure 2



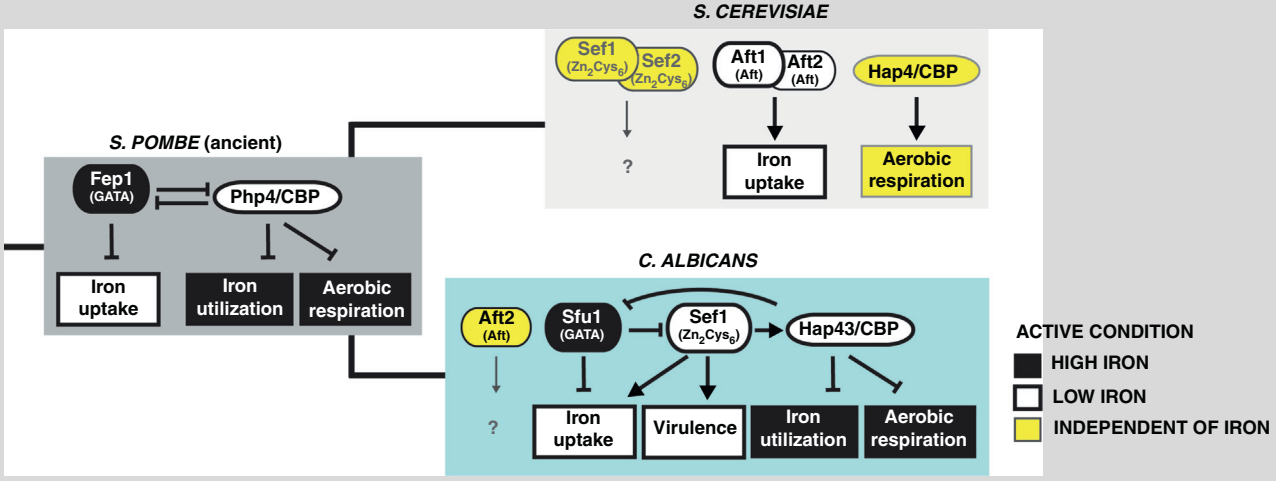
C. albicans regulation of iron homeostasis, virulence, and commensalism. In *C. albicans*, the transcription factors controlling iron homeostasis are also required for virulence or commensalism. *SEF1* is induced in the bloodstream, where iron is scarce, and is required for virulence in a murine bloodstream infection model. Its direct binding targets include genes for all three of its iron uptake systems, as well as known virulence factors, similar to the responses of certain bacterial pathogens that use iron depletion as a cue for virulence gene expression. By contrast, *C. albicans SFU1* is induced in iron-replete environments such as the GI tract and represses genes for iron uptake factors. It remains to be determined whether iron surfeit serves as a cue for the expression of commensalism factors.

How does *C. albicans* defend against iron-related toxicity in areas of iron excess, while retaining the capacity for aggressive iron uptake in host niches of iron depletion? One answer lies in its evolution of a unique transcriptional regulatory circuit for maintaining iron homeostasis (Figure 2). In most characterized fungi (apart from *S. cerevisiae*), a highly conserved GATA family transcription factor represses genes for iron uptake factors when environmental iron is replete (see Box 1) [57–64]. *C. albicans* maintains an ortholog of this factor, Sfu1 [65,66], which directly represses genes for iron uptake factors, including components of the hemoglobin uptake system, the reductive iron uptake system, and the Sit1 siderophore transporter [67••]. Indeed, *SFU1* is essential for defense against high iron *in vitro* and for normal commensal fitness in the mammalian gut [67••].

