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Review article

Nutritional immunity: targeting fungal zinc homeostasis

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ABSTRACT

Transition metals, such as Zn^{2+}, are essential dietary constituents of all biological life, including mammalian hosts and the pathogens that infect them. Therefore, to thrive and cause infection, pathogens must successfully assimilate these elements from the host milieu. Consequently, mammalian immunity has evolved to actively restrict and/or pool metals to toxic concentrations in an effort to attenuate microbial pathogenicity - a process termed nutritional immunity. Despite host-induced Zn^{2+} nutritional immunity, pathogens such as *Candida albicans*, are still capable of causing disease and thus must be equipped with robust Zn^{2+} sensory, uptake and detoxification machinery. This review will discuss the strategies employed by mammalian hosts to limit Zn^{2+} during infection, and the subsequent fungal interventions that counteract Zn^{2+} nutritional immunity.

1. Introduction

Fungal pathogens represent an enormous burden on human health, affecting billions and responsible for more than 1.5 million deaths annually, more than malaria, tuberculosis or HIV (Brown et al., 2012). The most prevalent of these pathogens include *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, all of which are opportunistic, causing disease primarily in individuals with perturbations in immune function (Brown et al., 2012). Both *A. fumigatus* and *C. neoformans* are environmentally acquired, initially disseminating in lung tissue following infectious spore inhalation (Hohl and Feldmesser, 2007; Kronstad et al., 2011). *C. albicans*, on the other hand, is usually a commensal constituent of the human mycobiome, persistently colonising the oral, gastrointestinal and genitourinary cavities, but can rapidly convert into a pathogen in response to deviations in host factors (Pappas, 2006). This conversion of its fungal lifecycle is facilitated by the expression of numerous virulence attributes, resulting in a broad variety of infections, ranging from superficial mucosal to systemic candidiasis (Mayer et al., 2013). Whereas superficial infections are extremely common and treatable, bloodstream infections are life-threatening with a reported mortality rate of 34%, despite antifungal therapy (Nguyen et al., 1995). Worryingly, multidrug-resistant fungal species have emerged, demanding the immediate development of new therapeutic applications, targeting largely unexploited fungal pathways, for example, micronutrient homeostasis (Arendrup et al., 2017).

Transition metals such as Fe^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+} are essential for all biological life. Therefore, a significant aspect of microbial infection is the constitutive assimilation of micronutrients from the host milieu. In this arena, metal ions occupy a surprisingly vital role for both host and pathogen alike, particularly Zn^{2+}. On the host side, Zn^{2+} is essential for optimum immune function, required for monocyte chemotaxis, cytokine production and phagocytosis (Prasad 2008, 2009). Even mild Zn^{2+} deficiency results in perturbed host protection, thereby enhancing susceptibility to fungal infections (Salvin et al., 1987; Singh et al., 1992). On the pathogen side, Zn^{2+} serves as a cofactor for a multitude of proteins, some of which are essential drivers of virulence as will be discussed shortly for *C. albicans*. These few examples underline the importance of Zn^{2+} for weaponising both host and pathogen, with each rival grappling to retain and secure Zn^{2+} to gain a competitive advantage.

Amongst fungi, the molecular mechanisms governing cellular Zn^{2+} trafficking have been extensively investigated in *Saccharomyces cerevisiae* for roughly three decades, laying the basis for Zn^{2+} homeostasis not only in fungal organisms but in eukaryotes in general. In pathogenic fungi, Zn^{2+} metabolism and its influence on virulence and host-pathogen outcome are only beginning to be appreciated, with the largest body of work centered on *Candida*, *Cryptococcus* and *Aspergillus* species (Table 1). Therefore, insight into the mechanistic strategies employed by mammalian immunity to control microbial infection through Zn^{2+} manipulation and subsequent fungal interventions will be the framework of this review. Moreover, due to its high disease prevalence, a detailed

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outline of Zn^{2+} metabolism in *C. albicans* will be provided and compared to the well-characterised Zn^{2+} metabolic machinery of *S. cerevisiae*. To this end, the PubMed database was utilised for the extraction of relevant publications using Zn^{2+} nutritional immunity and fungal Zn^{2+} homeostasis search terms. Studies published before 2000 were filtered and any citations found in relevant studies were considered for investigation.

In summary, this review aims to provide a comprehensive overview of the tactics exploited by mammalian hosts to restrict microbial growth through perturbations in fungal Zn^{2+} homeostasis and describes distinct countermeasures utilised by fungi to overcome extracellular Zn^{2+} depletion.

2. Nutritional immunity and the significance of Zn^{2+}

Mammalian hosts have evolved to exploit the essentiality and toxicity of metal nutrients to attenuate microbial growth. This process, known as nutritional immunity, involves mechanisms of metal starvation and/or poisoning and is crucial for protection against infection (Hood and Skaar, 2012). Nevertheless, successful pathogens have co-evolved with their hosts, establishing powerful machinery comprised of metal sensors, transporters, and scavengers to maximally exploit host resources, facilitating the circumvention of nutritional immunity.

