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Mini-review

The Zinc Transport Systems and Their Regulation in Pathogenic Fungi

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Abstract Zinc is an essential micronutrient required for many enzymes that play essential roles in a cell. It was estimated that approximately 3% of the total cellular proteins are required for zinc for their functions. Zinc has long been considered as one of the key players in host-pathogen interactions. The host sequesters intracellular zinc by utilizing multiple cellular zinc importers and exporters as a means of nutritional immunity. To overcome extreme zinc limitation within the host environment, pathogenic microbes have successfully evolved a number of mechanisms to secure sufficient concentrations of zinc for their survival and pathogenesis. In this review, we briefly discuss the zinc uptake systems and their regulation in the model fungus *Saccharomyces cerevisiae* and in major human pathogenic fungi such as *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus gattii*.

Keywords Fungi, Virulence, Zap1, Zinc, ZIP family transporter

Zinc is an important micronutrient required by many enzymes for their catalytic functions in cells. In the model fungus *Saccharomyces cerevisiae*, it was estimated that approximately 3% of the total proteins require zinc for their functions [1]. These proteins include alkaline phosphatase, Cu-Zn superoxide dismutase, alcohol dehydrogenase, and several regulatory proteins that possess structural domains, such as the zinc finger domain, the LIM domain and the RING finger domain, which require zinc for stabilization [2, 3]. However, excess zinc is detrimental to cells. Unlike iron and copper, zinc is redox inactive, and hence, does not mediate free radical-induced cellular damage. However, excess zinc has been shown to cause cell toxicity by binding to inappropriate sites in proteins. For example, excessive zinc interferes with the functions of mitochondrial aconitase in cellular respiration [4]. Therefore, cells must possess mechanisms to acquire zinc from the environment and to tightly control intracellular zinc homeostasis.

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Zinc has long been considered as one of the key players in host-pathogen interactions. As a means of nutritional immunity, the mammalian host sequesters intracellular zinc, using multiple cellular zinc importers and exporters. Zinc level in the host environment is also limited by calprotectin, which is an antimicrobial protein with high zinc-binding affinity [5]. To overcome zinc sequestration within the host, pathogenic microbes have successfully evolved a number of mechanisms to secure sufficient zinc concentration for their survival and pathogenesis within the host niche. Emerging evidences have illustrated critical roles of host-bacterial pathogen interactions in this regard. One well-known contributor is the ZnuABC transport system in bacterial pathogens, which plays a major role in the high-affinity zinc transport in several bacterial pathogens including *Campylobacter jejuni*, *Salmonella enterica*, *Haemophilus ducreyi*, *Escherichia coli*, *Brucella abortus*, and *Streptococcus pyogenes*. It was observed that eliminating the function of the ZnuABC transport systems reduced virulence in the experimental animal model [6]. The acquisition of zinc also plays an important role in the virulence of pathogenic fungi; however, the mechanism underlying this process is still largely unclear. This review briefly discusses the zinc uptake systems and their regulation in the model fungus *Saccharomyces cerevisiae* and in the major human pathogenic fungi such as *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus gattii*.

THE ZINC TRANSPORTERS AND THEIR REGULATION IN *SACCHAROMYCES CEREVISIAE*

Numerous studies have investigated the mechanisms of

zinc uptake and homeostasis in *S. cerevisiae*. The first isolated gene associated with zinc transport and homeostasis was Zrc1, which contributes to detoxification of intracellular zinc in the cytoplasm [7]. *ZRC1* encodes a protein with six transmembrane domains; it is localized in the vacuolar membrane and is responsible for vacuolar zinc transport in *S. cerevisiae* [8]. Together with Zrc1, zinc-replete cells transport cytoplasmic zinc into the vacuole, using another vacuolar zinc transporter, Cot1, which also confers tolerance to cobalt [9]. Vacuolar zinc levels are also modulated by Zrt3, a member of the Zrt- and Irt-related protein (ZIP) family of zinc transporters, which was shown to mobilize stored zinc from the organelle to the cytoplasm when the cells were depleted of zinc [10]. These findings suggested that the combinatorial role of zinc transporters in the vacuole is critical for intracellular zinc homeostasis and that a cellular organelle also contributes to zinc homeostasis in *S. cerevisiae*. In addition to the vacuole, zinc transporters have been reported in other organelles as well. Examples include Msc2 and Zrg17, which are members of the cation diffusion facilitator family of zinc transporters localized in the endoplasmic reticulum (ER). Msc2 and Zrg17 form the heterodimeric complex, which transports zinc into the ER, which is required for the proper functioning of the organelle [11, 12].

