Iron and copper as virulence modulators in human fungal pathogens

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/mmi.12653

Summary:

Fungal pathogens have evolved sophisticated machinery to precisely balance the fine line between acquiring essential metals and defending against metal toxicity. Iron and copper are essential metals for many processes in both fungal pathogens and their mammalian hosts, but reduce viability when present in excess. However, during infection, the host uses these two metals differently. Fe has a longstanding history of influencing virulence in pathogenic fungi, mostly in regards to Fe acquisition. Numerous studies demonstrate the requirement of the Fe acquisition pathway of *Candida*, *Cryptococcus*, and *Aspergillus* for successful systemic infection. Fe is not free in the host, but is associated with Fe-binding proteins, leading fungi to develop mechanisms to interact with and to acquire Fe from these Fe-bound proteins. Cu is also essential for cell growth and development. Essential Cu-binding proteins include Fe transporters, superoxide dismutase (SOD), and cytochrome *c* oxidase. Although Cu acquisition plays critical roles in fungal survival in the host, recent work has revealed that Cu detoxification is extremely important. Here, we review fungal responses to altered metal conditions presented by the host, contrast the roles of Fe and Cu during infection, and outline the critical roles of fungal metal homeostasis machinery at the host-pathogen axis.

INTRODUCTION

All forms of life identified thus far utilize metals to perform essential functions. When incorporated into proteins, metals can play a plethora of structural and enzymatic roles (Kim et al., 2008a; Nevitt et al., 2012; Samanovic et al., 2012). Metal coordination clusters are structural components of many essential proteins, while the ability of metals to transfer electrons is utilized in many enzymatic processes (Kim et al., 2008a). Many metal-binding proteins are conserved along the tree of life, suggesting that metal use was an early development in the course of evolution. While many important metals exist in biology, the metals discussed in this review, iron (Fe) and copper (Cu), play pivotal roles during fungal infection (Kim et al., 2008a; Kronstad et al., 2012; Kronstad et al., 2013; Nevitt et al., 2012; Samanovic et al., 2012).

Historically, Fe has been the most well-studied metal at the host-pathogen interface (Hood and Skaar, 2012). The two common states of Fe are Fe(II) and Fe(III). Fe-sulfur (Fe-S) clusters are present in complex coordination schemes and provide structural stability for a number of proteins, provide active centers for redox reactions, are heavily conserved in energy production through various metabolic pathways, and are used by various cytochrome enzymes that transfer electrons to drive respiration (Nevitt, 2011; Sheftel et al., 2010). Furthermore, as a crucial component of hemoglobin, Fe is an essential cofactor for a plethora of enzymes and is responsible for oxygen transport and circulation in higher

multicellular organisms. The two states of Cu are Cu(I) and Cu(II). Like Fe, Cu directly participates in many vital biological processes in cells. Cu is also essential for cellular respiration, SOD activity, and melanin formation in fungal pathogens (Kim et al., 2008a; Nevitt et al., 2012; Samanovic et al., 2012). Importantly, Fe uptake is partially dependent on Cu, resulting in an interesting interplay between the two metals (Askwith et al., 1994). Cu transport in higher eukaryotes is not as well-understood as Fe transport; however; the major plasma proteins such as albumin and ceruloplasmin contain most of the Cu found in the blood (Chiarla et al., 2008). While essential for life, these metals can be detrimental to the cell if levels are not properly maintained. The Irving-Williams series shows that Fe and Cu have a high affinity to displace other metals from their cognate coordination sites, which disrupts protein function (Irving, 1948). With the most potential to de-stabilize proteins, Cu can even disrupt Fe-S clusters and render proteins inactive (Macomber and Imlay, 2009). Both Cu and Fe can participate in Fenton reactions, in which the reduced forms of the metals can react with hydrogen peroxide to form oxygen-radical species that are highly reactive in the cell. The redox activities of Fe and Cu provide favorable chemical centers for a variety of biological ligands. Switching between oxidized and reduced forms of Fe and Cu is essential for catalytic functions. Of the many Fe- or Cu-binding proteins, SOD and cytochrome *c* represent classical examples of utilization of Fe and Cu oxidation states for chemical reactions. Electrons reduce Fe(III) and Cu(II) to Fe(II) and Cu(I), respectively, on cytochrome *c* to facilitate the binding of oxygen. SOD uses Fe(II) and Cu(I) to convert O_2^- to O_2 in the presence of H^+.

Because metals are essential for life, pathogens must maintain proper metal homeostasis for survival and successful pathogenesis. This means that pathogens must be able to acquire enough metal to successfully grow and propagate, while preventing toxicity in a hostile environment. In fact, mammalian hosts use the dual essentiality/toxicity of these metals in an attempt to weaken and kill invading pathogens (Hood and Skaar, 2012; Kronstad et al., 2013; Samanovic et al., 2012). Nutritional immunity is a term that has been developed to describe the battle over nutrients between a pathogen and its host (Hood and Skaar, 2012). The sequestration of Fe from pathogens has been well-characterized, countered by robust mechanisms of metal acquisition by pathogens (Skaar, 2010). Conversely, hosts have developed ways to use Cu as an antimicrobial weapon, forcing pathogens to deal with excess Cu (Ding et al., 2013; Hood and Skaar, 2012; Samanovic et al., 2012).

In this review, we focus on the role of Fe and Cu in the context of infections by three widely studied human fungal pathogens, *Candida albicans*, *Cryptococcus neoformans,* and *Aspergillus fumigatus*, and explore how these fungi have developed ways to either acquire sequestered Fe, or resist the toxicity of Cu. All of these fungi commonly infect immunocompromised individuals usually due to AIDS, organ transplantation, or immunosuppressive cancer therapies. However, these organisms experience different environmental niches, whether in the environment, as a commensal organism, or during infection, which results in complex and different metal requirements during different phases of growth. Together, these fungal pathogens account for a large burden of infection in immunocompromised individuals, and more efficacious treatments must be

developed. By understanding the theme of metal homeostasis in these organisms, we may identify new drug targets or strategies to fight these pathogens and skew the balance in favor of the host.

