Investigation of Cryptococcus neoformans magnesium transporters reveals important role of vacuolar magnesium transporter in regulating fungal virulence factors

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Abstract

Cryptococcus neoformans is an important opportunistic fungal pathogen in humans. Recent studies have demonstrated that metals are critical factors for the regulation of fungal virulence in hosts. In this study, we systemically investigated the function of C. neoformans magnesium transporters in controlling the intracellular Mg balance and virulence-associated factors. We identified three Mg transporters in C. neoformans: Mgt1, Mgt2, and Mgt3. While we could not detect a Mg2+-related growth phenotype in mgt1 and mgt3 knockout strains, a GAL7p-Mgt2 strain showed significant Mg-dependent growth defects in the presence of glucose. Further analysis demonstrated that MGT2 is a homolog of MNR2 in Saccharomyces cerevisiae, which is localized to the vacuolar membrane and participates in intracellular Mg transport. Interestingly, a transcriptome analysis showed that Mgt2 influenced the expression of 19 genes, which were independent of Mg2+. We showed that melanin synthesis in C. neoformans required Mg2+ and Mgt2, and that capsule production was negatively regulated by Mg2+ and Mgt2. Repressing the expression of MGT2-induced capsule, which resulted in an increased fungal burden in the lungs. Cumulatively, this study sets the stage for further evaluation of the important role of Mg homeostasis in the regulation of melanin and capsule in C. neoformans.

1 INTRODUCTION

Cryptococcus neoformans is a major fungal pathogen in humans. It primarily afflicts immunodeficient and immunocompetent individuals, causing about 600,000 deaths per year globally (Idnurm et al., 2005; Kronstad et al., 2011; May, Stone, Wiesner, Bicanic, & Nielsen, 2016). Cryptococcus infections begin in the lung tissue. By unknown mechanisms, fungal cells disseminate into the central nervous system, resulting in lethal meningitis (Idnurm et al., 2005; Kronstad et al., 2011; May et al., 2016). Recent epidemiological studies have revealed that infections of Cryptococcus species are a significant health issue in the Pacific North West in Northern America, in addition to Europe, Africa, and China (Byrnes et al., 2010, 2011; Liu et al., 2017; May et al., 2016).
Two major fungal virulence factors have been extensively studied. These are the generation of a cell-surface polysaccharide capsule structure and melanization using L-DOPA as a substrate (Idnurm et al., 2005; Kronstad et al., 2011). The capsule structure is critical for shedding the fungal cells from the host immune cells and manipulating the responses of immune cells to C. neoformans invasion (May et al., 2016; Zaragoza et al., 2009). Melanin formation in C. neoformans protects fungal cells from many environmental stresses, such as UV radiation and free-radical toxicity (Wang & Casadevall, 1994; Williamson, 1997). Numerous studies have demonstrated that metals, such as copper (Cu) and iron (Fe), regulate the production of the capsule structure and melanin pigment (Bairwa, Hee Jung, & Kronstad, 2017; Chun & Madhani, 2010; Ding, Festa, Sun, & Wang, 2014; Do et al., 2016; Jung, Sham, White, & Kronstad, 2006; Lian et al., 2005). It is known that the key melanin biogenesis protein (Lac1) utilizes Cu⁺ as a cofactor (Williamson, 1997). Disrupting key regulators of Cu homeostasis of C. neoformans influences melanin formation. For example, disrupting the Cu⁺ transporter genes CTR1 and CTR4, the Cu⁺ chaperone ATX1, or the P-type ATPase CCC2 impairs the development of dark brown pigments in C. neoformans (Chun & Madhani, 2010; Ding et al., 2011; Walton, Idnurm, & Heitman, 2005; Waterman et al., 2007, 2012). Fe, the most well studied metal in C. neoformans, plays extremely important roles in modulating melanin and the capsule (Jung et al., 2006; Lian et al., 2005; Tangen, Jung, Sham, Lian, & Kronstad, 2007). A master Fe homeostasis regulator in C. neoformans, transcription factor Cir1 was demonstrated to inhibit melanin formation by repressing expression of LAC1 and LAC2. It also induces capsule biogenesis by activating capsule production-related genes (Jung et al., 2006). Moreover, C. neoformans cells utilize melanin as a Fe reduction system (Nyhus, Wilborn, & Jacobson, 1997). Given the important functions of these metals in regulating fungal virulence factors, human fungal pathogens have evolved sophisticated acquisition and defense machineries to cope with metal deficiency and overload conditions at the pathogen-host axis (Bairwa et al., 2017; Ding et al., 2014).