Zrt1 and Zrt2, members of the ZIP family of metal transporters, mediate zinc uptake in the plasma membrane of *S. cerevisiae* [13, 14]. Zrt1 and Zrt2 are the high-affinity and low-affinity plasma membrane zinc transporters, respectively. Zrt1 was originally isolated as the homolog of *Arabidopsis thaliana* iron transporter Irt1. However, the mutant lacking *ZRT1* showed a growth defect only in the zinc-limited medium, suggesting that *ZRT1* is required for growth of the cells in zinc-depleted conditions. *ZRT1* encodes the protein containing putative eight transmembrane domains and a metal-binding domain, and shows high-affinity zinc transporter activity. Further, a previous study showed that the level of *ZRT1* transcription was highly upregulated in the cells grown in zinc-depleted conditions compared to that of the cells grown in a medium with high zinc concentration, suggesting that its expression is regulated by zinc [13]. *ZRT2* encodes the low-affinity zinc transporter protein and is responsible for zinc uptake in cells grown in zinc-replete conditions [14]. Although Zrt2 contributes to zinc accumulation, its role in zinc uptake seemed marginal. Further, it was suggested that the driving force for zinc uptake by Zrt1 and Zrt2 might be associated with electrical potential, which is generated across the plasma membrane by the plasma membrane ATPase. A transmembrane gradient of another ion may also trigger zinc uptake by Zrt1 and Zrt2 [13, 14].

In *S. cerevisiae*, the expression of the zinc uptake systems is primarily regulated by the C_2H_2-type zinc finger transcription factor Zap1 at the transcriptional level. Genome-wide transcription analysis revealed that among the 400 genes that are regulated by zinc, approximately 80 genes including

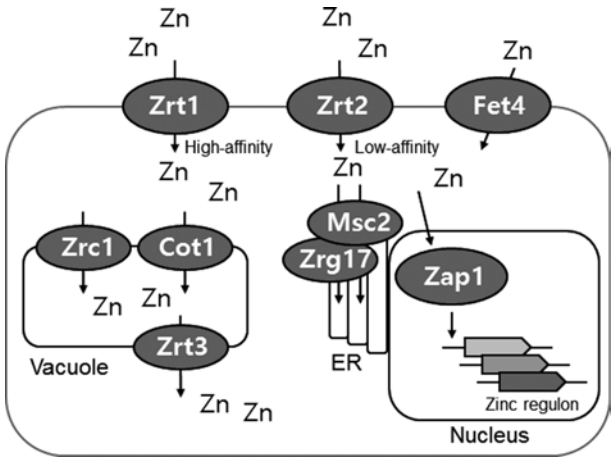

Fig. 1. Proteins involved in zinc uptake and homeostasis in *Saccharomyces cerevisiae*. ER, endoplasmic reticulum.

ZRT1 and *ZRT2* are direct target genes of Zap1 [15, 16]. Zap1 activates the transcription of Zrt1 and Zrt2 by binding to the zinc-response element in their promoter region, and its binding affinity is controlled by zinc levels [17]. Further, vacuolar zinc transporters Zrt3 and Zrc1 are directly regulated by Zap1 [16]. In addition to transcriptional regulation by Zap1, post-translational regulation also controls the Zrt1 activity. Upon zinc limitation, Zrt1 is expressed, *N*-glycosylated, and localized in the plasma membrane. However, under high zinc concentrations, Zrt1 is ubiquitinated by the Rsp5 ubiquitin-protein ligase, and the Ubc4 and Ubc5 ubiquitin-conjugating enzymes followed by rapid internalization via endocytosis, and degraded in the vacuole [18, 19]. Moreover, it has been suggested that transcriptional and post-translational regulations may separately control Zrt1-mediated zinc transporter activity. The Zap1-mediated transcriptional regulation governs the Zrt1 activity upon moderate changes of zinc levels while post-translational regulation controls the activity when the cells face extreme changes in zinc concentrations [2]. Thus, multiple components and regulatory mechanisms control zinc uptake and homeostasis in *S. cerevisiae* (Fig. 1).

THE ZINC TRANSPORTERS AND THEIR REGULATION IN *ASPERGILLUS FUMIGATUS*

Zinc uptake and homeostasis is also important in the physiology and virulence of *A. fumigatus*, which infects the lungs of a susceptible individual, causing pulmonary aspergillosis [20]. *A. fumigatus* possesses the ZIP family zinc transporters, ZrfA and ZrfB, which are membrane zinc transporters highly homologous to Zrt1 of *S. cerevisiae* [21]. Expressions of both ZrfA and ZrfB are regulated by environmental zinc concentrations. It has been reported that the expressions of ZrfA and ZrfB are upregulated by zinc deficiency, while a high concentration of zinc downregulates their expressions. Furthermore, the results of the growth assay performed using the *zrfA* mutant, the