Iron and pathogenic fungi

While the niches and lifestyles of these three fungi differ, the strategy to sequester Fe ions employed by the host is similar, which is indicative of the typical response of the host to fungal pathogens. *C. albicans C. neoformans* and *A. fumigatus* typically face low Fe levels in the host (Amich et al., 2013; Jung and Do, 2013; Kronstad et al., 2013; Moore, 2013; Saikia et al., 2014; Schrettl and Haas, 2011; Seifert et al., 2008). *C. albicans,* a commensal organism that can colonize the high-Fe environment of the gastrointestinal (GI) tract, faces low Fe found in the bloodstream and tissues when progressing to infection (Chen et al., 2011). During infection, fungal cell intrusion leads to the sequestration of Fe by the host in an attempt to further starve invading pathogens (Ganz, 2009). Fe sequestration may occur in the phagosomal compartment through the action of metal transporters (Ganz, 2009) or binding by Fe-containing proteins in the bloodstream. A role for Fe excretion in pathogenic fungi has not been identified, which supports the hypothesis that Fe is withheld during infection and points to Fe uptake as a major player in infection.

Much of what is known about Fe acquisition in fungi was first studied in *Saccharomyces cerevisiae*, however pathogenic fungi utilize very distinct methods in their mechanisms of Fe acquisition, including uptake Fe from ferritin, hemoglobin or via siderophores. Fe uptake mechanisms consist of reductive and nonreductive pathways. The high-affinity reductive Fe uptake pathway is used to

import oxidized Fe(III) (as found in ferritin and transferrin), which is reduced to soluble Fe(II) by Fe reductases (the Fre family of proteins) and then imported by a complex of Fe permease (Ftr) and multicopper ferroxidase (Fet) proteins. The Ftr and Fet proteins are inextricably tied together, and are required for mutual stability at the cell membrane. In *S. cerevisiae* and other organisms, Ftr1 will only transport oxidized Fe directly delivered from Fet3. Fet3 binds Cu, which is delivered through Ccc2. In *C. albicans*, Ftr1 is essential for murine bloodstream infections, suggesting an important role in Fe uptake during infection (Ramanan and Wang, 2000). However, Disrupting Fet3 or Ccc2 showed no significant change in fungal virulence in mice (Eck et al., 1999; Weissman et al., 2002). *Cryptococcus neoformans* utilizes the small molecule 3-hydroxyanthranilic acid (3HAA), as well as melanin, to reduce Fe (Nyhus et al., 1997). Reduction by 3HAA and melanin are not mutually exclusive: 3HAA accounts for nearly 50% of the Fre activity (Nyhus et al., 1997). A strong connection exists between melanin formation and Fe acquisition (Coulanges et al., 1997; Howard, 1999). In *C. neoformans*, the phenoloxidase for melanin biosynthesis is regulated by Fe levels (Jacobson and Compton, 1996; Polacheck et al., 1982). In fact, many genes involved in melanin formation are required for Fe uptake, such as *LAC1*, *ATX1,* and *CCC2* (Nyhus et al., 1997; Walton et al., 2005). Furthermore, genes involved in Fe transcriptional regulation (*CIR1*), reductive Fe uptake (*FRE4*), and siderophore-mediated Fe acquisition (*SIT1*) are influencers of *Cryptococcus* melanin formation (Jung et al., 2006; Saikia et al., 2014; Tangen et al., 2007). The *SIT1* deletion mutant induces melanin formation by effecting protein kinase A (Tangen et al., 2007), and Cir1 is known to regulate a range of genes important

for melanin formation (Jung et al., 2006). While no direct reports have been made on the roles of *Aspergillus* melanin in Fe acquisition, it has been shown that a master regulator of secondary metabolites (*laeA*) co-regulates expression of *sidE* and fungal pigmentation (Bok and Keller, 2004; Perrin et al., 2007). However, a recent study demonstrated that *sidE* is not involved in siderophore-mediated Fe homeostasis (Steinchen et al., 2013).

Multiple potential orthologs of Fre proteins exist in fungi, suggesting a complex and possibly redundant network of these proteins. *Candida* Cfl1 was found to regulate Fe reduction, oxidative stress resistance, and virulence (Xu et al., 2014). An important recent study has characterized eight *Cryptococcus* Fre proteins (Fre1, Fre2, Fre3, Fre4, Fre5, Fre6, Fre7, and Fre201) (Saikia et al., 2014). Expression of the corresponding Fre genes is regulated by Fe and Cu, reinforcing the cross talk between Fe and Cu homeostasis. Of these reductases, Fre2 is involved in the utilization of Fe from heme and transferrin, and participates in fungal virulence. Fre4 is associated with melanin production (Saikia et al., 2014). In addition to Fre proteins, the main permeases and ferroxidase of *Cryptococcus neoformans* that play a role in virulence are Cft1 and Cft2, and Cfo1, respectively (Jung et al., 2009). In *Aspergillus*, the reductive Fe assimilation system plays a very important role in high-affinity Fe uptake. The Fe assimilation system consists of FreB (ferric reductase), FetC (ferroxidases) and FtrA (Fe permease) (Blatzer et al., 2011; Haas, 2012; Schrettl et al., 2004). Unlike the Fe permease from *Candida* and *Cryptococcus*, *Aspergillus* FtrA was found to be dispensable for fungal virulence, in the presence of the siderophore-mediated Fe uptake system (Schrettl et al., 2004; Schrettl et al., 2007). In *S.*

cerevisiae, Ccc1 (CccA in *A. fumigatus*) transports intracellular Fe into the vacuolar space for storage and detoxification (Gsaller et al., 2012; Li et al., 2001). When Fe is low, vacuolar storage Fe is released by the Fe permease-oxidase complex (Fth1-Fet5) into the cytosol space (Cheng et al., 2013; Urbanowski and Piper, 1999). The functions of Ccc1 and Fth1-Fet5 complex in *C. albicans* and *C. neoformans* in mediating Fe homeostasis have not been revealed.

Besides reductive Fe uptake machinery, pathogenic fungi developed nonreductive Fe acquisition, that involves transportation of Fe-binding proteins (hemoglobin, ferritin or transferrin) or siderophores. *C. albicans* expresses receptors for hemoglobin (Rbt5), ferritin (Als3), and an unknown transferrin receptor (Almeida et al., 2008; Knight et al., 2005; Weissman and Kornitzer, 2004). The uptake of Fe from ferritin appears to be specific to hyphae, is an important early developmental step to evade killing by the host, and suggests a possible role for Fe in hyphal growth (Knight et al., 2005). Fe from ferritin must be reduced by Fre proteins as mentioned above. As the sole Fe source in vitro, transferrin is able to support the growth of *C. albicans*, and Fe uptake from this source requires cell contact with transferrin, as well as reduced Fe, fed through the high-affinity uptake system. However, a transferrin receptor remains unidentified in *C. albicans*. A third common source of Fe to *C. albicans* is hemoglobin released from blood erythrocytes by *C. albicans'* hemolytic activity (Manns et al., 1994). Uptake of this source of Fe is mediated through the Rbt5 receptor, and Fe is subsequently released by the heme oxygenase Hmx1 (Navarathna and Roberts, 2010; Weissman and Kornitzer, 2004). Interestingly, the Rbt5 protein is highly conserved in nearly all *Candida* species, but absent