Behind Fe^{2+}, Zn^{2+} is the most common trace metal in the human system. Bioinformatics analysis have estimated that 5% of prokaryotic proteins and 9% of eukaryotic proteins require Zn^{2+} as a cofactor to function properly (Andreini et al., 2009). This is because, unlike Fe^{2+} and Cu^{2+}, Zn^{2+} is redox inert under physiological conditions and therefore very stable. The biological functions of Zn^{2+} are diverse and are generally divided into three distinct classes: structural, catalytic, and regulatory (Maret and Li, 2009). Inside cells, Zn^{2+} primarily exhibits a structural role, conferring stability to a plethora of proteins. Moreover, due to its Lewis acid strength and fast ligand exchange rate, Zn^{2+} is incorporated into the catalytic centres of many enzymes, covering all six enzyme commission classes (Vallee and Auld, 1990). Lastly, Zn^{2+} demonstrates a regulatory role via the activation/inactivation of a wide range of signalling proteins, thereby mediating numerous cellular signalling networks (Kitamura et al., 2006; Schothorst et al., 2017; Taylor et al., 2012; Yamasaki et al., 2007).

The *C. albicans* genome comprises 6215 genes in total, 315 of which occupy the Gene Ontology term 'Zn^{2+} ion binding' (Skrzypek et al., 2017). Although many of these genes are of unknown function, some are indispensable for fungal virulence, metabolism, and gene regulation. For instance, superoxide dismutases (SODs) are fundamental for the neutralisation of superoxide radical anions during fungal interaction with immune cells. Three of the six putative SODs (Sod1, Sod4, and Sod6) are thought to be Cu^{2+}/Zn^{2+}-dependent metalloenzymes and fungal strains lacking any one of them exhibit attenuated virulence, underscoring their importance during infection (Fradin et al., 2005; Frohner et al., 2009; Hwang et al., 2002; Martchenko et al., 2004; Miramon et al., 2012). In addition, some virulence-associated transcription factors exhibit Zn^{2+} fingers, required for protein folding and subsequent DNA recognition and binding. These include the pH responsive Rim101 and the repressor of filamentous growth Nrg1. Deletion of either *RIM101* or *NRG1* attenuates fungal virulence (Davis et al., 2000; Murad et al., 2001; Nobile et al., 2008). Other Zn^{2+}-requiring transcription factors govern central fungal biological processes such as sugar metabolism (Suc1), cell wall remodelling (Cwt1) and ergosterol biosynthesis (Upc2) (Kelly and Kwon-Chung, 1992; MacPherson et al., 2005; Moreno et al., 2003; Silver et al., 2004). Therefore, *C. albicans* must have access to a sufficient quantity of Zn^{2+} to optimally fuel commensal, as well as pathogenic lifecycles.

3. Host mechanisms of Zn^{2+} nutritional immunity

An average adult is estimated to harbour 1.4–2.3 g of Zn^{2+}, the majority of which, roughly 99.9%, is compartmentalised intracellularly within human tissue, predominantly skeletal muscle, bone, and liver (Bleackley and Macgillivray, 2011; Jackson, 1989). The remaining ~0.1% of Zn^{2+} is present in blood serum, either chelated by alpha2-macroglobulin (~20%) or albumin (~80%) (Foote and Delves, 1984; Jackson, 1989). Thus, under normal conditions, these mechanisms of constitutive restriction result in practically no free accessible Zn^{2+}. Under inflammatory conditions, the host undergoes rapid global Zn^{2+} readjustments at systemic, local, and intracellular levels to disrupt microbial Zn^{2+} homeostasis and control infection.

In response to inflammation, intestinal Zn^{2+} assimilation is inhibited, and systemic Zn^{2+} levels are further restricted via hepatic sequestration (Gaetke et al., 1997; Liuzzi et al., 2005). This event occurs rapidly, decreasing serum Zn^{2+} levels (hypozincemia) by over 50% within only a few hours and is driven by the cell surface Zn^{2+} transporter, Zip14, in an interleukin 6-dependent manner (Gaetke et al., 1997; Liuzzi et al., 2005). Both mice deficient in either Zip14 or interleukin 6 fail to elicit hypozincemia (Aydemir et al., 2012; Liuzzi et al., 2005). Interestingly, hepatic Zip14 upregulation is concomitant with an increase in metallothionein 1 (MT1) levels (Liuzzi et al., 2005). This observation suggests that MT1 subsequently serves to buffer the resulting Zip14-mediated increase in intracellular Zn^{2+}.

Another important layer of Zn^{2+} nutritional immunity occurs at the site of inflammation through the expression and release of Zn^{2+}-sequestering molecules. Of key importance are the S100 family, a 25-member low molecular weight proteins, characterised by two EF-hand calcium-binding domains (Schaub and Heizmann, 2008). By far, the most-detailed mechanism of host-induced localised Zn^{2+} sequestration occurs via calprotectin (Corbin et al., 2008; Nakashige et al., 2016). Calprotectin is a heterodimer consisting of two subunits, S100A8 and S100A9, and is highly associated with inflammation (Edgeworth et al., 1991; Eversole et al., 1993; Korndorfer et al., 2007). This antimicrobial protein constitutes roughly 40% of the cytoplasmic neutrophil protein content, is significantly upregulated in response to inflammatory and Zn^{2+}-limiting conditions and can be detected at concentrations exceeding 1 mg/ml in tissue abscesses (Edgeworth et al., 1991; Eversole et al., 1993; Mazzatti et al., 2008). Upon release, calprotectin elicits antimicrobial activity towards a wide range of bacterial and fungal pathogens through Zn^{2+} chelation (Amich et al., 2014; Bianchi et al., 2011; Clohessy and Golden, 1995; Corbin et al., 2008; Hood et al., 2012; Urban et al., 2009). Consistent with its role, genetic inactivation of putative Zn^{2+}-binding residues attenuates calprotectin's antimicrobial capacity (Gaddy et al., 2014; Kehl-Fie et al., 2011). In addition to Zn^{2+}, calprotectin has been observed to chelate other essential metal nutrients, such as Fe^{2+}, Mn^{2+}, Cu^{2+}, and Ni^{2+}, thus extending its known metal-binding repertoire (Besold et al., 2018; Hayden et al., 2013; Nakashige et al., 2015, 2017).