zrfB mutant, and the *zrfA zrfB* double mutant suggested that both *ZRFA* and *ZRFB* are required for the growth of *A. fumigatus* in the zinc-limited conditions, although the contribution of *ZRFA* was marginal. Environmental pH has also been shown to influence the regulation of expressions of *ZRFA* and *ZRFB*. The promoters of *ZRFA* and *ZRFB* contain PacC-like binding sites, and transcription of *ZRFA* and *ZRFB* was more significantly induced in the acidic zinc-limited conditions than in the neutral and alkaline conditions. These observations indicated that, unlike *S. cerevisiae*, the ZIP family membrane zinc transporters ZrfA and ZrfB in *A. fumigatus* are sensitive to the environmental pH and play roles mainly in the acidic zinc-deficient conditions [21].

In addition to *ZRFA* and *ZRFB*, four more genes that encode the ZIP family zinc transporters were identified from the genome of *A. fumigatus*. Among these genes, only *ZRFC* showed zinc-dependent regulation. In contrast to *ZRFA* and *ZRFB*, which play a role in zinc transport under acidic conditions, *ZRFC* was suggested to be the major zinc transporter under alkaline conditions. Furthermore, experiments conducted using immunocompromised mice revealed that ZrfC is the major zinc transporter in the host tissue and that ZrfC is the protein that partially supports the growth of the fungus against the Zn/Mn chelating effect of calprotectin [22]. These experiments also suggested that ZrfA and ZrfB may be dispensable for virulence when ZrfC is present. The homolog of *S. cerevisiae* Zap1 has been identified in *A. fumigatus* and named ZafA, which transcriptionally regulates the expressions of ZrfA, ZrfB, and ZrfC. It was shown that ZafA functionally complemented Zap1 in *S. cerevisiae* and was required for the growth of *A. fumigatus* in the zinc-limited conditions. Furthermore, the *zafA* mutant was unable to survive in mice, suggesting that *ZAFA* is required for virulence [23].

THE ZINC TRANSPORTERS AND THEIR REGULATION IN *CANDIDA ALBICANS*

Several studies have suggested diverse roles of zinc uptake, homeostasis, and regulation in the morphological transition, biofilm formation, and virulence of *C. albicans*, and many of these have focused on understanding the functions and regulation of Csr1/Zap1 in the fungus, which is the homolog of *S. cerevisiae* Zap1. Csr1/Zap1 in *C. albicans* was first identified by Kim *et al.* [24]. They suggested that Csr1/Zap1 is the homolog of *S. cerevisiae* Zap1 and showed that Csr1/Zap1 is a high copy number suppressor of *ZRT2* in *S. cerevisiae*. They also showed that the mutant lacking *CSR1/ZAP1* alleles shows deficient growth in the zinc-depleted conditions, and is unable to form germ tubes and hyphae, suggesting that Csr1/Zap1 contributes not only to zinc uptake and homeostasis but also to the morphological transition in *C. albicans* [24]. Subsequently, the influence of Csr1/Zap1 in biofilm formation was suggested. In particular, the *csr1/zap1* mutant accumulated 1.5- to 2-fold more soluble β-1,3 glucans than the wild-type, suggesting its negative regulatory role in producing extracellular matrix for biofilm formation [25]. Transcriptome analysis using microarrays revealed that a total of 232 and 272 genes are up- and downregulated in the *csr1/zap1* mutant, respectively. Downregulated genes include *ZRT1*, *ZRT2*, and *ZRT3*, which are homologous to *S. cerevisiae* zinc transporters, the results of which agreed with the significantly reduced growth of the *csr1/zap1* mutant in the zinc-limited conditions. Moreover, binding of Csr1/Zap1 to the promoters of *ZRT1*, *ZRT2*, and *ZRT3* was confirmed by the genome-wide chromatin immunoprecipitation analysis [25]. Similar to Csr1/Zap1 in *C. albicans*, another pathogenic *Candida* species, *C. dubliniensis* also possesses the *S. cerevisiae* Zap1 homolog named Csr1. As in *C. albicans*, Csr1 is a transcriptional activator for *ZRT1*, *ZRT2*, and *ZRT3*, and the *csr1* mutant exhibits growth defects in the zinc-limited conditions. However, unlike *C. albicans*, the *csr1* mutant was able to form germ tubes and undergo morphological transition, although it showed attenuated virulence [26].