from nonpathogenic fungi, such as *Saccharomyces cerevisiae* (Ding et al., 2011a). Deletion of *ALS3* and *RBT5* led to decreased virulence in an oral epithelial infection model (Navarathna and Roberts, 2010). These three Fe inputs may possibly compensate for each other in various modes of infection, making it difficult to parse out specific roles during infection. *Cryptococcus* has been shown to house machinery capable of extracting Fe from heme, while *Aspergillus* appears to be unable to acquire heme iron (Cadieux et al., 2013; Schrettl et al., 2004). However, no specific receptors have been identified in *Cryptococcus*. Therefore, the source of Fe moving through the high-affinity uptake pathway for these two organisms remains elusive, which is possibly due to redundancy among systems.

While one commonality between these three fungi is their use of siderophores to acquire Fe, only the *Aspergillus* siderophore uptake system is involved in fungal virulence (Seifert et al., 2008), whereas *C. albicans* siderophore uptake is involved in infecting human oral mucosa, and the *C. neoformans* siderophore transporter plays no function in the host (Heymann et al., 2002; Tangen et al., 2007). *A. fumigatus* is able to produce, secrete, and take up its own siderophores. The siderophores Fusarinine C (FsC) and triacetylfusarinine C (TAFC) are excreted from the fungus, and upon coordinating Fe, TAFC is taken up by the siderophore-Fe transporter mirB (Haas et al., 2003; Raymond-Bouchard et al., 2012). Ferricrocin and hydroxyferricrocin are intracellular-producing siderophores that are important for sexual development and conidiation by their function of reducing the Fe in the conidia, leaving them more susceptible to host insults that require Fe-associated proteins to resist,

such as the protection against oxidative stress by catalase A (Eisendle et al., 2006; Schrettl et al., 2007; Wallner et al., 2009). To accomplish full attenuation of virulence, both extracellular and intracellular siderophores must be inactivated, by the disruption of *sid*A, which catalyzes the first step reaction for both types of siderophores. Partial fungal virulence is attenuated when genes involved in siderophore biosynthesis are disrupted, including *sidI, sidH, sidF,* or *sidD* for extracellular, as well as *sidC* for intracellular, siderophore production (Hissen et al., 2005; Schrettl et al., 2004; Schrettl et al., 2007). *C. neoformans* and *C. albicans* both encode SIT homologues, but these two fungi appear to have taken an energetically more favorable route and do not produce their own siderophores. Mutation of Sit1 in either organism, however, does not have a significant affect on virulence and only affects epithelial cell invasion in *C. albicans* (Heymann et al., 2002; Tangen et al., 2007). This may be due to the activation of other Fe acquisition machineries, which compensates for Fe uptake from siderophores. Furthermore, by scavenging siderophores, successful infections by these pathogens may be tied to the presence of other siderophore-producing organisms that are found in the environment and the host. Given that pathogenic fungi are widely distributed in the environment, it is possible that siderophore-mediated Fe uptake is critical for *C. neoformans* and *C. albicans* when colonizing outside the host or in different host niches. Furthermore, immunocompromised hosts may have a higher propensity for co-infection, with a different opportunistic pathogen producing optimal siderophores suited for *C. neoformans* or *C. albicans* uptake. However, co-infection experiments have rarely been performed.

As a possible exception to the common requirement for Fe uptake in the host, commensal *C. albicans* must resist Fe toxicity in the gut (Chen et al., 2011). A transcription factor, Sfu1, is a repressor for Fe uptake genes, including genes encoding Fre proteins, high-affinity Ftr proteins, siderophore transporters, and heme receptors. The gut is an iron-rich environment. As a commensal fungus, *C. albicans* dramatically induces expression of *SFU1* to systemically shut down all aspects of Fe uptake machinery to overcome intracellular Fe toxicity. This allows for colonization that can, in turn, lead to a later infection. Homologs of *Candida* Sfu1 were identified in *Aspergillus* (SreA) and *Cryptococcus* (Cir1) (Jung et al., 2006; Schrettl et al., 2008). The SreA-mediated Fe regulation in *Aspergillus* resembles that of Sfu1 in *Candida*. However, the disruption of *sreA* accumulates intracellular Fe contents, while the mutant does not affect *Aspergillus* virulence in mice. In addition, *Aspergillus* HapX regulates Fe starvation and virulence (Schrettl et al., 2010). *Cryptococcus* Cir1 was demonstrated to act as both repressor and activator in Fe regulation, as well as an important virulence modulator in mice, which clearly indicates the differential regulation of Fe homeostasis among the three pathogens. *Cryptococcus* HapX has been demonstrated to influence Fe regulation and virulence (Jung et al., 2010). *C. albicans* Hap43 (a homolog of HapX) is repressed by Sfu1 and required for low Fe cell growth and virulence (Baek et al., 2008). It has been shown that Cth1 and Cth2 regulate Fe homeostasis regulators in *S. cerevisiae,* by modulating RNA stability under Fe deficiency (Puig et al., 2005; Puig et al., 2008). However, this Fe regulation mechanism has never investigated in pathogenic fungi.

Copper and pathogenic fungi

Many fungal Cu-binding proteins are important for virulence, including SOD, cytochrome oxidase, laccase, and many others, suggesting that some Cu acquisition is needed for infection (Kim et al., 2008a; Nevitt et al., 2012; Samanovic et al., 2012). Moreover, it has been implied that Cu is capable of influencing hyphal formation in *C. albicans* (Marvin et al., 2003). In the environment, Cu is required for competent mating and capsule production by *C. neoformans* (Lin et al., 2006; Williamson, 1994). Importantly, Cu mediates Fe uptake via a high-affinity Cu-dependent Fe transporter, Fet3, tightly tying together roles for Cu and Fe (Askwith et al., 1994). However, in contrast to Fe, host Cu levels are typically increased in response to infection and inflammation in what is thought to be an attempt to eradicate pathogens (Ding et al., 2013; Samanovic et al., 2012; Wagner et al., 2005; White et al., 2009).