SFU1 is not required for virulence in the bloodstream, however; rather, an *sfu1* knockout mutant exhibits enhanced fitness in competitive infections in this environment, presumably because of enhanced expression of iron uptake factors [67••]. At least three different transcription factors mediate the fungal response to iron sequestration in the bloodstream. Rim101 is a Cys_2His_2 zinc finger transcription factor required for expression of iron uptake genes under neutral or alkaline conditions [68•]. Rim101 also activates known virulence genes [69,70] and is required for virulence in bloodstream [71] and oropharyngeal [72] infection models. Similarly, two transcription factors that form a circuit with Sfu1 are

Box 1 Evolution of iron homeostasis in fungi.

Most characterized fungal species maintain cellular iron homeostasis by regulating the expression of iron uptake and iron utilization via a simple transcriptional switch. As depicted for the prototypical model yeast, *S. pombe*, a GATA family transcription factor with iron-sensing activity (Fep1) is expressed when environmental iron is replete. Fep1 directly represses iron uptake genes and *PHP4*, the gene for the regulatory subunit of the CCAAT binding complex. When environmental iron is low, Php4 directly represses genes for nonessential iron-utilizing processes as well as *FEP1*. A common ancestor to the *Candida* and *Saccharomyces* lineages gained two transcription factors (Sef1 and Aft1 precursors), leading to rewiring of the circuit. In *C. albicans*, Sef1 was intercalated into the existing circuit and associated with virulence genes in addition to iron uptake genes. The coregulation of iron uptake genes by both Sfu1 and Sef1 creates a feed forward loop predicted to buffer the expression of coregulated genes against transient fluctuations in environmental iron. In *S. cerevisiae*, the GATA factor was lost, and regulation of iron uptake genes was transferred to Aft1 and Aft2 (produced by a whole genome duplication event in the *Saccharomyces* lineage).



also required for virulence in the bloodstream (Figure 2): Hap43, a highly conserved regulatory component of the CCAAT binding protein (CBP) complex [73•,74•] (whose *Aspergillus fumigatus* and *Cryptococcus neoformans* orthologs likewise promote virulence in these species [75,76]) and Sef1, a Zn_2Cys_6 zinc knuckle transcriptional activator that was introduced relatively recently into the *C. albicans* lineage [67••,77•]. Under iron-depleted conditions, the Hap43/CBP complex directly represses *SFU1* and genes for nonessential iron-utilizing processes, such as aerobic respiration and iron-sulfur cluster assembly [67••,73•,74•,78]. Under these same conditions, Sef1 directly activates *HAP43*, genes for all three modes of iron uptake, as well as virulence genes thought to act independently of iron [67••]. Sef1 also affects fitness in a gastrointestinal infection model [67••], suggesting that at least some regions of the gut are effectively depleted for iron.

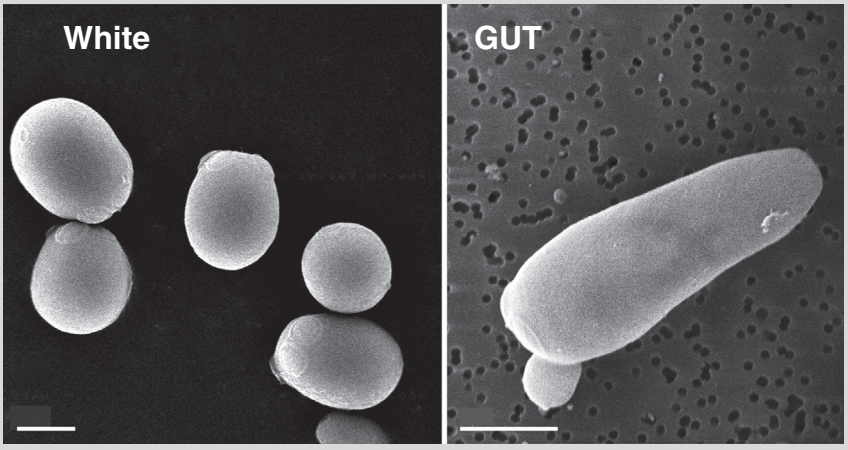
Reminiscent of Rim101, Sef1 integrates the expression of iron uptake and virulence genes, and this transcriptional regulator is itself regulated at multiple levels. When environmental iron is replete, *SEF1* transcription is directly inhibited by Sfu1, as described above. Remarkably, Sfu1 also directly inhibits Sef1 protein activity by sequestering it in the cytoplasm, where it is rapidly degraded [79•]. Under iron-depleted conditions, however, Sef1 is phosphorylated by the protein kinase, Ssn3, and transported into the nucleus, where it induces the transcription of iron uptake genes [79•]. We hypothesize that the introduction of Sef1 into *C. albicans* iron homeostasis, along with mechanisms controlling its expression and protein activity, allowed for finer control of a critical gene regulon in the context of fluctuating host iron levels.

