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The intersection of host and fungus through the zinc lens

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In this review, we summarize data regarding the influence of zinc on host defenses to human pathogenic fungi and how the fungus acquires zinc to sustain biological functions. Mammals have evolved several extracellular and intracellular mechanisms to withhold zinc from the fungus. Specific immune cells release zinc binding proteins such as calprotectin to capture the metal and deny it to the fungus. Intracellularly, several zinc binding proteins such as metallothioneins starve the fungus of zinc. The net result in both situations is depriving the fungus of a crucial micronutrient. To combat this struggle, fungi have developed means to capture zinc and store it. The mechanisms of transport for various fungi are discussed herein.

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Introduction

The trace metal zinc is essential for many biological processes including gene expression, proper protein folding, enzyme activity, and intracellular signaling. In eukaryotes, zinc-binding proteins constitute ~10% of the proteome [1]. The metal must be obtained from the environment or from the diet to supply cells with the required quantities. To maintain the proper amount of zinc without causing overload, fungi and mammals contain numerous importers and exporters. In *Candida albicans*, for example, there are nine transporters whereas mammals contain over twenty [2,3••]. The sheer number of transporters dedicated to handling zinc highlights the impact that this metal exerts on the physiology of a cell. This metal is crucially important for the mammalian immune system to perform its operations optimally [4]. Too much or too little leads to immune dysfunction. Zinc becomes a prized commodity when fungi confront the mammalian immune system. A struggle ensues in which the fungus must overcome a hostile environment that seeks to deprive it of zinc — a process known as nutritional immunity [5]. In this context, our review will highlight publications that discuss the progress made in understanding how the host regulates zinc availability and how the fungus adapts to this nutritionally hostile environment.

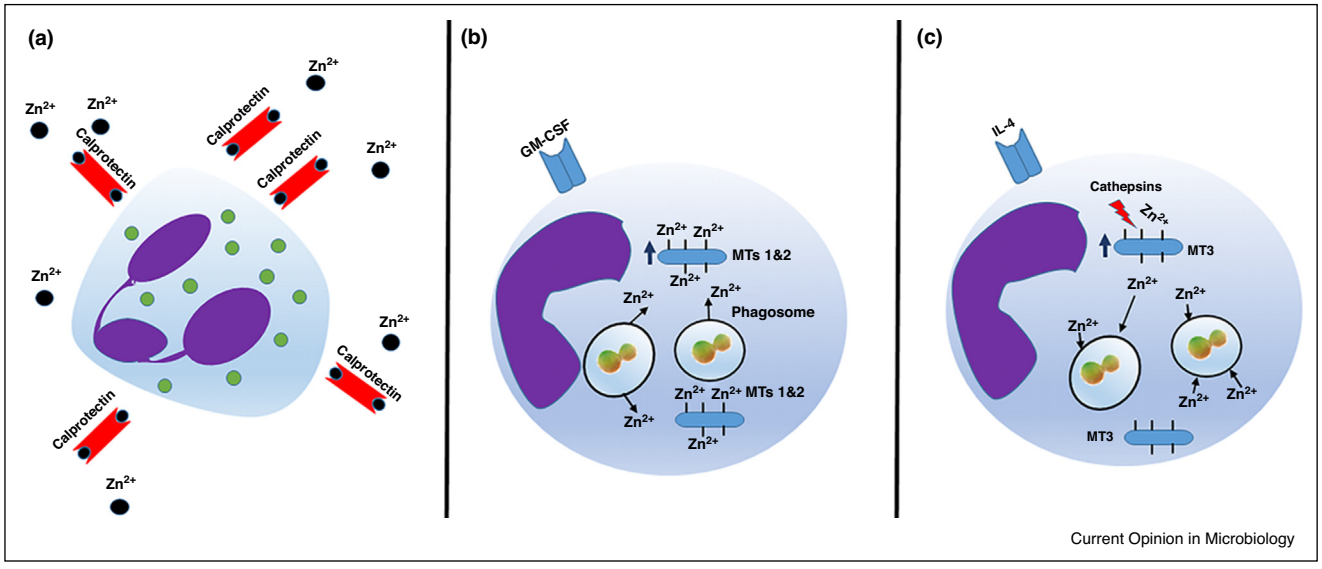
Host manipulation of zinc to combat fungal infection

Extracellular trapping

Several molecules from mammalian immune cells limit access to zinc; they include calprotectin (S100A8/9), psoriasin (S100A7), and calgranulin C (S100A12) [6]. Among these, calprotectin is best known for its antifungal properties. The sources of the protein are neutrophils, monocytes/macrophages, keratinocytes, and plasmacytoid dendritic cells. In neutrophils, calprotectin constitutes ~40% of the cytoplasmic protein [7]. Although it binds zinc, Mn, iron, and Cu, its antimicrobial activity appears to be chiefly associated with zinc and Mn sequestration [8]. The chelating property of calprotectin depends on access to the extracellular environment. Herein, calprotectin confronts a higher concentration of Ca^{2+} which is required for proper folding and thus, optimal zinc binding. This molecule lacks a secretion signal, and its presence extracellularly generally occurs when cells undergo dissolution [8].