Cu homeostasis research in the model yeast *S. cerevisiae* has facilitated our understanding for Cu acquisition and utilization in human pathogenic fungi (Puig and Thiele, 2002; Rees and Thiele, 2004). Given that Cu widely exists as Cu(II), cells employ two ferric/cupric reductases (Fre1 and Fre2) involved in Cu reduction. Reduced Cu is then transported by three high-affinity transporters (Ctr1, Ctr2, and Ctr3). While Ctr1 and Ctr3 are localized on the plasma membrane, and acquire Cu from the environment, Ctr2 is found on the vacuolar membrane and pumps Cu into the cytosol (Pena et al., 2000; Rees et al., 2004; Rees and Thiele, 2007). The Ctr transporter family is highly conserved across all fungal species. However, the fact that some fungi have lost one or two copies of genes encoding Ctr proteins is curious (Ding et al., 2011b). Phylogenetic analysis implies that the loss of *CTR3* is a common phenomenon among other Cu

transporter genes in the Baidiomycota, Zygomycota, and Saccharomycotina lineages (Ding et al., 2011b). For example, *S. cerevisiae* Ctr1 and Ctr3 are known to be independent and redundant Cu transporters. In *C. albicans*, deleting Ctr1 leads to cell growth defects under both Cu- and Fe- deficient conditions, giving rise to mitochondrial respiratory defects, and resembling *ctr1Δ ctr3Δ* cells in *S. cerevisiae*. Very interestingly, gene dosage of *CTR1* is important for hyphal formation in *C. albicans*, as loss of one or both alleles leads to hyperfilamentation on non-hyphal-inducing conditions (YPD agar) (Marvin et al., 2003).

C. neoformans encodes two functional Cu starvation inducible Cu-transporters that are functionally redundant in conditions tested (Ding et al., 2013). These two Cu transporters functionally compensate for Cu uptake for melanin formation (Chun and Madhani, 2010; Ding et al., 2011b; Waterman et al., 2012). The promoter sequence from *CTR4*, a homolog of *CTR3* from *S. cerevisiae*, was first applied as an inducible overexpression system in *C. neoformans* (Ory et al., 2004). Initiation of target gene transcription by the *CTR4* promoter is regulated by Cu concentration, resembling that from *S. cerevisiae* (Ding et al., 2013; Ding et al., 2011b; Ory et al., 2004; Wang et al., 2012; Waterman et al., 2007). Recent studies extensively demonstrated that, during cryptococcal pulmonary infection, fungal cells sense a gradual elevation of Cu in the environment and the activity of the *CTR4* promoter remains constant (Ding et al., 2013). Although *C.* encounters elevated Cu levels in the lung, *CTR4* is still expressed, indicating a potential function of *CTR4* in virulence (Ding et al., 2013; O'Meara et al., 2014; Waterman et al., 2012). Many interesting studies have been conducted recently, demonstrating that low Cu induces *Cryptococcus* cell

size, and an unexpected nutrition-dependent phenotype of *CTR4* was described (Raja et al., 2013; Waterman et al., 2012). However, the size enlargement in the presence of BCS was not observed in previous studies (Waterman et al., 2007; Waterman et al., 2012).

In contrast to the Ctr-family importers, Ccc2 is a highly conserved P-type ATPase Cu exporter that is conserved from bacteria to mammals and delivers Cu through the hydrolysis of ATP (Cankorur-Cetinkaya et al., 2013; Fu et al., 1995; Hung et al., 1997; Walton et al., 2005). The protein receives Cu from the Cu chaperone, Atx1, via a direct protein-protein interaction, and pumps Cu into the late- or post-Golgi compartment to load Cu into an Fe transporter (Fet3) and, presumably, other Cu-dependent proteins (Kim et al., 2008a; Nevitt et al., 2012). Disruption of *CCC2* in *C. albicans* has no effect on fungal pathogenicity in the animal model, which suggests a complimentary pathway for Cu loading in these mutants (Weissman et al., 2000). However, in *Cryptococcus neoformans,* deleting either Atx1 or Ccc2 significantly impairs melanin formation, and potentially influences fungal virulence (Walton et al., 2005).

Although Cu acquisition machinery in *Aspergillus* species has not been fully investigated, phylogenetic analysis and homology searches indicate that *Aspergillus* genomes contains homologs of Ctr proteins, Ccc2, and Atx1 (Ding et al., 2011b; Upadhyay et al., 2013). Recently, Upadhyay et al. demonstrated that the enzymatic activity of DHN melanin laccases (Abr1 and Abr2) from *Aspergillus fumigatus* requires Cu, resembling that from *Cryptococcus neoformans*. Deleting either *abr*1 or *abr2* significantly reduces DHN melanin formation in *Aspergillus*

fumigatus. However, the expression of laccase in *Aspergillus fumigatus* and *Cryptococcus neoformans* are regulated by distinct mechanisms. Expression of *LAC1* from *Cryptococcus neoformans* is induced by Cu, but the induction of *abr1* and *abr2* occurs in low Cu concentration (Jiang et al., 2009). In addition, expression of *LAC1* is regulated by the master Cu homeostasis regulator (Cuf1), whereas expression of *abr1* and *abr2* is positively controlled during conidiophore development via BrlA. In the same study, an *Aspergillus* Cu transporter, CtpA, was identified. Disruption of CtpA impairs DHN melanin formation, though the mutant shows increased pathogenicity in the *Galleria mellonella* model (Upadhyay et al., 2013).

Intracellular Cu is utilized by Cu/zinc (Zn) SODs (Kim et al., 2008a). The delivery of Cu to SODs is accomplished by the Cu chaperone Ccs1. Cu-containing SODs become active and strong antioxidant agents to convert superoxide into O_2 and H_2O_2. SODs were found to be an important virulence factor in nearly all pathogenic fungi (Cox et al., 2003; Hwang et al., 2002; Lambou et al., 2010; Narasipura et al., 2003). SOD1 from *C. albicans* and *C. neoformans* directly participates in fungal pathogenicity. The killing of SOD1-mutant cells by macrophages is enhanced in vitro, while fungal virulence is greatly attenuated in vivo (Cox et al., 2003; Narasipura et al., 2003). Disruption of *C. neoformans* SOD1 leads to decreased expression of many *Cryptococcus*-specific virulence factors, including laccase, urease, and phospholipase (Cox et al., 2003). The *C. albicans* genome contains six genes encoding SOD proteins (SOD1 to SOD6), of which SOD1, SOD4 and SOD6 are Cu/Zn SODs (Frohner et al., 2009; Gleason et al., 2013; Martchenko et al., 2004), whereas SOD5 is a Cu-

only SOD (Gleason et al., 2014). While SOD1 is a cytosolic protein, SOD4, SOD5, and SOD6 were found to localize on the cell surface via GPI anchors (Frohner et al., 2009). Upon encountering superoxide stresses, such as NAPDH oxidase-mediated O_2^- species generation, predominant antioxidant proteins SOD4 and SOD5 rapidly break down O_2^- on cell surfaces. Holdom and coworkers purified Cu/Zn SOD in *A. fumigatus*, and demonstrated that universal SOD inhibitors were able to abolish the activity of purified protein (Holdom et al., 1996). Furthermore, SODs were found in cell culture supernatant, implying the secretion of SODs by *Aspergillus* cells. In agreement with this observation, SODs from *A. fumigatus* are recognized by infected human sera, serving mainly as antigens for IgA (Hamilton et al., 1995; Holdom et al., 2000). Given that three *C. albicans* SOD proteins were found extracellularly linked by GPI anchors, experiments to test whether these proteins are released during infection are of interest.