From iron homeostasis to developmental switches

Similar to free-living fungi, *C. albicans* must adapt to stark differences in the abundance of iron and other essential micronutrients within different niches of the mammalian host. Where this metal is scarce, *C. albicans* succeeds by extracting iron from host iron-sequestering molecules. Ferritin and transferrin are utilized by means of an ancient reductive iron uptake system, which *C. albicans* has customized with the introduction of additional homologs of each component and a novel ferritin receptor, Als3. Specific roles for the ferric reductase, multicopper ferroxidase, and iron permease homologs remain to be defined, but a plausible hypothesis is that different alleles are optimized for uptake of iron from different host compartments. Homologs of Rbt5 may serve analogous roles in hemoglobin utilization. Also, the recent discovery of a role for Pra1 as a 'zincophore' speaks to the importance of micronutrients other than iron and implies the existence

Box 2 *C. albicans* **white and GUT cell types differ in the expression of iron uptake genes.**

White cells (left) exhibit a round-to-oval cell morphology and are the default *C. albicans* cell type. White cells are virulent in murine bloodstream models of virulence. GUT (Gastrointestinally IndUced Transition) cells (right) correspond to a recently described developmental state that is triggered by passage of *C. albicans* yeasts through the mammalian gastrointestinal tract. GUT cells exhibit elongated cell morphology and enhanced commensal fitness but are attenuated for virulence in the bloodstream. Consistent with these functional specializations, white and GUT cells express inverse sets of iron-related genes, with *SEF1* and iron uptake genes induced in white cells and *SFU1* induced in GUT cells. Shown are scanning electron micrographs of white and GUT cells, with scale bars corresponding to 2 μm (images from Chen and Noble, unpublished).



of as yet undiscovered mechanisms targeting these alternative nutrients.

Some bacterial pathogens use iron depletion as a kind of location marker, signifying entry into the mammalian host and triggering the expression of virulence factors [46]. *C. albicans* appears to use a similar logic, such that key transcriptional regulators of iron uptake genes such as Sef1 and Rim101 also activate the expression of virulence factors. Moreover, the coupling of virulence with nutrient uptake functions extends to the iron and zinc uptake effectors, Als3 and Pra1, which play additional, direct roles in virulence.

Finally, in its commensal role within the mammalian gastrointestinal tract, *C. albicans* encounters much higher levels of iron. It is possible that, by the converse of the logic described above, high iron levels may signify the gastrointestinal milieu and the need to express commensalism factors. My research group recently discovered that passage of *C. albicans* through the host digestive tract induces a developmental switch to a novel commensal cell type (Box 2) [80••]. 'GUT' (gastrointestinally induced transition) cells strongly outcompete previously defined virulent ('white') and sexually competent ('opaque') cell types within a mouse gastrointestinal infection model and express a distinct transcriptome [80••]. Compared to white and opaque cells, GUT cells upregulate *SFU1* and downregulate *SEF1* and iron uptake genes, as well as altering the expression of metabolic genes to match the nutrient composition of the distal mammalian GI tract [80••]. Future experiments will be required to determine what additional factors promote the commensal lifestyle and whether they are triggered by levels of iron, zinc, and other critical nutrients.

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- •• of outstanding interest

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