In addition to the regulatory roles of Csr1/Zap1, a recent study investigated in detail how *C. albicans* sequesters zinc from the environment, and found that the fungus possesses the protein pH-regulated antigen 1 (Pra1) and utilizes it as an extracellular zinc scavenger. Upon zinc depletion, Pra1 is secreted extracellularly, binds to zinc and, in turn, delivers zinc to the *C. albicans* Zrt1 homolog at the cell membrane. The Pra1-Zrt1-mediated zinc sequestration was considered a 'zincophore system' in *C. albicans* and was shown to be required for zinc acquisition within host endothelial cells [27].

THE ZINC TRANSPORTERS AND THEIR REGULATION IN *CRYPTOCOCCUS GATTII*

It has long been known that *C. neoformans* is the predominant cause for cryptococcosis in immunocompromised individuals such as acquired immune deficiency syndrome patients. However, to date, no study has focused on analyzing zinc uptake and regulation in the fungus. However, zinc uptake and regulation have been studied in *C. gattii*, which mainly infects immunocompetent individuals. The homolog of *S. cerevisiae* zinc regulatory transcription factor Zap1 was identified in *C. gattii* and its functions were characterized. Similar to other fungi, Zap1 positively regulates the expression of *ZIP1* and *ZIP2*, which are the ZIP family zinc transporters in *C. gattii*. The mutant lacking *ZAP1* showed impaired growth in the zinc-limited conditions and accumulated more intracellular reactive oxygen species compared to the wild-type. Furthermore, the *zap1* mutant displayed attenuated virulence in a murine model of cryptococcosis, which could be attributed to a defect in zinc transport and increased reactive oxygen species in the mutant cells within the host tissue. These findings suggested that, as in other fungi, Zap1 plays critical roles in zinc transport, homeostasis, and virulence in *C. gattii* [28].

A recent study by Schneider Rde *et al.* [29] more closely investigated the roles of zinc transporters in *C. gattii*, and suggested that the fungus possesses at least four genes encoding ZIP family zinc transporters, namely *ZIP1*, *ZIP2*, *ZIP3*, and *ZIP4*. Because *ZIP3* and *ZIP4* were homologs of *S. cerevisiae ZRT3* and *YKE4*, respectively, the study mainly focused on the functional characterization of plasma membrane zinc transporters *ZIP1* and *ZIP2*, which are homologs of *S. cerevisiae ZRT1* and *ZRT2*. As suggested previously [28], the expressions of *ZIP1* and *ZIP2* were regulated by zinc availability. The mutant lacking *ZIP1* showed significant growth defect in zinc-limited medium, the phenotype of which was restored by either exogenously added zinc or reconstitution of the wild-type *ZIP1* gene. Moreover, the zinc uptake assay using the intracellular zinc indicator dithizone showed reduced intracellular zinc accumulation in the mutant lacking *ZIP1*. All these results confirmed that Zip1 is indeed the major zinc transporter in *C. gattii*. Interestingly, unlike its *A. fumigatus* homolog *ZRFA*, *ZIP1* was required for growth in zinc-limited conditions in both acidic and alkaline pH, suggesting its pH independency [29].

In contrast to the *zip1* mutant, no phenotype associated with zinc uptake and homeostasis was observed from the *zip2* mutant except upregulation of *ZIP1* in the mutant, which suggested the possible compensatory effect by *ZIP1* in absence of *ZIP2*. However, interestingly, the *zip2* mutant reduced macrophage survival rate, implying that *C. gattii* requires a Zip2 function to survive within the host macrophage. While the *zip1* and *zip2* mutants displayed virulence comparable to the wild-type, the *zip1 zip2* double mutant completely abolished the virulence in murine model of cryptococcosis. These results suggested that both *ZIP1* and *ZIP2* are required for virulence of *C. gattii* [29].

CONCLUSION

Zinc acquisition and its regulation are important in the physiology and virulence of fungi. Highly conserved membrane zinc transporters belonging to the ZIP family have been identified not only from the model fungus *S. cerevisiae* but also from the major human pathogenic fungi *A. fumigatus*, *C. albicans*, and *C. gattii*, and were shown to mediate zinc transport and homeostasis. The homologs of the zinc-responsive transcription factor Zap1 were also identified from the fungi, as described in this review, suggesting that the core zinc uptake and regulatory machinery are evolutionarily conserved and play an essential role. However, the influence of the environmental conditions on the expressions of zinc transporters might slightly differ among fungi. For example, while the expressions of *ZRFA*, *ZRFB*, and *ZRFC* in *A. fumigatus* were regulated by pH, *ZIP1* in *C. gattii* showed pH-independent expression, suggesting that apart from the Zap1 regulatory system, another distinct regulatory mechanism may also govern zinc transport in different fungi; this finding, however, warrants further investigation. Moreover, a series of studies have shown that zinc acquisition and regulation contribute greatly to the virulence of pathogenic fungi, suggesting that this pathway could be a novel target for antifungal therapeutics.

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