One of the primary functions of Cu is to mediate Fe acquisition. Deletion of Cu transporters has been shown to cause reduction of cell growth under Fe-deficient environments (Askwith et al., 1994), because the activity of high affinity Fe transporter (Fet3) depends upon Cu. In fact, *C. albicans* and *C. neoformans* mutants that harbor defects in Cu homeostasis show severely reduced cell growth in the presence of Fe chelators (Ding et al., 2011b; Marvin et al., 2003; Walton et al., 2005). In *A. fumigatus*, inactivation of *sidA* causes cell growth defects in the presence of Cu or Fe chelators (Blatzer et al., 2011). Expression of genes encoding Cu-binding proteins is regulated by Fe homeostasis. *CCC2*, *CTR2,* and *ATX1* are transcriptionally activated by Fe chelation and *CCC2* or *ATX1* knockout strains in *S. cerevisiae, C. albicans,* and *C. neoformans* all

demonstrate Fe deficiency growth sensitivities (Walton et al., 2005; Weissman et al., 2002). Given that the host creates Fe limitation conditions when fungal infection is detected, the tightly linked relationship between Fe and Cu homeostasis indicates that Cu homeostasis may play an important role at the host-pathogen axis. Recent works demonstrated that *C. neoformans CTR4* is expressed in lung tissue, and the level of *CTR4* RNA isolated from fungal cells recovered from the lung is induced 1.3-fold over that under normal cell growth conditions (YPD) (Ding et al., 2011b; O'Meara et al., 2014; Waterman et al., 2012). Cells harboring a *CTR4* deletion using an auxotrophic marker (*URA5*) demonstrated an auxotrophic phenotype, and such mutants exhibit less fungal virulence in the host (Waterman et al., 2012). How *CTR4* influences nutrition acquisition in *Cryptococcus neoformans* is still unclear.

Despite the important roles of Cu in fungal biology, excess Cu is highly toxic to the cell. Recent work has demonstrated that the host employs Cu for antimicrobial purposes. An *Escherichia coli* Cu exporter mutant is more sensitive to macrophage killing, while proteins that respond to high Cu are required for *Mycobacterium tuberculosis* virulence (White et al., 2009; Wolschendorf et al., 2011). Furthermore, *Yersinia* species have shown the ability to secrete the siderophore yersiniabactin to protect from host Cu during infection (Chaturvedi et al., 2012). In line with the evidence for Cu overload during bacterial infections, *Cryptococcus neoformans* senses high Cu stresses by inducing expression of metallothioneins (MTs), but not the Ctr4 transporter, during pulmonary infection in mice (Ding et al., 2013).

MTs are ubiquitous Cu detoxification proteins found in organisms from prokaryotic to mammalian cells. The critical component for MT function is cysteine residues that coordinate multiple Cu ions (Babula et al., 2012; Palacios et al., 2011). Fungal cells detoxify Cu through the binding of Cu via Cys-thiolate bonds Expression of fungal MTs is specifically induced by high Cu conditions (Ding et al., 2013; Ding et al., 2011b; Szczypka and Thiele, 1989; Thiele, 1988). *S. cerevisiae*, *C. albicans,* and *C. neoformans* contain two redundant MTs, respectively (Ding et al., 2013; Ding et al., 2011b; Oh et al., 1999; Riggle and Kumamoto, 2000). While MTs from *S. cerevisiae* and *C. albicans* are small cysteine-rich proteins, *C. neoformans* MTs are much larger proteins. Mutation of both *C. neoformans* MT genes demonstrated attenuated virulence during pulmonary colonization (Ding et al., 2013). Using live animal imaging techniques, *C. neoformans* MT promoter-luciferase was shown to be steadily induced in luciferase activity as infection developed up to 14 days in the lung, while a low Cu responsive reporter (*CTR4* promoter-luciferase) remained low and constant. These results indicate that fungal cells encounter a high Cu environment in the primary site of infection. Further analysis indicated that the Cu-binding capacity of MTs is essential for fungal survival in the lung (Ding et al., 2013). Although *C. neoformans* detoxification serves as a critical virulence factor in the lung, Cu acquisition machinery may demonstrate important functions in other tissues. For example, in situ hybridization analysis indicates the presence of *CTR4* expression in the brain (Waterman et al., 2007). MTs have been described in *Candida* and *Aspergillus*, but the role of these proteins during infection has not been elucidated (Goetghebeur and Kermasha, 1996; Goetghebeur et al., 1995).

While MTs appear to be the major players in Cu resistance in *C. neoformans*, Cu export is utilized by *C. albicans*. Weissman and coworkers discovered a P-type ATPase (Crp1) as a plasma membrane-localized Cu exporter in *C. albicans* (Weissman et al., 2000). Crp1 contains GMXCXXC and CXXC consensus motifs that receive free Cu or fetch chaperone-bound Cu and that pump Cu extracellularly through the transmembrane channel. *CRP1* is transcriptionally up-regulated by high Cu conditions, and a *crp1* mutant shows massive intracellular Cu accumulation and a Cu-sensitive growth phenotype. It must be mentioned that *CRP1* also appears to be unique to *Candida,* which implies that pathogenic fungi have evolved differently to manage Cu toxicity. Another *Candida albicans* membrane protein that phenotypically resembles that of Crp1 is the integral membrane protein, Sur7 (Douglas et al., 2012). *SUR7* mutant cells demonstrated growth sensitivity in media containing a high Cu concentration. Fungal virulence of the mutant is greatly impaired; however, the contribution of Sur7 to fungal virulence may be due to many factors. In addition to the reduction of cell growth on in high Cu, *SUR7* mutants also show an enhanced phagocytic ratio and cell growth defects in the presence of H_2O_2, diamide, and menadione. Sur7 is a conserved protein in *Aspergillus* species, but a role for Sur7 as a virulence factor has not been revealed in these species.