Of all-important metals in biology, magnesium (Mg) is one of the most abundant metals in the cell and it plays critical roles as an enzymatic cofactor and structural component of biological molecules. Many mammalian disorders are associated with Mg²⁺ imbalance in the cell, such as depression, anxiety, agitation, and insulin resistance (Abdulatif, Ahmed, Mukhtar, & Badawy, 2013; Boyle, Lawton, & Dye, 2016, 2017; Sereiko, Szopa, & Poleszak, 2016; Sereiko et al., 2013). In fungal cells, supplementation of Mg²⁺ in the growth medium protects Saccharomyces cerevisiae cells from dehydration (Trofinova, Walker, & Rapoport, 2010). Mg²⁺ is also involved in mitotic spindle formation in Schizosaccharomyces pombe (Uz & Sarikaya, 2016). In S. cerevisiae, the Mg²⁺ transporters Mr1 and Mr2 were identified as plasma membrane-bound Mg importers. The knockout strains for ALR1 and ALR2 display blocked Mg²⁺ uptake, which reduces intracellular Mg²⁺ concentrations and subsequently inhibits cell growth in normal growth condition (da Costa, Cornish, & Keasling, 2007). S. cerevisiae Mnr2 is a vacuolar Mg²⁺ transporter that pumps Mg²⁺ from the vascular lumen into the cytosolic space (Pisat, Pandey, & Macdiarmid, 2009). Mg²⁺ is required for mitochondrial function in fungi. Three mitochondrial Mg²⁺ transporters have been identified in S. cerevisiae. Lpe10 and Mrs2 are Mg²⁺ importers and Mme1 acts as a mitochondrial Mg²⁺ exporter. Inactivation of any mitochondrial Mg²⁺ transporters reduces Mg²⁺ accumulation in the mitochondrion and causes defects in respiration (Cui et al., 2015; da Costa et al., 2007).

Although Mg²⁺ homeostasis plays vital functions in fungal biological processes, the role of Mg²⁺ in regulating virulence factors in human fungal pathogens remains largely unknown. In this study, we examined the function of three C. neoformans Mg²⁺ transporters with respect to the regulation of fungal virulence-associated factors. We demonstrated that a vacuolar Mg²⁺ transporter (Mgt2) in C. neoformans positively regulated Mg²⁺ delivery and melanin formation, but negatively controlled capsule production. Using transcriptome analysis, we showed that knockdown of MGT2 differentially regulated the expression of 118 genes. Of these, 19 genes were influenced by MGT2 knockdown independently of exogenous Mg. In animal infection models, the mgt2 mutant displayed an elevated fungal burden in lung tissues as a result of an enlarged capsular structure.

2 | MATERIAL AND METHODS

2.1 | Fungal strains and growth conditions

The wild-type strain H99 was used to generate Mg transporter mutants. Mutants were generated by homologous recombination and transformed using ballistic transformation as described previously (Toffaletti, Rude, Johnston, Durack, & Perfect, 1993). Fungal cells were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C. To activate the C. neoformans GAL7 promoter, YPGal was used (1% yeast extract, 2% peptone, 2% galactose). After biositic transformation, fungal cells were plated onto YPDSorb plates (1% yeast extract, 2% peptone, 2% galactose, 2% agar, 1 mol/L sorbitol). The transformant selection was performed using YPD agar supplemented with 200 μg/ml of G418. A final concentration of 50 mmol/L MgSO₄ was used for GAL7p-MGT2 cell growth.