Table 1. Pathogenic fungi with published studies on zinc metabolism.

Fungal organism	Effect of Zn^{2+} limitation on growth	Effect of perturbed Zn^{2+} homeostasis on virulence	Reference
C. albicans	Decreased	Decreased	Crawford et al. (2018); Kim et al. (2008); Malavia et al. (2017)
C. dubliniensis	Decreased	Decreased	Böttcher et al. (2015)
A. fumigatus	Decreased	Decreased	Amich et al. (2014); Moreno et al. (2007); Vicentefranqueira et al. (2005)
C. neoformans	Decreased	Decreased	Do et al. (2016); Urban et al. (2009)
C. gatti	Decreased	Decreased	Schneider Rde et al. (2012); Schneider et al. (2015)

Calprotectin is also a crucial antimicrobial constituent of neutrophil extracellular traps (NETs) (Urban et al., 2009). NETs are large, thread-like complexes released by neutrophils following NETosis (a unique form of programmed cell death) to neutralise neighbouring pathogens (Brinkmann et al., 2004; Urban et al., 2006). Calprotectin-mediated Zn^{2+} chelation by NETs was observed to be the major fungicidal element against *C. albicans*, *C. neoformans*, *A. fumigatus* and *Aspergillus nidulans* (Bianchi et al., 2011; Clark et al., 2016; McCormick et al., 2010; Urban et al., 2009).

Additional members of the S100 family that exhibit Zn^{2+}-chelating antimicrobial properties include psoriasin (S100A7) and calgranulin C (S100A12) (Glaser et al., 2005; Haley et al., 2015). Psoriasin is usually present in its disulphide-reduced form on the surface of the skin and confers potent fungicidal activity against numerous fungi, including *A. fumigatus*, but surprisingly not *C. albicans* (Brodersen et al., 1999; Hein et al., 2015). However, psoriasin was recently demonstrated to bind *C. albicans* cell well glucans, thereby perturbing fungal adhesion (Brauner et al., 2018). Calgranulin C is primarily expressed and secreted by neutrophils (Pietzsch and Hoppmann, 2009). This antimicrobial protein exhibited Zn^{2+} (and Cu^{2+}) chelation and attenuated *Helicobacter pylori* virulence and viability *in vitro* (Haley et al., 2015; Moroz et al., 2003, 2009), but its antifungal activity is yet to be examined.

In addition to systemic and localised Zn^{2+} readjustments, Zn^{2+} manipulation in response to microbial invasion is further exploited at an intracellular level. Interestingly, parasitised macrophages can utilise mechanisms of both Zn^{2+} depletion and intoxication to kill pathogens within the phagolysosomal compartment (Botella et al., 2011; Subramanian Vignesh et al., 2013; Winters et al., 2010). On the one hand, macrophages have been shown to starve *Histoplasma capsulatum* of Zn^{2+} (Subramanian Vignesh et al., 2013; Winters et al., 2010). On the other hand, the elimination of *Mycobacterium tuberculosis* through Zn^{2+} intoxication has been reported (Botella et al., 2011; Wagner et al., 2005). Interestingly, *C. albicans* appears to encounter Zn^{2+} starvation following internalisation by macrophages, as indicated by the significant upregulation of the cell surface Zn^{2+} transporter-encoding gene *ZRT2* (Crawford et al., 2018; Lorenz et al., 2004). The method of microbial killing adopted by macrophages remains to be elucidated but may depend on numerous factors such as the cytokine environment, pathogen encountered, and location of infection.

Despite host-induced Zn^{2+} manipulation, fungal pathogens are still capable of maintaining Zn^{2+} homeostasis and therefore must be equipped with robust Zn^{2+} detoxification and assimilation machinery. We next describe the mechanistic approaches exploited by fungi to maintain Zn^{2+} homeostasis at optimal levels, with a primary emphasis on *S. cerevisiae* and *C. albicans*.

4. Regulation of Zn^{2+} homeostasis

The regulatory mechanism of Zn^{2+} homeostasis is shown in Figure 1. The transcriptional activator Zap1 primarily regulates Zn^{2+} homeostasis in yeast in Zn^{2+} depleted environment. In Zn^{2+} replete environment it is repressed. Zap1 has seven C_2H_2-type Zn fingers called Znf1-Znf7. Five of these Zn^{2+} finger Znf3 - Znf7 located at the *C terminus* contain the DNA binding domain (DBD) and are involved in binding to specific Zn^{2+} responsive elements (ZREs) in the promoter region of Zap1 target genes (Bird et al., 2000). Zap1 also has two transcriptional activator domains: AD1 and AD2.