Calprotectin has a range of antifungal activity but is principally active against extracellular organisms (Figure 1a). Thus, calprotectin inhibits growth of *Candida* sp.; this effect is reversed by addition of zinc, Mn, or Cu [9,10]. Mice deficient in calprotectin are susceptible to a challenge with *C. albicans*. Calprotectin slows the growth of *Aspergillus fumigatus*, and this protein is essential for clearing infection in experimental corneal infection [11••]. The absence of calprotectin in neutrophils does not modify ingestion or killing of conidia. Thus, the activity of calprotectin is on limiting hyphal growth. Calprotectin in plasmacytoid dendritic cells limits the growth of *A. fumigatus*. This is a suicidal event since the fungus kills host cells which subsequently release calprotectin [12]. Neutrophils kill *Cryptococcus neoformans* by oxidative and non-oxidative mechanisms. The cytosolic

Figure 1



The many faces of zinc sequestration. **(a)** Neutrophils release calprotectin upon cell death. This molecule binds environmental zinc to sequester it from extracellular pathogens such as *Candida* and *Aspergillus.* **(b)** GM-CSF activates macrophages to inhibit intracellular growth of *Histoplasma capsulatum* by enhancing expression of metallothioneins (MTs) 1 and 2 that bind zinc and deprive this metal from phagosomes containing the fungus. **(c)** Interleukin-4 exerts the opposite effect in that it induces expression of MT3. Cathepsins cause detachment of zinc from this metal binding protein. Subsequently, this metal is transported to the phagosome and fortifies *H. capsulatum* yeast cells survival in phagosomes.

fraction of these cells exerts anti-*Cryptococcal* activity largely through release of calprotectin [13]. Since unopsonized encapsulated cryptococci are extracellular, it is calprotectin in the extracellular environment that contributes to limiting fungal growth.

Neutrophil extracellular traps (NETs) form when neutrophils discharge chromatin and other intracellular contents extracellularly. NETs exert broad antimicrobial properties and as the name implies, ensnare microorganisms and limit their mobility [14]. Calprotectin is a major constituent of this meshwork. Although NET formation does not require calprotectin, this zinc-binding protein contributes to the antifungal properties of NETs. In this regard, NETs from calprotectin expressing, but not calprotectin-deficient neutrophils exhibit anti-*Candida* activity [9]. *In vivo*, NETs develop in mice in response to lung or soft tissue infection with *C. albicans*, and these structures contain calprotectin [9]. The conclusion is that NETs incorporate calprotectin *in vivo*, and this moiety is important for anti-*Candida* activity.

Chronic granulomatous disease (CGD) is an inherited disorder in which individuals manifest defective assembly of the NADPH oxidase system. Neutrophils from these patients do not form NETs, implicating reactive oxygen species in NET formation. The inability to form NETs is one explanation as to why these individuals are susceptible to *Aspergillus* sp. Complementation of the oxidase into CGD neutrophil restores NET formation and anti-*Aspergillus* activity and growth limitation of this fungus depends on zinc sequestration by NETs [15–17]. However, NETs are principally beneficial for the extracellular hyphal form rather than the ingested conidia, and therefore this meshwork of neutrophil contents likely is a secondary killing mechanism if the conidia escape killing upon ingestion.

Although calprotectin is the subject of most of the literature of zinc-chelating host proteins, S100A12 and S100A7 also manifest zinc-associated antifungal properties. S100A12 is a homodimer originally identified in porcine neutrophils and subsequently in humans. The protein is present in neutrophils, constituting ~5% of the total cytosolic protein, and chelates both zinc and Ca. Like calprotectin, S100A12 requires Ca to coordinate its zinc-binding capacity. *In vitro*, it inhibits the growth of several *Candida* spp. [18•]. However, since the quantity of this protein in neutrophils is markedly less than that of calprotectin, it is difficult to know the relative importance *in vivo*.