2.2 | Plasmid construction and mutant construction

The generation of C. neoformans strains in which Mg transporter genes were disrupted was performed as follows. All primers used for the generation of mutants are listed in Table S1. For the targeted disruption of the MGT1 or MGT3 open reading frame, the upstream sequence of MGT1 or MGT3 was amplified using primer pair Cdp280/Cdp281 or Cdp288/Cdp289, respectively. The PCR products were cloned between the Apal and XmaI restriction sites in a NEO³ plasmid. The downstream sequence of MGT1 or MGT3 was amplified using primer pair Cdp282/Cdp283 or Cdp290/Cdp291, respectively. The resulting PCR products were cloned between the BamHI and XbaI restriction sites of the NEO³ plasmid. The resulting plasmids were sequenced, and the inserts were amplified using primer pair Cdp23/Cdp24 to generate the knockout integration cassette. The PCR products were purified, concentrated, and delivered using a PDS-1000/He System (Bio-rad). Transformants were picked and genomic DNA was
isolated for PCR confirmation using primer pair CDP428/CDP131 for MGT1 and CDP962/CDP131 for MGT3.

The C. neoformans mgt2 mutant was constructed by replacing the MGT2 endogenous promoter with the GAL7 promoter. The upstream sequence from MGT2 was amplified using primers CDP284 and CDP285, and then this was cloned between the SpeI and SacII restriction sites in a NEO R plasmid. The C. neoformans GAL7 promoter or MGT2 ORF (first ~1 kb of genomic DNA sequence) were amplified using primer pair CDP611/CDP612 or CDP613/CDP614, respectively. The resulting PCR fragments were purified and mixed, and overlapping PCRs were performed to join the GAL7 and MGT2 fragments using primers CDP611 and CDP614. The overlapping PCR product was purified and cloned between the SpeI and SacII restriction sites in the NEO R plasmid. The integration cassette was amplified using primers CDP23 and CDP24. The PCR fragment was transformed into C. neoformans by biolistic transformation. After 4 h of recovery incubation, cells were transferred onto YPGal agar supplemented with G418. Transformants were confirmed by PCR using primers CDP632 and CDP131 and by Southern blotting.

For the construction of an overexpression strain of Mgt2, the C. neoformans TEF1 promoter was used to constitutively drive gene expression at the CMT2 locus. The upstream or downstream sequence of CMT2 was amplified from genomic DNA using primer pair CDP732/CDP733 or CDP738/CDP739, respectively. The upstream fragment was then cloned between the HindIII and EcoRI restriction sites in a NAT R plasmid, and the downstream fragment was cloned between the Xbol and SacII restriction sites in the same plasmid. The C. neoformans TEF1 promoter was amplified using primers CDP2204 and CDP2205, and the MGT2 ORF was amplified using primers CDP2206 and CDP2207. The fragments of the TEF1 promoter and the MGT2 ORF were joined by overlapping PCR using primers CDP2204 and CDP287. The resulting fragment was cloned at the Xbol site in the plasmid. The integration cassette was transformed using biolistic transformation. Successful integration at the CMT2 locus was confirmed using primers CDP861 and CDP131.

For the expression of S. cerevisiae ALR1 or MNR2 in the GAL7-MGT2 strain, a C. neoformans GPD1 expression plasmid (pXL-HYG) was used as described previously. The genomic DNA sequence from S. cerevisiae ALR1 or MNR2 was amplified using primer pair CDP1699/CDP1700 or CDP1954/CDP1955, respectively. The resulting PCR products were cloned into the PacI site in the pXL-HYG plasmid. The plasmids with the correct gene orientation were transformed in the C. neoformans GAL7-MGT2 strain.

2.3 Genomic DNA isolation and Southern blotting

Genomic DNA isolation and Southern blotting were performed as previously described (Ding & Butler, 2007). Briefly, 5 ml of cell culture (YPD medium or YPGal medium) was washed twice with water and then resuspended in lysis buffer (100 mM Tris-HCl pH 8.0, 50 mmol/L EDTA, 1% SDS, 100 mM NaCl, 2% Triton X-100). Cells were disrupted using a bead beater (Biospec). The supernatant was mixed with 7 mol/L ammonium acetate (pH 7.0). Genomic DNA was extracted using chloroform and precipitated with isopropanol. DNA pellets were then washed, dried, and resuspended in water. Next, 1 μg of DNA was used for PCR. Genomic DNA (20 μg) was treated with RNase A and digested with Stul and SacII overnight for Southern hybridization analysis. Southern blotting was performed using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche), and the experimental procedure was carried out according to the manufacturer’s manual. The DIG-labeled DNA probe was synthesized by PCR using primers CDP1045 and CDP612 (Table S1).