Regulation of Zn^{2+} homeostasis is well studied in *S. cerevisiae*. The transcriptional activator Zap1 primarily regulates Zn^{2+} homeostasis in yeast in Zn^{2+} depleted and replete environment and its own expression *S. cerevisiae*. However in *C. albicans* Sut1 activates Zap1 which then activates the expression of several genes in Zn^{2+} deficient conditions. Zap1 induces the expression of Zrt2 and Zrc1 and others. Zap1 also interacts with Rim101, to induce the expression of Sap6, Pra1, and Zrt1 expression. Pra1 and Sap6 bind Zn^{2+} facilitating its internalization. The regulation of Yek4 is unknown whereas Msc2, and Cot1 regulation is not dependent on Zap1. Adapted from (Soares et al., 2020).

The large Zn responsive domain (ZRD) of AD1 confers Zn^{2+} responsiveness to AD1 (Herbig et al., 2005) whereas ZRD for AD2 is located within Znf1 and Znf2 and similarly confers Zn responsiveness to AD2. In a Zn^{2+} depleting environment, Zn^{2+} binds to the AD2 of Znf1 and Znf2 or in the cysteine/histidine rich region in AD1 inactivating the ADs resulting in reduced expression of the target genes. In a Zn^{2+} surplus environment, Zap1 loses its DNA binding activity and then dissociate from ZREs of the target genes (Bird et al., 2003). This is indicating that Zn^{2+} independently regulates AD1, AD2 and DNA-binding domains and a combination of these 3 mechanisms may account for the global Zn responsiveness to Zap1 (Bird et al., 2000; Eide, 2003, 2020; Frey et al., 2011; Wilson and Bird, 2016). *ZAP1* deletion mutants did not show distended vacuoles and flocculence typical of Zn^{2+} deficient cells, indicating that vacuolation and flocculation play a homeostatic role in Zn^{2+} deficient cells. These make Zap1 a unique target for developing new antifungal agents.

In *S. cerevisiae*, Zap1 regulates its own expression by binding to the ZREs on its own promoter (Zhao et al., 1998). This gets Zap1 transcript and protein levels to increase encouraging the induction and expression of target genes. Zap1 plays an important role in Zn^{2+} homeostasis through the regulation of genes involved in Zn^{2+} uptake and metabolism (Frey et al., 2011). In *C. albicans*, Zap1 expression has been shown to be induced by Sut1. Zap1 expression induces Zn^{2+} Zip family plasma membrane transporters, Zrt2 and Zrt3 and a zincosome Zn^{2+} importer, Zrc1. A zincosome is a cellular organelle into which Zn^{2+} is stored. Zap1 interacts with Rim101 to induce the expression of a proteinase, Sap6, a zincophore, Pra1, and another member of Zip family plasma membrane transporters, Zrt1. In *C. albicans*, Zrt1 and Pra1 have similar promoter sequence and are regulated by the transcriptional activators Zap1 and Rim101. Zap1 and Rim101 have been shown to facilitate *C. albicans* responses to the availability of Zn^{2+} and pH conditions, respectively (Bensen et al., 2004; Nobile et al., 2009). Furthermore, Zap1 and Rim101 play a role in filamentation in *C. albicans*, thus associating the process of yeast dimorphism with Zn^{2+} homeostasis and pH (Kim et al., 2008; Ramon et al., 1999). Rim101 is orthologous to *A. fumigatus* PacC and it is involve in virulence (Ramón and Fonzi, 2003).

Pra1 is a known zincophore that binds Zn^{2+}, or detaches Zn^{2+} bound to other proteins during infection and delivers it to the cell surface (Citiulo et al., 2012; Loboda and Rowinska-Zyrek, 2017). Zrt1 interacts with Pra1 to form a dimer that finally transports Zn^{2+} into the cytoplasm. Sap6 is a virulence protein associated with biofilm formation in *C. albicans* and also functions as a Zn^{2+} scavenger therefore mediating Zn^{2+} internalization (Kumar et al., 2017). *C. albicans* cells with high expression of Pra1 and Zrt1 show increased Sap6-induced autoaggregation (Kumar et al., 2017) suggesting that Pra1 and Zrt1 may share commom promoter sequences. Sap6 and Pra1 are secreted proteins.

Zn^{2+} regulation by *C. albicans* in a Zn^{2+} depleted environment is thought to follow the same course seen in *S. cerevisiae* (Figure 1). The transcriptional activator for Zn^{2+} regulation in *C. albicans* is also Zap1 or alternatively Csr1. *C. albicans* Zap1 has 7 Zn^{2+} fingers but does not have any identified ADs sequences (Böttcher et al., 2015; Kim et al., 2008). Zap1 also binds to its promoter and regulates its own expression a function that may be conserved in fungi (Böttcher et al., 2015). Infection profiles have shown that Sut1 interacts with Zap1 in the same pathway to regulate Zn^{2+} homeostasis. This was shown in *C. albicans* by finding that overexpression of Zap1 or Zrt2 restores wild type pathogenicity to a *sut1*Δ mutants (Xu et al., 2015).