S100A7 (psoriasin) is a homodimer found principally in skin. The reduced form of this molecule inhibits the growth of *A. fumigatus* and *Trichophyton rubrum*. Reduced S100A7 penetrates the fungus and induces killing by chelating zinc [19]. In contrast to these studies, S100A7 binds to β-glucan of *C. albicans* and prevents adhesion of

the fungus to epithelial cell surfaces resulting in activation of epithelial cells [20•].

Clinically employed antimicrobial agents restrict zinc. Atovaquone, which expresses antifungal and antiparasitic activity, diminishes zinc content of *C. albicans*, *Aspergillus* sp., and *Fusarium* sp. Growth inhibition of the latter two correlates with decreased intracellular free Zinc [21•]. Halofantrine, an antimalarial agent, reduces growth of *C. albicans* commensurate with its capacity to diminish free zinc in the fungus [22]. The precise mechanisms by which these compounds alter zinc amounts are unknown. The impact of certain antimicrobial agents on their intended targets may be attributable to reducing intracellular zinc levels.

Intracellular sequestration

H. capsulatum yeast cells seek occupancy in macrophages where they thrive and thwart attack by host cells. Intracellular residence poses a dramatic shift in environment for this fungus as it transitions from soil that contains more zinc than macrophages (free zinc in the pM range). One cytokine that activates the anti-*Histoplasma* activity of macrophages is granulocyte macrophage colony-stimulating factor (GM-CSF). It stimulates zinc influx into the cytosol of macrophages and concomitantly, production of two zinc-binding proteins metallothioneins 1 and 2. These metallothioneins sequester zinc, thereby reducing free metal in host cells and consequently yeast cells (Figure 1b). Concomitantly, GM-CSF induces upregulation of a voltage-gated proton channel, Hv1 that enables the function of the NADPH oxidase [23]. Zinc is a known inhibitor of this channel and therefore, the decrement of available zinc by metallothionein sequestration increases reactive oxygen species. The combination of amplified ROS and zinc-deficient yeast cells enhances fungal killing. Alternatively, production of non-protective interleukin-4 induces zinc accumulation by *H. capsulatum* to fortify intracellular survival of the fungus (Figure 1c) [24••].

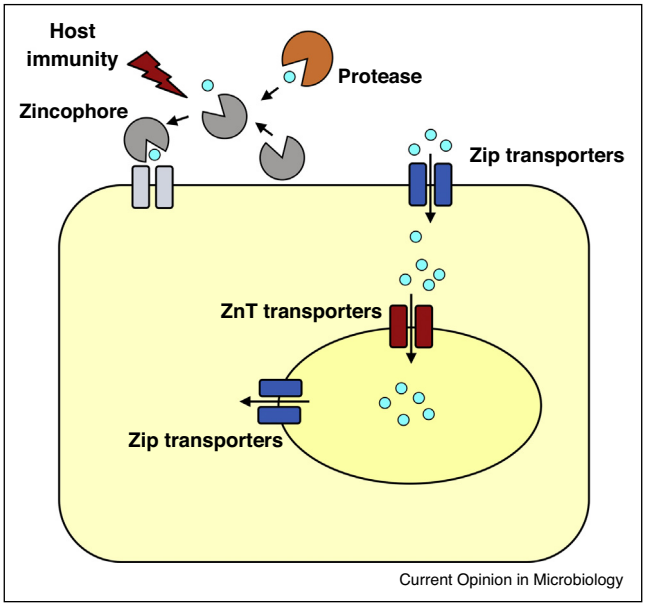
Maintenance of zinc homeostasis in fungi

To counteract the restrictive conditions imposed by nutritional immunity, pathogenic microbes possess effective mechanisms for scavenging zinc from the environment of the host. Unlike bacterial pathogens, which predominantly rely on znuABC transporters for high affinity zinc uptake and virulence, fungi do not appear to encode ABC transporters for zinc. Rather, fungi encode two unrelated classes of zinc transporter: ZIP (Zrt/Irt-like protein), which transport zinc from the extracellular milieu into the cytoplasm; and ZnT (Zinc transporter) which transport from the cytoplasm. Some, but not all fungi, encode secreted zinc binding proteins called zincophores (Figure 2).