Other metals and pathogenic fungi

Despite the important roles of Fe and Cu in fungal virulence, other metals, such as Zn and Mn, also demonstrate essential functions. Numerous biological processes require Zn and Mn. In particular, Zn is critical for the regulation of gene

474 expression. Approximately 44% of transcriptional factors are Zn-dependent
475 proteins, and 50% of eukaryotic proteins are Zn-binding proteins (Hood and
476 Skaar, 2012). Zn and Mn are involved in the activity of SODs to protect fungal
477 cells from oxidative stresses (Hwang et al., 2003; Lamarre et al., 2001).
478 Interestingly, Mn has been demonstrated to regulate hyphal formation in fungi
479 (Asleson et al., 2000). During pathogenic invasions, Zn and Mn levels are
480 extremely low in the host due to the efficient chelation of these metals by immune
481 cells (Corbin et al., 2008; Hood and Skaar, 2012). Following phagocytosis by
482 macrophages, NRAMP1 is expressed on the phagosomal membrane and
483 mediates Fe and Mn export from the phagosomal compartment. Furthermore, in
484 response to infection, neutrophils release calprotectin to chelate Zn and Mn
485 (Corbin et al., 2008). In *S. cerevisiae*, Zn homeostasis is regulated by a
486 transcription factor, Zap1, which is found to repress genes responsible for Zn
487 uptake (Zhao et al., 1998). Homologs of Zap1 have been identified in *Candida*,
488 *Cryptococcus,* and *Aspergillus* species, named Csr1/Zap1, Zap1, and ZafA. The
489 disruption of ZafA or Zap1 in *Aspergillus* or *Cryptococcus* leads to attenuation of
490 fungal virulence in mice (Kim et al., 2008b; Moreno et al., 2007). *Candida* Zap1
491 controls extracellular matrix production during biofilm formation (Nobile et al.,
492 2009). Recently, a Zn scavenger protein (zincophore), Pra1, was identified, and
493 is secreted during endothelial invasion to obtain Zn in *Candida albicans*. A
494 homolog for *C. albicans* Pra1 was found in *Aspergillus* but not in *C. neoformans*
495 (Citiulo et al., 2012).

496 Given the importance of Zn in life, Zn acquisition is predicted to be essential
497 during systemic infection in pathogenic fungi. However, unlike Fe and Cu

transporters, Zn transporters are poorly investigated in human pathogenic fungi. ZrfA and ZrfB transporters have been identified in *Aspergillus*, with the ability to absorb Zn in acidic Zn-deficient conditions, whereas ZrfC mediates Zn uptake in conditions of high pH (Amich et al., 2010; Amich et al., 2014). Genome searches in *Candida* and *Cryptococcus* clearly reveal the presence of Zn transporter homologs, but their functions have not been studied. Interestingly, the *Cryptococcus* Fe regulator, Cir1, has been shown to regulate a Zn transporter Zrt2 (Jung et al., 2006).

Conclusion:

Fungal metal homeostasis is no doubt one of the most important virulence modulators. Although the host employs Fe/Zn/Mn chelation and Cu-releasing strategies to minimize fungal replication and pathogenicity, three major human pathogenic fungi have evolved efficient but distinct methods to counter metal-mediated killing. The ultimate goal behind the different methods for managing metal stresses for these fungi is the same: to maximize Fe acquisition and neutralize Cu toxicity. Fungal cells possess multiple mechanisms to obtain Fe; these include transport of Fe from heme and ferritin, and extracting Fe from siderophores. Drug development targeted to specifically prevention of Fe uptake by pathogens is a promising direction. Conversely, by boosting Cu delivery to pathogens, novel therapies may be developed. In fact, Cu has historically been used as an anti-microbial agent. More recently, Cu surfaces in healthcare settings has been proposed to dramatically reduce nosocomial infections, while Cu-binding molecules have been used to treat superficial fungal infections of the

skin. Despite Cu detoxification being essential for fungal virulence, Cu acquisition clearly plays an important role in numerous biological processes. Further studies regarding metal homeostasis in host niches are critical for understanding fungal virulence. Future work in the field of metal homeostasis in pathogenic fungi will help us to decipher the mechanism of fungal infection at the host-pathogen axis and, most importantly, to develop potential therapies.

Figure legends:

Figure 1: Iron homeostasis in *Saccharomyces cerevisiae* and major human pathogenic fungi

This figure illustrates main proteins involved in iron (Fe) uptake in four fungal species. Experimentally characterized proteins are labeled in black, whereas uncharacterized proteins with homologs in other fungal cells are labeled in red, and corresponding gene identifications (IDs) are included for *C. albicans*, *C. neoformans*, and *A. fumigatus*. Fe^{3+} is reduced to Fe^{2+} by highly conserved Fe reductase (Fre) proteins on the cell surface. Fre4 in *Cryptococcus neoformans* is involved in melanin formation (Saikia et al., 2014). *Aspergillus* FreB is a homolog of Fre proteins from other fungi, and serves as a reductase for ferric ion (Blatzer et al., 2011). Fe^{2+} is then bought into cells through the high-affinity iron transporter complex Ftr1/Fet3 (Cft1/Cfo1 in *Cryptococcus* and FtrA/FetC in *Aspergillus*) (Blatzer et al., 2011; Haas, 2012; Jung et al., 2009; Schrettl et al.,

2004). Intracellular Fe is pumped by Ccc1 (CccA in *A. fumigatus* and orf19.6948 in *C. albicans*) into vacuoles for storage (Gsaller et al., 2012; Li et al., 2001). Ccc1 (CNAG_05154) in *Cryptococcus neoformans* remains uncharacterized. Upon the requirement of Fe, Fth1 (orf19.4802 in *Candida albicans*, CNAG_02959 in *Cryptococcus neoformans*, and no homolog in *Aspergillus fumigatus*) and Fet5 (CNAG_02958 in *Cryptococcus neoformans* and no homolog in *Aspergillus fumigatus*) complexes release Fe into the cytosolic space from vacuoles (Cheng et al., 2013; Urbanowski and Piper, 1999). The function of Fet3 and Fet5 depends upon Cu (Nevitt, 2011). *Cryptococcus* Fre2 and Fre4 are involved in uptake of Fe from heme and melanin formation, respectively (Saikia et al., 2014).