5. Zn^{2+} uptake and compartmentalisation

The study of Zn^{2+} cell biology began in 1957 with the discovery of methalothionein protein in 1957 and the study of the protein was studied for next 30 years by many researcher which represented the development of this area of research. This took another dimension in the mid-1990s

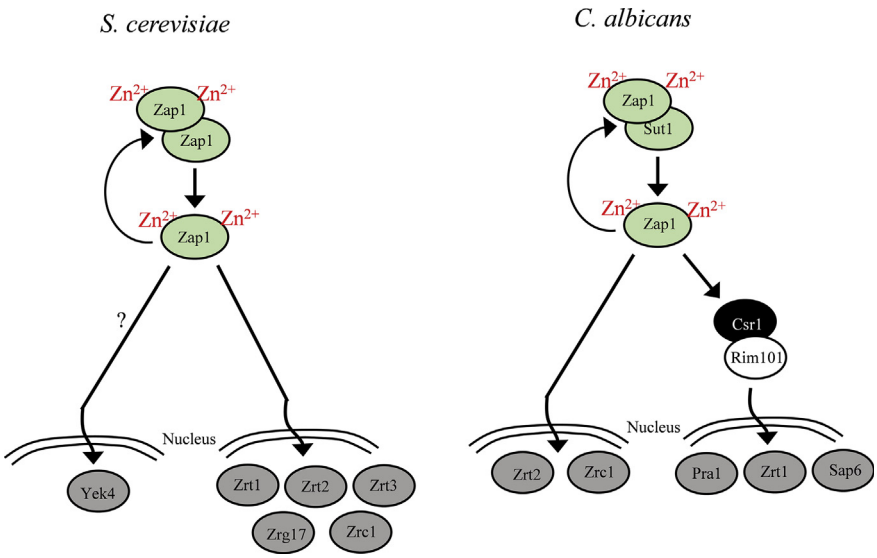

Figure 1. Regulation of Zn homeostasis in *S. cerevisiae* and *C. albicans*.

with the discovery of Zn^{2+} transporters able to transport Zn^{2+} across the cell membrane (Table 2). As will be seen below, subsequent studies in the following decades have revealed more about Zn^{2+} transporters, their families and mechanism of interaction and Zn^{2+} utilisation.

Zn^{2+} uptake and intracellular compartmentalisation is mediated by several protein families in yeast (Table 2 and Figure 2). The process is well understood in *S. cerevisiae* and follows similar pathways in *C. albicans* with few exceptions including that the Zn^{2+} transporters in *S. cerevisiae* are not pH regulated and that Zrt1 is the major Zn^{2+} transporter (Zhao and Eide, 1996a) while Zrt2 is a low affinity Zn^{2+} transporter (Zhao and Eide, 1996b).

Zrt1 and Zrt2 are members of the ZIP family transport proteins that mediate Zn^{2+} transport across the plasma membrane. In *C. albicans*, Zrt1 is a surface bound protein essential for reassociating secreted Pra1-bound-Zn^{2+} to the fungal cell surface, suggesting that Zrt1 is a Pra1 receptor (Citiulo et al., 2012). Zrt1 is expressed in neutral and alkaline environment and it is essential for Zn^{2+} transport under those pH environments. Zn^{2+} uptake in *C. albicans* in an acidic environment is facilitated by Zrt2. Zrt2 is essential for growth and it is thought to be the major Zn^{2+} importer in *C. albicans*. It also supports growth in alkaline Zn^{2+} limiting environment (Crawford et al., 2018) making it a versatile Zn^{2+} transporter. *C. albicans* has a similar Zn^{2+} cellular import pathway to that

Table 2. *S. cerevisiae* and *C. albicans* Zn binding proteins and transporters.

Name	Type	Function	Reference
S. cerevisiae			
Zrt1	ZIP	High-affinity Zn^{2+} uptake system	Zhao and Eide (1996a)
Zrt2	ZIP	Low-affinity Zn^{2+} uptake system	Zhao and Eide (1996b)
Zrt3	ZIP	Vacuolar Zn^{2+} exporter	MacDiarmid et al. (2000)
Yke4	ZIP	ER bi-directional Zn^{2+} transporter	Kumánovics et al., 2006
Atx2	ZIP	Golgi Mn^{2+} homeostasis	Lin and Culotta (1996)
Zrc1	ZnT	Vacuolar Zn^{2+} importer	MacDiarmid et al. (2003)
Cot1	ZnT	Vacuolar Zn^{2+} importer	MacDiarmid et al. (2003)
Msc2	ZnT	ER Zn^{2+} importer	Ellis et al. (2004); Ellis et al. (2005)
Zrg17	ZnT	ER Zn^{2+} importer	Ellis et al. (2004); Ellis et al. (2005)
Mmt1	ZnT	Mitochondrial Fe^{2+} importer	Li and Kaplan (1997); Li et al. (2014)
Mmt2	ZnT	Mitochondrial Fe^{2+} importer	Li and Kaplan (1997); Li et al. (2014)
Fet4	-	Fe^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+} uptake system	Li and Kaplant, 1998; Waters and Eide (2002)
Pho84	-	Zn^{2+}/phosphate uptake system	Jensen et al. (2003)
Zps1	-	Predicted Zn^{2+} binding cell wall protein	Wu et al. (2008)
C. albicans			
Zrt1	ZIP	High-pH Zn^{2+} uptake system	Crawford et al. (2018)
Zrt2	ZIP	Broad-spectrum pH Zn^{2+} uptake system	Crawford et al. (2018)
Zrt3	ZIP	Unknown (ScZrt3 orthologue)	Crawford et al. (2018)
orf19.5428	ZIP	Unknown (Atx2 orthologue)	Crawford et al. (2018)
Zrc1	ZnT	Zincosomal Zn^{2+} import	Crawford et al. (2018)
orf19.3874	ZnT	Unknown (no orthologue present in yeast)	Crawford et al. (2018)
orf19.3769	ZnT	Unknown (Zrg17 orthologue)	Crawford et al. (2018)
orf19.3132	ZnT	Unknown (Msc2 orthologue)	Crawford et al. (2018)
orf19.52	ZnT	Unknown (Mmt2 orthologue)	Crawford et al. (2018)
Pra1	-	Zn^{2+} binding protein (Zps1 orthologue)	Citiulo et al. (2012)