ZIP transporter zinc uptake: species-specific contributions to virulence

Our knowledge of zinc transport in fungi originated in the model organism, *Saccharomyces cerevisiae* which encodes

Figure 2



Zinc capture and transport in fungi. Many fungal pathogens encode orthologues of the *Candida albicans* zincophore zinc scavenger, but the gene has been lost in several important pathogenic species. The zincophore exhibits multiple interaction with host immunity (complement system, neutrophils). In *C. albicans*, the protease Sap6 additionally delivers zinc. All investigated species encode Zip transporters which can capture zinc from the environment. Internalised zinc is shuttled into organelles (E.R., vacuole, zincosomes) via ZnT transporters which can be subsequently mobilized by intracellular Zip transporters.

two plasma membrane zinc importers: the high affinity Zrt1 and the low affinity Zrt2. In human fungal pathogens, zinc transporters were first characterized in *A. fumigatus* which contains at least five predicted cell surface ZIP-type zinc importers [25]. *zrfA* and *zrfB* are the *A. fumigatus* orthologues of ScZrt1 and ScZrt2. ZafA, an orthologue of the *S. cerevisiae* Zap1 transcriptional regulator, induces expression of these two importers in response to zinc limitation in acidic environments [26]. At neutral pH, PacC, a master regulator of pH responses, represses expression of these two genes [27]. *A. fumigatus zrfA*Δ and *zrfB*Δ deletion mutants exhibit growth defects in zinc-limiting acidic media [28]. Under conditions of neutral-alkaline pH, a third zinc transporter gene, *zrfC*, is induced by ZafA activation and PacC derepression and is required for growth in neutral-alkaline zinc-limited media [27]. The remaining two genes, *zrfD* and its unnamed paralogue Afu8g04010 remain uncharacterized.

Despite belonging to the Saccharomycotina, *C. albicans* zinc transporters are more similar to those of *A. fumigatus* in terms of regulation and functionality. *ZRT1* and *ZRT2* transcripts are regulated by zinc availability [3••], by Zap1 (although Zap1-dependent regulation in response to zinc

has not yet been demonstrated) [29,30] and by pH via Rim101 [31]. Zrt1 also acts as the Pra1 zincophore receptor (see below). CaZrt2 is orthologous to ScZrt2 and AfZrfB. Although modestly upregulated by acidic pH, CaZrt2 is functional in neutral-alkaline medium as a *zrt1*Δ mutant grows in neutral-alkaline media and zinc uptake by a *zrt2*Δ mutant is reduced by 50% at neutral pH. In acidic conditions, zinc uptake and growth is abolished by deletion of *ZRT2* [32••]. The conservation of zinc import mechanisms shared between *C. albicans* and *A. fumigatus* suggests that pH-dependent regulation and functionality of zinc import may be common within the Ascomycetes. This is most likely due to the biochemistry of histidine residues which, along with cysteine and, to a lesser extent aspartic and glutamic acid serve to coordinate zinc ions. The pK_a value of histidine is around 6.5, meaning that, in acidic environments, it becomes positively charged and loses its capacity to coordinate divalent cations [33].

Zinc transporters have also been characterized in two dimorphic fungal pathogens. *H. capsulatum* encodes orthologues of all-but AfZrfA zinc transporters from *A. fumigatus* [25]. Of these, HcZrt2 shares sequence similarity with AfZrfB and is necessary for zinc uptake, fungal growth, and virulence [34]. The AfZrfC/CaZrt1 orthologue in *Blastomyces dermatitidis* is important for survival *in vivo* [35].

C. neoformans and *Cryptococcus gattii*, encode two putative zinc importers: Zip1 and Zip2. In *C. gattii* only a *zip1*Δ/*zip2*Δ double deletion mutant exhibited virulence attenuation [36]. In contrast, a *C. neoformans zip1*Δ mutant was hypovirulent in mice; the *zip1*Δ/*zip2*Δ mutant is only slightly less virulent than the single mutant [37]. This correlates with the *in vitro* behavior of these mutants: *Cnzip1*Δ, but not *zip2*Δ exhibited decreased zinc assimilation, impaired growth under zinc limitation, and defects in intramacrophage proliferation.

The fungal-specific zincophore locus

C. albicans scavenges zinc via a proteinaceous zincophore system consisting of secreted Pra1 and cell surface-associated Zrt1 [38]. Environments of low zinc and neutral pH induce expression of these genes which share an upstream intergenic/promoter region. The Pra1 protein is secreted and sequesters host-cell derived zinc. This is likely via a series of zinc coordinating binding motifs [39]. Following zinc binding, secreted Pra1 re-associates with the fungal cell via Zrt1. Thermodynamic modelling of Pra1-Zn(II)-Zrt1 indicates that Pra1-bound Zinc may be transferred to Zrt1, for subsequent cellular assimilation [40].