2.4 RNA isolation, real-time PCR, and transcriptome analysis

RNA samples used for quantitative real-time PCR and transcriptome library construction were isolated using TRIzol reagent (Thermo Fisher Scientific) followed by Turbo DNase I treatment (Thermo Fisher Scientific) to eliminate DNA contamination. Next, 1 μg of total RNA was reverse-transcribed into cDNA using the Go Script Reverse Transcription System (Promega). Real-time PCR was performed using a CFX Connect Thermocycler (Bio-Rad). The data were analyzed using 2-ΔΔCt method. ACT1 was used as a loading control. Primers are listed in Table S1. For RNA-seq analysis, 3 μg of total RNA were processed using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Next, mRNA was purified from total RNA using the polyT oligo-attached magnetic beads. An Illumina proprietary fragmentation buffer was used for mRNA fragmentation. First-strand cDNA was synthesized with random hexamer primers using SuperScript II followed by second-strand cDNA synthesis using RNase H and DNA polymerase I. Overhang sequences were then blunted and adenylated at the 3’ end of the cDNA sequence. Subsequently, cDNA sequences 200 bp in length were selected using the AMPure XP system (Beckman Coulter) and enriched using Illumina PCR Primer Cocktail in a 15-cycle PCR reaction. The PCR products were purified and their integrity was confirmed using the Agilent high sensitivity DNA assay on a Bioanalyzer 2100 (Agilent). The sequencing library was then sequenced on a Hiseq platform (Illumina) by Shanghai Personal Biotechnology Co. Ltd. Sequenced reads were mapped using TopHat (http://ccb.jhu.edu/software/tophat/), and the transcript assembly was performed using Cufflinks (http://cufflinks.cbcb.umd.edu) (Trapnell et al., 2012). Genes with differential expression were analyzed using DESeq, as previously described (Chen et al., 2014). Genes of the mutant strains with fold changes greater or less than 1.5 in comparison to that of the wild-type strain were considered to be upregulated or downregulated, respectively.

2.5 Microscopy

The capsule was observed using a Nikon Eclipse Ni-E microscope with the 100× objective (Nikon). Lung tissue mucicarmine and hematoxylin/eosin staining were visualized using a Leica DM4000B LED microscope (Leica) with the 20× objective. Protein localization of GFP-Mgt2 was performed by inducing cells in medium supplemented with galactose and 200 μM EDTA for 6 h. The GFP signal was observed using a Leica TCS-SP8 confocal microscope under the 63× oil objective (Leica).
2.6 Animal virulence assay, fungal burden, and histopathology

All animal experiments were reviewed and ethically approved by the Research Ethics Committees of the College of Life and Health Sciences of Northeastern University. The animal infection experiments were performed as previously described (Sun et al., 2014). Briefly, 6–8-week-old female C57BL/6 mice were used for survival rate and fungal burden analysis, and they were purchased from Changsheng Biotech (China). For the survival rate assay, mice were anesthetized and intranasally administered 10⁵ fungal cells in 50 µl of PBS. Animals were monitored twice a day for morbidity. For the fungal burden analysis, infected mice at 14 days post infection were killed by exposure to CO₂. Lung and brain tissues were collected, weighed, and homogenized in 5 ml of PBS. The suspensions were diluted with PBS, and serial dilutions were plated onto YPGal agar at 30°C for 3 days. For the histopathological assay, lung tissues were collected at 14 days post infection and fixed with paraformaldehyde. Fixed lung tissues were frozen and processed using a Cryostat Microtome Leica CM1850 (Leica). Tissue sections (10 µm in thickness) were stained with mucicarmine or hematoxylin/eosin (Sigma).