of *A. fumigatus,* a pathogenic mould. *A. fumigatus*, ZrfC and ZrfB (orthologues of Zrt1 and Zrt2) are required for Zn^{2+} uptake in neutral/alkaline, and acidic pH, respectively (Amich et al., 2010; Vicentefranqueira et al., 2005). Thus, *C. albicans* Zrt1 and Zrt2 and their *A. fumigatus* orthologues are promising unique targets for the development of new effective antifungals. Furthermore, pyrvinium pamoate has been shown to reduce cellular Zn^{2+} and it is a potential means of developing new antifungals, that targets this unique metal metabolic processes in fungi or that can increase fungi susceptibility to host nutritional immunity (Simm & May, 2019).

ZIP family transporters include Zrt1, Zrt2 and Zrt3, and CDF family transporters include Zrc1, Cot1, and Msc2-Zrg17 heterodimer (Ellis et al., 2005; Simm et al., 2007). While these Zn^{2+} plasma membrane transport proteins can bind Zn^{2+} individually, Zrt1 has been shown to function in Zrt1-Pra1 interaction to bind Zn^{2+}. Pra1 is expressed and secreted from *C. albicans* cell surface during host cell invasion. It functions as siderophore (zincophore in *C. albicans*) and binds Zn^{2+} either in the form of free Zn^{2+} or from Zn^{2+}-binding proteins in the host cell and reassociates with Zrt1 on the surface of *C. albicans* cell. Zn^{2+} transportation into *C. albicans* cytoplasm is thought to occur through Zrt1-Pra1 interaction (Citiulo et al., 2012). Zrt1 is abundant in hyphal tip. Zn^{2+} transporters from other protein family include Fet4 and Pho84. Sap6 is secreted during fungal growth in Zn^{2+} depleted environment. It is also thought to function as zincophore probably through the degradation of bound metalloproteins within fungal aggregation (Kumar et al., 2017). Zn^{2+} compartmentalization is mediated by Zrc1, Cot1 and Msc2-Zrg17. Zrc1 is required for vacuolar Zn^{2+} transport in *S. cerevisiae* (Li and Kaplant, 1998) but is required for zincosomal Zn^{2+} compartmentalisation and detoxication in *C. albicans* (Crawford et al., 2018). Vacuolar Zn^{2+} is thought to be bound by a ligand (L) to induce Zn^{2+} detoxication and storage (Simm et al., 2007). Zrt3 mobilizes this stored Zn^{2+} in Zn^{2+} limited cells (MacDiarmid et al., 2000). The Zap1 transcription factor (coloured circle) is activated in a Zn^{2+}-depleted environment and then targets and induces the expression of genes involved in Zn^{2+} transport into and out of the cytoplasm. Adapted from (Frey et al., 2011; Soares et al., 2020; Wilson et al., 2012).

The CDF family transporters transport Zn^{2+} from cell cytoplasm into organelles to maintain essential metabolic processes that are Zn^{2+} dependent (Eide, 2006) and also decrease Zn^{2+} cytoplasmic levels in case of "Zn shock" (Choi et al., 2018). Zrc1 and Cot1 are associated with vacuolar Zn^{2+} transport for Zn^{2+} detoxication and storage (Simm et al., 2007). The mechanism of Zn^{2+} detoxication is not well understood. In a recent study, however, 108 yeast single-gene deletion mutants identified to be sensitive to 6 mM $ZnCl_2$ were used to probe the toxic mechanisms of Zn^{2+}. Data showed that higher Zn^{2+} stress induces increased intracellular reactive oxygen species (ROS) levels. This suggests the involvement of ROS in regulating ROS homeostasis in response to high Zn^{2+}. Zn^{2+} toxicity may lead to oxidative damage, thus activating the expression of various antioxidant defence proteins (Zhao et al., 2020).

Zrt3 is shown to transport Zn^{2+} from the vacuole into the cytoplasm, thus at the time of Zn^{2+} depletion in the cell, Zrt3 mobilises vacuolar Zn^{2+} for cell function (MacDiarmid et al., 2000). *Sc*Yke4 a member of the ZIP family transports Zn^{2+} in and out of the secretory pathway. It is a bidirectional Zn^{2+} transporter (Kumánovics et al., 2006). Other Zn^{2+} transporters such as Msc2-Zrg17 heterodimer, are involved in transporting Zn^{2+} into the secretory pathway (Bird and Wilson, 2020; Ellis et al., 2005).

In a recent study, Wilson's lab tried to understand Zn^{2+} compartmentalisation in *C. albicans*. They used both *in silico* and primary research data to show that Zrc1 is predominantly required for zincosomal Zn^{2+} compartmentalisation in response to both slight variations in Zn^{2+} availability or potentially toxic levels of Zn^{2+}. Therefore, Zrc1 plays an important role in *C. albicans* adaptation to environmental Zn^{2+}. In *S. cerevisiae*, Zrc1 mediates Zn^{2+} entry into the vacuole (Li and Kaplant, 1998). However, Zrc1 still plays similar roles in both organisms since it is involved in intracellular compartmentalisation of Zn^{2+} when both organisms are exposed to toxic level of the metal. Zrc1 is also shown to be essential for liver colonization, since hepatocytes increase Zn^{2+} uptake during infection (Crawford et al., 2018; Liuzzi et al., 2005). *In silico* analysis has identified in *C. albicans* genome, *S. cerevisiae* Zrt3 homologue, named also Zrt3, though no function has been assigned to it yet (Crawford et al., 2018).