Although the main Fe acquisition pathway is highly conserved in all four fungi, pathogenic fungi are different in many circumstances. *Candida* and *Cryptococcus* are able to utilize heme and ferritin, respectively, as a sole Fe source, whereas *Aspergillus* cannot (Cadieux et al., 2013; Navarathna and Roberts, 2010; Schrettl et al., 2004; Weissman and Kornitzer, 2004). In *Candida albicans*, Rbt5/Rbt51 are heme transporters (Weissman and Kornitzer, 2004). Heme is brought into cells by endocytosis, and is then delivered into the vacuoles. This process is mediated by the ESCRT pathway (Weissman et al., 2008). *Candida albicans* Als3 is a hyphal-expressed protein and is responsible for ferritin uptake (Almeida et al., 2008). *Cryptococcus* can also use heme and ferritin as a source of Fe, though respective receptors have not been identified (Almeida et al., 2008; Jung et al., 2010; Jung et al., 2008). Heme is thought to bind by a receptor protein, and the binding requires Cig1 (Cadieux et al., 2013). Similar to that of *Candida*, the heme utilization process in *Cryptococcus* requires

the ESCRT pathway and Vps23 (Hu et al., 2013). Melanin and 3-hydroxyanthranilic acid (3-HAA) from *Cryptococcus* can serve as Fe reductants on the cell surface (Nyhus et al., 1997).

Different from *Candida* and *Cryptococcus*, *Aspergillus* can produce intra- and extracellular siderophores. Non-siderophore producing fungi can extract Fe from siderophores, and the recognition and binding of siderophores are through Sit1 and Arn proteins in *Saccharomyces* and *Candida*, and Sit1 in *Cryptococcus* (Heymann et al., 2002; Tangen et al., 2007). *Aspergillus* siderophore transporters mirA and mirB deliver heterologous siderophores and native siderophores, respectively (Haas et al., 2003).

Saccharomyces species utilize Aft1/Aft2 to regulate Fe acquisition processes (Nevitt, 2011). *C. albicans* Sef1 positively regulates expression of Fe acquisition, and the activity of Sef1 relies on phosphorylation by Ssn3. Sfu1 is a repressor, which can block the binding of Sef1 to the promoter region and facilitate the degradation of Sef1 (Chen and Noble, 2012). In *Cryptococcus*, Cir1 is a major regulator of Fe transporter expression, and acts as a repressor and activator in Fe regulation (Jung et al., 2006). *Aspergillus* HapX is required for Fe deficiency adaptation and fungal proliferation in the host, and *Cryptococcus* HapX has a small effect on fungal virulence (Jung et al., 2010; Schrettl et al., 2010). *C. albicans* Hap43 is repressed by Sfu1 and required for iron uptake and virulence (Baek et al., 2008). In *Saccharomyces*, once cells encounter Fe deficiency, two CCCH Zn finger proteins (Cth1 and Cth2) are expressed to specifically degrade RNA molecules encoding proteins involved in Fe-dependent

pathways, but the Cth1/Cth2 mechanism has not been revealed in other pathogenic fungi (Puig et al., 2005; Puig et al., 2008).

Figure 2: Cu homeostasis in *Saccharomyces cerevisiae* and major human pathogenic fungi

In general, fungal Cu acquisition is highly conserved. Environmental Cu^{2+} is converted by Fre proteins to generate Cu^{+}, which is easily taken up by the high affinity Cu transporters Ctr1 and Ctr3 (Samanovic et al., 2012). In *Candida* and *Aspergillus*, only one Cu transporter has been identified to date (Marvin et al., 2003; Upadhyay et al., 2013). Vacuolar Cu is pumped into cytosol by Ctr2 (Rees and Thiele, 2007). Although homologs of Ctr2 are present in the genome of *C. albicans* (orf19.4720), *C. neoformans* (CNAG_01872) and *A. fumugatus* (AFUB_040930), the function of Ctr2 remain unknown. It has been demonstrated that intracellular Cu is delivered to Sod1 by Ccs1 in *S. cerevisiae* and *Candida albicans* (Culotta et al., 1997; Gleason et al., 2013). The delivery of Cu to Ccc2 by Atx1 was only demonstrated in *S. cerevisiae* and *C. neoformans* (Walton et al., 2005). Overload Cu is chelated by metallothioneins (MTs) (Cup1/Crs5 in *Saccharomyces*, Cup1/Crd2 in *Candida*, and Cmt1/Cmt2 in *Cryptococcus*) (Ding et al., 2013; Ding et al., 2011b; Oh et al., 1999; Riggle and Kumamoto, 2000). *Aspergillus* species have been shown to possess MTs and SODs, but corresponding genes have not been identified (Goetghebeur and Kermasha, 1996; Goetghebeur et al., 1995; Holdom et al., 1996). Sur7 has been identified as a Cu detoxification protein on the cell surface in *C. albicans* (Douglas et al.,

2012). Homologs of Sur7 are present in the genome of *S. cerevisiae* and *A. fumigatus* (AFUB_019400), but their functions in detoxifying Cu have not been revealed. While only one SOD protein has been reported in *Cryptococcus*, *Candida* SOD4/SOD5/SOD6 are GPI-anchored proteins, whereas SODs from *Aspergillus* are found to be secreted proteins (Cox et al., 2003; Frohner et al., 2009; Gleason et al., 2013; Martchenko et al., 2004). In *Candida* species, Cu detoxification is slightly complicated, involved in using P-type ATPase, Crp1, as a Cu exporter (Weissman et al., 2000). *Cryptococcus* and *Aspergillus* utilize Cu to produce melanin. However, *Aspergillus* melanin producers (Abr1 and Abr2) are found to be hyphal-associated proteins (Upadhyay et al., 2013). Expression of Cu acquisition genes is regulated by Mac1 in *Saccharomyces* and *Candida*, and expression of Cu detoxification genes is mediated by Ace1 in *S. cerevisiae*(Thorvaldsen et al., 1993). A putative gene (*CUP1*) was identified in the genome of *C. albicans*, but remains uninvestigated. *Cryptococcus* Cu homeostasis is quite different, as Cuf1 regulates both Cu detoxification and acquisition via unknown mechanisms (Ding et al., 2013; Ding et al., 2011b). The Cu regulation of *Aspergillus* has not been identified.

Acknowledgments:

C.D and R.F would like to thank the training received from DSID program (Duke Scholar in Infectious Disease) at Duke University. C.D is funded by National Science Foundation of China (3130974).