2.7 Melanin formation and capsule induction

Melanin was quantified using L-DOPA agar medium. Overnight cell cultures were diluted to an A₆₀₀ of 1.0 and spotted onto L-DOPA plates supplemented with various concentrations of MgSO₄ (0 g/L, 0.25 g/L, 0.5 g/L, or 1 g/L). The plates were incubated at 37°C until black pigment was observed. Photographs were then taken. The capsule induction was performed using two induction conditions. Cells were either incubated in DMEM supplemented with 10% FCS at 37°C and 5% CO₂ for 3 days, or in ACSF medium supplemented with 0.1% glucose at 37°C for 3 days (Rathore, Raman, & Ramakrishnan, 2016). The capsule was stained with India ink.

2.8 Atomic absorption spectroscopy

To quantify intracellular Mg concentrations, the wild-type and Gal7p-MGT2 cells were inoculated in YPGal medium overnight. The cultures were diluted to an A₆₀₀ of 0.2 in 10 ml of YPD medium and incubated on an orbital shaker at 30°C for 6 hr. Cells were then washed twice with 10 ml of 1 mmol/L EDTA and twice with deionized water to remove Mg ions from the medium and cell surface. The washed cells were collected in preweighed Eppendorf tubes, and the tubes were incubated at 80°C overnight to eliminate residual water. The dried cells were weighed. Next, 400 ml of 70% nitric acid was added to digest the cells at 100°C for 30 min. The resulting suspensions were diluted with 1% nitric acid. Mg concentrations were quantified using a JENA Atomic Absorption Spectroscopy ZEE nit 700P (JENA). The operation procedure was performed according to the manufacturer’s manual. A standard curve was first constructed using an Mg atomic spectroscopy standard solution (Sigma). The Mg concentrations were then calculated using the standard curve.

3 RESULTS

3.1 Identification C. neoformans Mg transporters

Previous studies in baker’s yeast identified five Mg²⁺ transporters, including two plasma membrane-bound Mg transporters (Alr1 and Alr2), a vacuolar Mg²⁺ transporter (Mnr2), and two mitochondrial Mg²⁺ transporters (Lpe10 and Mrs2) (Figure 1a) (Bui, Gregan, Jarosch, Ragnini, & Schweyen, 1999; Cui et al., 2015; da Costa et al., 2007; Pisat et al., 2009). To identify Mg²⁺ transporters in C. neoformans, a protein phylogenetic analysis was employed. Three S. cerevisiae Mg²⁺ transporter homologs were found in a proteome database for C. neoformans, including CNAG_03901, CNAG_03502, and CNAG_00010. These were re-named Mgt1, Mgt2, and Mgt3, respectively. The phylogenetic analysis showed that Mgt1 grouped with S. cerevisiae mitochondrial Mg²⁺ transporters, Mgt2 had a close evolutionary relationship with the vacuolar Mg²⁺ transporter, and Mgt3 was similar to plasma membrane Mg²⁺ transporters (Figure 1b). Because the gene expression of many metal transporters is regulated by environmental metal levels, real-time PCRs were carried out to measure the endogenous expression levels of MGT1, MGT2, and MGT3 in response to elevated Mg²⁺ conditions (Figure 1c). Our data showed that the expression of C. neoformans Mg²⁺ transporter-encoding genes was not regulated by environmental Mg levels.

To determine the function of C. neoformans Mg²⁺ transporters in maintaining Mg²⁺ balance, Mg²⁺ transporter knockout strains were generated using the targeted homologous integration method, to obtain mgt1Δ, mgt3Δ, and mgt1Δ mgt3Δ. In our hands, we could not disrupt MGT2 after multiple attempts, implying an essential function for MGT2 under normal yeast growth conditions. We therefore substituted the MGT2 promoter with a C. neoformans galactose-regulated promoter (GAL7, CNAG_06052: the gene coding for galactose-1-phosphate uridyl transferase), which was inserted before the start codon of the MGT2 ORF. This generated a GAL7p-MGT2 strain in which the expression of MGT2 was activated by galactose and repressed by glucose (Figure 1d). A DNA probe from the GAL7 promoter was used for Southern blot analysis to evaluate the precise integration of the GAL7 promoter cassette in the genome (Figure 1e). For the wild-type strain, a 2400-bp band was detected, representing the wild-type GAL7 promoter with the Stul-SacII restriction-digested fragment. The correct GAL7 cassette integrations yielded a smaller band of 1800 bp. The Southern blot analysis demonstrated successful construction of the GAL7p-MGT2 strain in almost all transformation isolates (Figure 1e).