Zincophore activity has been described only for *C. albicans*, but the locus is found in multiple fungal species. In *A. fumigatus*, low environmental zinc and high pH also positively regulate the *PRA1* and *ZRT1* orthologues, *aspF2* and *zrfC*, respectively. The *A. fumigatus aspF2-zrfC* and the *B. dermatitidis PRA1* orthologue are required for fungal growth under zinc limitation at neutral pH [35]. The *S. cerevisiae* Pra1 orthologue Zps1 is upregulated by zinc starvation [41]. The fact that Pra1 orthologues are important for growth under zinc restriction in multiple species implies conserved function. The syntenic arrangement of the *PRA1-ZRT1* locus occurred before the divergence of the Ascomycota and Basidiomycota, suggestive of a highly successful adaptation [38]. Although Pra1 orthologues have been identified in approximately 85% of sequenced fungal species [42], many extant species appear to have lost the gene. In environmental species this may be caused by adaptation to acidic environments [25] because Pra1-Zrt1 is non-functional at acidic pH [3••,38–40].

The activity of Pra1 extends beyond zinc binding. *C. albicans* Pra1 interacts with multiple components of the complement cascade, including factor H, factor H like protein, plasminogen, C3, C4b-binding protein [43–45]. In this context, Pra1 promotes immune evasion by preventing complement activation. Alternatively, Pra1 can be detrimental to the invading pathogen since it serves as a ligand for neutrophil $\alpha_M\beta_2$ and thus serves to recruit neutrophil migration. In a mouse model of disseminated candidiasis, a *pra1*Δ mutant is hyper-virulent, probably because it is less efficiently cleared by neutrophils [46,47]. The AspF2 orthologue from *A. fumigatus* binds factor H, factor H like protein and plasminogen [48]. Given these multi-level immune interactions, we speculate that Pra1 loss events in several important human fungal pathogens, including *Candida glabrata*, *Candida lusitaniae*, *Candida parapsilosis*, *H. capsulatum*, *C. neoformans*, and *C. gattii* may influence their interactions with host immunity [25,38,42].

The *C. albicans* Pra1/Zrt1-zincophore collaborates with the secreted aspartyl protease, Sap6, which is involved in zinc scavenging. This protease aggregates fungal cells and zinc, Zrt1 and Pra1 enhance this process. *sap6*Δ exhibits decreased zinc accumulation. These data suggest a model whereby Sap6 acts upstream in the zincophore pathway, delivering the substrate to Pra1/Zrt1 [49]. In support of this, exposure of *C. albicans* to perforin elicited a zinc starvation response with upregulation of *ZRT1*, *ZRT2*, *ZRT3* and *PRA1*. The most highly upregulated gene was *SAP6* [50], fitting with the concept that Sap6 is involved in Zinc acquisition.

Intracellular zinc trafficking in human fungal pathogens

Zinc is shuttled in and out of fungal organelles via the action of ZnT-transporters and Zip- transporters, respectively. *S. cerevisiae* transports zinc into the vacuole via the ZnT type transporter paralogues, Zrc1/Cot1, and this process is critical for tolerating zinc excess. In *Schizosaccharomyces pombe*, the Zrc1 orthologue, Zhf1 is required for zinc tolerance but

mediates zinc import into the endoplasmic reticulum [51,52]. In *C. neoformans* and *C. albicans*, Zrc1 is critical for adaptation to zinc excess via vacuole and zincosome sequestration, respectively. Zincosomes are small, vesicular-like zinc stores of unknown origin. However, Zrc1 is dispensable for virulence in mouse cryptococcosis but important for mouse liver colonization by *C. albicans* and virulence in a *Galleria*. This result suggests that *Cryptococcus* does not confront a high zinc environment whereas *C. albicans* does in the liver [53].

Zinc as a signaling molecule

Zinc exerts second messenger function in mammalian cells [54]. Zinc addition to zinc starved cells triggers Zrt1-dependent activation of the protein kinase A (PKA) target trehalase in *S. cerevisiae* [55]. Modulating environmental zinc levels or stimulating the PKA pathway via cyclic AMP agonists or phosphodiesterase-inhibition results in rapid mobilization of intracellular zinc pools in *C. albicans* suggesting signaling function in fungi as well [56].

In summary, both environmental and endogenous fungal pathogens face extremes in zinc availability due to the action of host nutritional immunity and must therefore possess countermeasures to deal with both micronutrient restriction and toxic zinc overload. Interestingly, of those species studied to date, each has quite different mechanisms and requirements of their zinc transport machinery for fitness and virulence making their molecular dissection in other species a fascinating field of study.

Conflict of interest statement

Nothing declared.

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