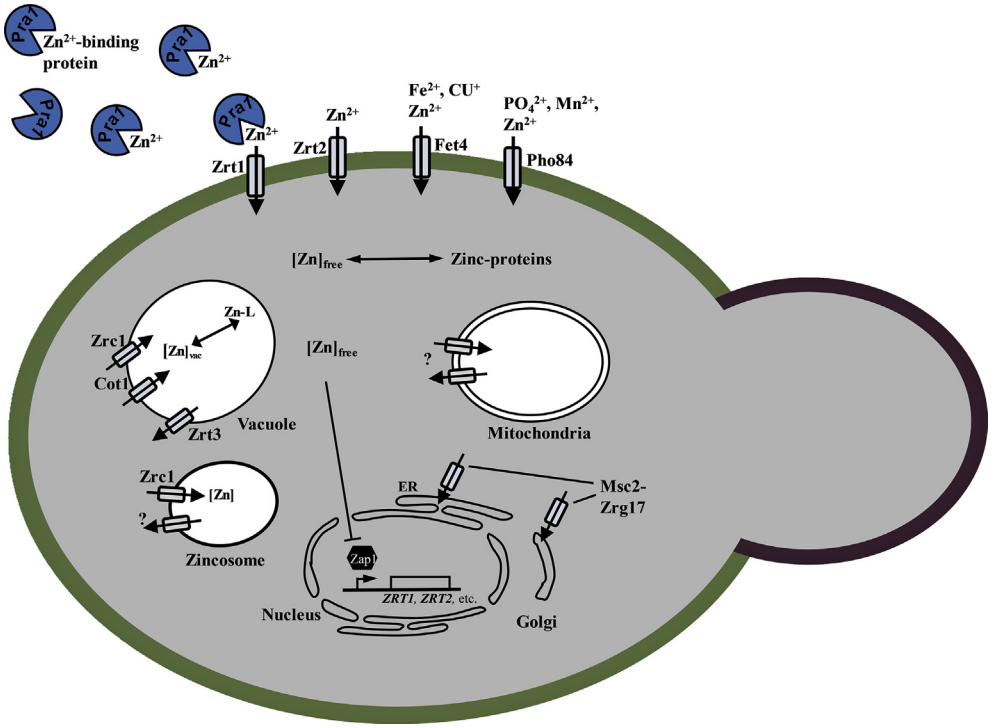

Figure 2. A schematic representation of Zn^{2+} scavenging and regulation by *S. cerevisiae* and *C. albicans*.

Another recent study gave new understanding into *C. albicans* Zn^{2+} scavenging during host infection by investigating a well characterised protein, the secreted aspartic proteinase, Sap6. Sap6 is an alkaline-pH-induced protein, associated with cell aggregation. Sap expression is induced in oral candidiasis together with known metal limitation genes, suggesting an important metal scavenging role during infection. The Pra1 zincophore system is only expressed and secreted in low Zn^{2+} and limited to neutral to alkaline pH which may be the reason for the low secretion levels in oral candidiasis. Sap6 may be an alternative zincophore system during oral candidiasis since it is secreted in acidic-to-neutral pH condition. Though the mechanism is not yet understood, Sap6 is thought to scavenge Zn^{2+} by binding to the metal for transport into the cytoplasm by surface localised plasma membrane transporters (Kumar et al., 2017).

Our initial knowledge of Zn^{2+} utilisation in *C. dubliniensis*, a less fatal species of *Candida*, was through the work of Brunke's lab. They functionally characterised Zap1 by generating a *C. dubliniensis zap1Δ* mutant. They investigated the protein's role in Zn^{2+} regulation and virulence. Their results showed that Zap1 is required for growth under Zn^{2+} depleted environments and is essential for *C. dubliniensis* virulence but not required for morphogenesis. They also showed that Zrt1 and Zrt2 role in Zn^{2+} uptake in *C. albicans* is similar in *C. dubliniensis*. In *C. dubliniensis*, Zrt2 is a more predominant Zn^{2+} transporter than Zrt1 which appears to play a secondary role in Zn^{2+} transport across the plasma membrane (Böttcher et al., 2015).