Using the spotting assay, the GAL7p-MGT2 strain showed severe growth defects when glucose was added to suppress the activity of the galactose promoter in the medium. This observation was in agreement with the difficulties related to generating an mgt2Δ strain (Figure 2a). The mgt1Δ, mgt3Δ, and mgt1Δ mgt3Δ strains had similar cell growth rates compared to the wild-type strain. The cell growth of the GAL7p-MGT2 strain was rescued when Mg was supplemented into the medium, but not with other metals (Figure 2a and b). Cumulatively, our data demonstrated that Mgt2 functions as a specific Mg transporter in C. neoformans. We speculated that the impairment in cell
growth was due to an intracellular Mg deficiency. Therefore, atomic absorption spectroscopy was used to measure cellular Mg levels. Our data showed a 50% reduction in the Mg\textsuperscript{2+} content in the GAL7p-MGT2 strain (Figure 2c). These results strongly suggested that the Mgt2 protein is a critical \emph{C. neoformans} Mg transporter involved in intracellular Mg accumulation.

### 3.2 Mgt2 is an \textit{Mnr2} homolog

Our phylogenetic analysis demonstrated that Mgt2 is a putative Mg\textsuperscript{2+} transporter and shares significant sequence similarity to \textit{Mnr2} from \emph{S. cerevisiae} (Figure 1b). To assess whether Mgt2 functionally resembles \textit{Mnr2}, a GAL7p-GFP-MGT2 strain was generated. This
strain expressed GFP-Mgt2 chimeric protein under the regulation of a galactose-inducible promoter (Figure 3a). The GAL7p-GFP-MGT2 strain generated similar growth phenotypes as that of the GAL7p-MGT2 strain on YPD (Yeast Peptone Dextrose), and the growth phenotype was complemented in the presence of galactose (Figure 3a), demonstrating that this was a function specific to GFP-Mgt2 protein. Confocal microscopy was used to show that GFP-Mgt2 was localized to the vacuolar membrane (Figure 3b). We also observed GFP signal in unidentified intracellular spaces. Because S. cerevisiae Mg²⁺ transporters have been shown to localize to the plasma membrane, vacuolar membrane, and mitochondria (Bui et al., 1999; Cui et al., 2015; Pisat et al., 2009), we employed a mitochondrial staining method using MitoTracker to ascertain whether Mgt2 was a mitochondrial-localized Mg²⁺ transporter in C. neoformans. However, no colocalization of Mgt2 and mitochondria was observed, indicating that Mgt2 is not a mitochondrial-associated Mg²⁺ transporter (Figure 3b). A vacuolar specific dye, FM4-64, was used to stain the GFP-MGT2 strain. Fluorescent signals corresponding to FM4-64 and GFP were colocalized on the vacuolar membrane, demonstrating that the Mgt2 is a vacuolar Mg²⁺ transporter (Figure 3c). An immunoblot assay was performed to measure the protein stability of Mgt2 in response to exogenous Mg²⁺ concentrations (Figure 3d). To further address Mgt2 function, S. cerevisiae MNR2 or ALR1 were cloned into a C. neoformans expression vector containing the constitutive promoter GPD1p. Vectors expressing S. cerevisiae MNR2 or ALR1 were transformed into the C. neoformans GAL7p-MGT2 strain. Fungal cells were spotted onto YPD agar medium. This revealed that the S. cerevisiae MNR2 gene functionally complemented the growth phenotype of the GAL7p-MGT2 strain on YPD, whereas ScALR1 did not (Figure 3e). These data strongly suggested that C. neoformans Mgt2 is a vacuolar Mg²⁺ transporter that functionally analogous to S. cerevisiae Mnr2.
3.3 | Mgt2 modulates gene expression independent of Mg