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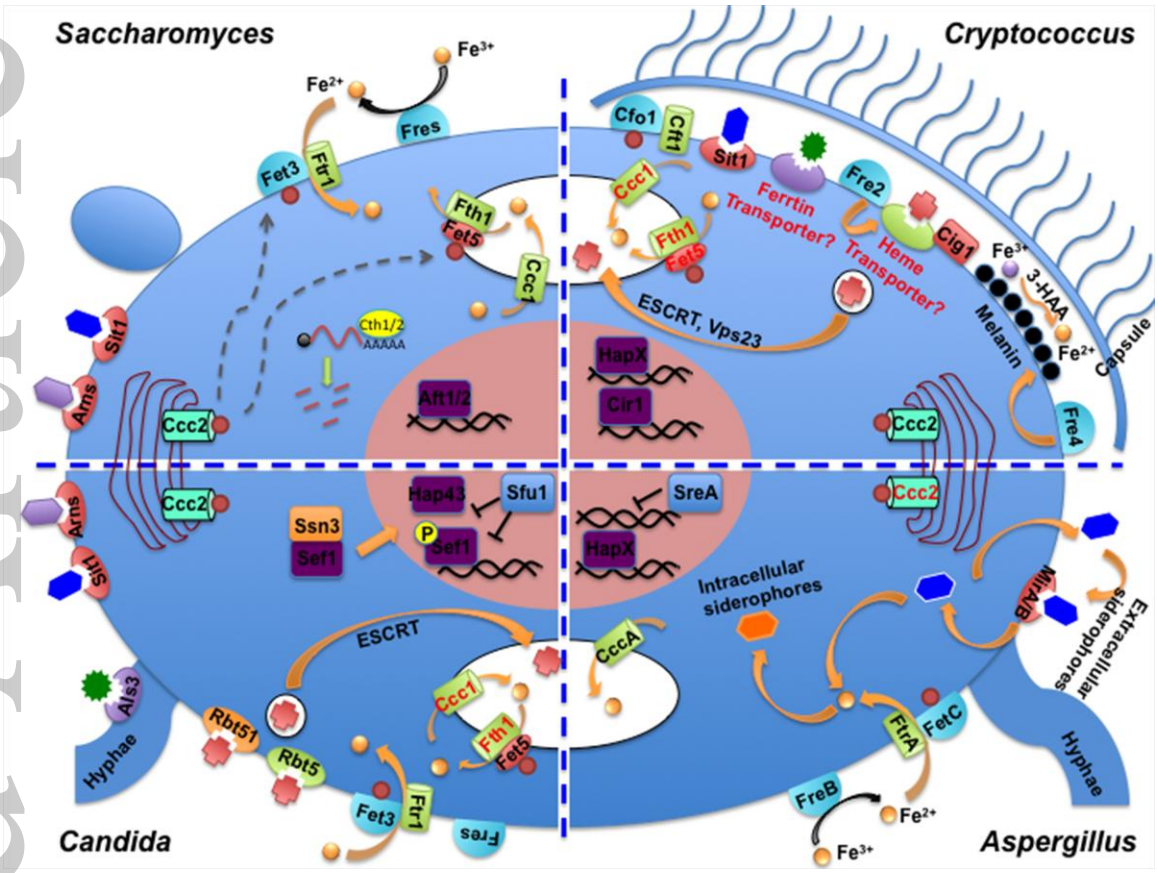

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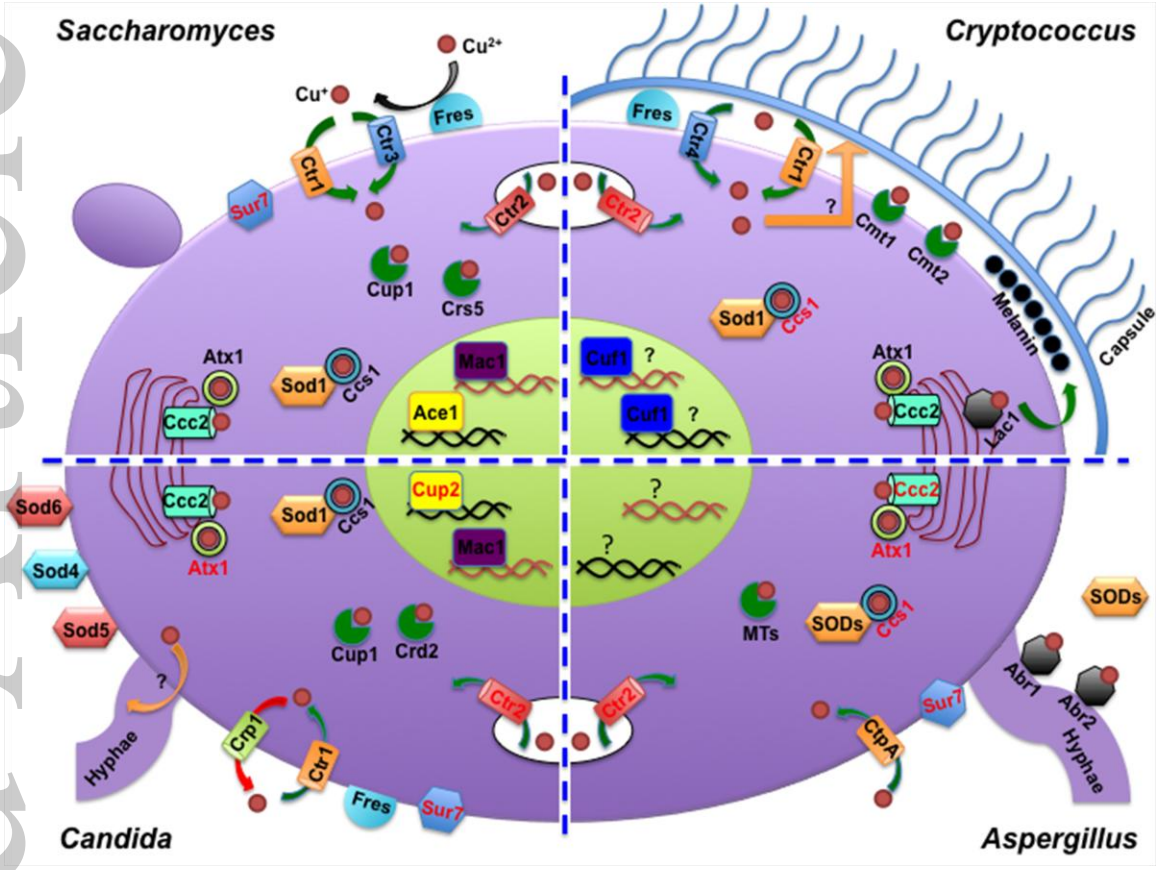

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