In *H. capsulatum*, an initial study identified a putative Zn^{2+} transporter, Zrt2 by comparing sequences of *S. cerevisiae* ZIP family with *H. capsulatum* genome (Dade et al., 2016). In a Zn^{2+} depleted environment, Zrt2 is expressed by *H. capsulatum* and it is also essential for virulence in a murine model of systemic histoplasmosis (Dade et al., 2016). Similar to Zrt2 in *C. albicans* Zrt2 is thought to be *H. capsulatum* high affinity Zn^{2+} transporter. However, no Pra1 orthologue has been found in *H. capsulatum* (Wilson et al., 2015). Recently, an elaborate *in silico* analyses revealed the presence of 8 genes related to *H. capsulatum* Zn^{2+} homeostasis. The genes included the transcription factor, Zap1 and members of CDF and ZIP family transporters. The transcriptional levels of *ZRT1*, *ZRT2* and *ZAP1* were induced in Zn^{2+} depleted environment (Assuncao et al., 2020). Proteomics analysis of cells grown in Zn^{2+} depleted environment at 24 h and 48 h showed 265 differentially expressed proteins. Some of the proteins are known to be involved in cell wall remodelling during stress. Interestingly, metabolic profile showed that glycolysis of glucose (fructose-6-phosphate) induces the biosynthesis of glycan and chitin in the cell wall. These results suggest that low metal availability increases the chitin and glycan content in fungal cell wall (Assuncao et al., 2020) a mechanism that has not been shown in yeast and other fungi (North et al., 2012; Wang et al., 2018). Data from the study also suggested that Zn^{2+} restriction could trigger oxidative stress, a mechanism shown to be involved in excess Zn^{2+} toxicity in yeast. On the contrary, Zn^{2+} depletion in *Paracoccidioides lutzii* suppresses the expression of membrane glycoproteins involved in cell wall synthesis leading to wall damage (de Curcio et al., 2017).

6. Distinct pathogenic strategies to evade host-induced Zn^{2+} manipulation

As previously discussed, one major strategy to counteract Zn^{2+} restriction is through the upregulation of Zn^{2+} transporters and scavengers. In fact, pathogens such as *Salmonella enterica* serovar Typhimurium and *Neisseria meningitidis* have evolved to not only survive under reduced Zn^{2+} availability but also shockingly capitalise on calprotectin-mediated Zn^{2+} chelation (Liu et al., 2012; Stork et al., 2013). Whereas the ZnuABC transporter confers *S.* Typhimurium resistance to calprotectin-sequestered Zn^{2+}, the CbpA receptor of *N. meningitidis* permits the hijacking and subsequent exploitation of calprotectin Zn^{2+} (Liu et al., 2012; Stork et al., 2013).

Other strategies to counteract nutritional immunity include circumventing the requirement of a particular metal, metal conservation and mobilisation of intracellular metal stores. *Borrelia burgdorferi* has evolved to substitute Mn^{2+} for Fe^{2+} in its essential metalloproteins, thus rendering Fe^{2+} restriction ineffective (Posey and Gherardini, 2000). Similarly, *C. albicans* Sod5 has lost Zn^{2+}-binding activation, evolving a Cu-only cofactor requirement (Gleason et al., 2014). This fungal adaption may confer the detoxification of reactive oxygen species under Zn^{2+} depletion.

Microbes also undergo metabolic reprogramming during periods of famine to conserve Zn^{2+} for essential processes only. In *S. cerevisiae*, the Fe^{2+}-requiring Adh4 substitutes the Zn^{2+}-requiring Adh1 under Zn^{2+} deficiency in a Zap1-dependent manner (Bird et al., 2006; Lyons et al., 2000; Wang et al., 2018; Wu et al., 2008). *C. albicans* may exploit an analogous strategy, as its *ADH*-encoding genes are also differentially regulated by Zap1 (Nobile et al., 2009).

Finally, eukaryotic organisms in particular may mobilise intracellular Zn^{2+} stores to circumvent extracellular Zn^{2+} depletion. Indeed, the vacuole of *S. cerevisiae* was reported to accumulate up to 100 mM of Zn^{2+} and found to be sufficient for the subsequent production of 8 generations of progeny under Zn^{2+} deficiency (Simm et al., 2007). Nevertheless, this attractive metal store and its position in maintaining Zn^{2+} homeostasis have yet to be fully dissected in fungal pathogens. In addition to the vacuole, zincosomes may facilitate fungal adaptation to limiting external Zn^{2+} as, in *C. albicans* at least, appear to be major sites of Zn^{2+} accumulation (Crawford et al., 2018).

7. Perspective

Our knowledge of Zn^{2+} homeostasis in fungi has grown at an unexpected rate over the past few years, for example we are now gaining understanding of the mechanisms of Zn^{2+} scavenging and compartmentalisation in *C. albicans* in response to toxic levels as well as Zn^{2+}-dependent colonisation of internal organs during infection. Several Zn^{2+} binding proteins and transporters have also been identified in several yeasts and fungi. However despite this progress, gaps remain in our understanding of intracellular Zn^{2+} transport in pathogenic yeast and moulds. For example, 1) No known or novel Zn^{2+} intracellular transporter has been identified for Zn^{2+} transport in and out of such important organelles as the mitochondria. Identifying the transporters can help understand Zn^{2+} metabolism in the organelle and possibly identify potential drug targets; 2) The main mechanisms of Zn^{2+} metabolism in fungi are yet to be fully understood. For example, Zn^{2+} toxicity and depletion have been associated with increased expression of cell surface bound glycoproteins and biosynthesis of cell wall polysaccharides: glycan and chitin. This mechanism though poorly understood has not been studied in yeast, research into this area will help understand stress tolerance/response in fungi and inform our understanding of the pathways and mechanisms involved. Molecules targeting these pathways can block Zn^{2+} metabolism and help host immune system clear the fungi pathogens; 3) The role of Zn^{2+} in virulence is not well understood, research into this area is needed to understand the full scale of Zn^{2+} involvement in fungal pathogenesis.

Our knowledge of Zn^{2+} regulatory mechanisms and uptake can be exploited as an efficient drug delivery system to combat fungi infection. Nutritional immunity is an exciting area of research and full of promises since some of the proteins involved in these processes are essential for growth and virulence and show little similarity to known human proteins.

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