The mgt2 knockout strain growth phenotype was complemented when exogenous Mg was supplemented in the growth medium. However, cell growth in the presence of Mg was only partially rescued, even at a very high level of Mg\(^{2+}\) (Figure 2a). This growth phenotype suggests that Mgt2 influences normal cell growth via a Mg\(^{2+}\)-independent pathway. To explore this hypothesis, we performed a high-throughput transcriptome analysis using RNA sequencing (RNA-seq). We compared the transcriptomes among the three conditions: wild-type cells in YPD medium, GAL7p-MGT2 cells in YPD medium, and GAL7p-MGT2 cells grown in YPD medium supplemented with 50 mmol/L MgSO\(_4\) (Figure 4a). In our data, repressing the expression of MGT2 resulted in differential expression of 118 genes. Of these, the expression of 84 and 34 genes was down or upregulated in the GAL7p-MGT2 cells, respectively (Table S2). Among all differentially expressed genes, expression of MGT2 was the most significantly downregulated in the GAL7p-MGT2 cells, demonstrating the accuracy of our RNA-seq data (Table 1, Table S2 and S3). We observed that genes encoding deacetylase, mitochondrial alternative oxidase, alternative cyclin Pcl12, Fe-S cluster assembly factor, and proteins involved in sugar metabolism and transport were significantly downregulated. Genes encoding ferric-chelate reductase, sulfate transporter, septon ring protein, and glucose-methanol-choline oxidoreductase were significantly upregulated (Table S2). On the other hand, supplementing Mg into the medium of GAL7p-MGT2 cells only caused 27 genes to be differentially expressed. Of these, expression of 7 genes and 20 genes was up or downregulated, respectively (Table S3). We detected that genes encoding alcohol dehydrogenase, alternative cyclin pcd12, carnitine...
O-acetyltransferase, and alternative oxidase were downregulated in the GAL7p-MGT2 strain when grown in high or low Mg conditions. Gene ontology analysis demonstrated that the addition of Mg into the medium of the GAL7p-MGT2 strain caused dramatic changes in the GO enrichment (Figure 4b) compared to that of GAL7p-MGT2 cells in YPD only. However, four GO terms remained unchanged in both conditions, regardless of Mg treatment, and these GO terms were significantly enriched in carbohydrate transport, single-organism
TABLE 1  RNA-seq analysis of differentially expressed genes in the GAL7-MGT2 strain supplemented with or without MgSO4

| Gene ID   | \(\log_2(\text{MGT2} - \text{v-H99})^a\) | \(\log_2(\text{MGT2} + \text{Mg} - \text{v-H99})^b\) | Description                   |
|-----------|--------------------------------------|----------------------------------------|--------------------------------|
| CNAG_03502| -4.28                                | -3.44                                  | Magnesium transporter          |
| CNAG_02489| -1.20                                | -2.37                                  | Alcohol dehydrogenase          |
| CNAG_03772| -0.92                                | -2.28                                  | Glucose transporter            |
| CNAG_07788| -1.62                                | -1.98                                  | Unknown                        |
| CNAG_05212| -2.08                                | -1.72                                  | Unknown                        |
| CNAG_07819| -1.33                                | -1.64                                  | Unknown                        |
| CNAG_05487| -1.65                                | -1.61                                  | Unknown                        |
| CNAG_07845| -1.84                                | -1.41                                  | Unknown                        |
| CNAG_05874| -1.55                                | -1.20                                  | Unknown                        |
| CNAG_00679| -1.32                                | -1.18                                  | Unknown                        |
| CNAG_00183| -1.65                                | -1.09                                  | Alternative cyclin Pcl12       |
| CNAG_01370| -0.92                                | -1.01                                  | Unknown                        |
| CNAG_06551| -0.84                                | -0.89                                  | Carnitine O-acetyltransferase  |
| CNAG_01065| -0.78                                | -0.72                                  | Unknown                        |
| CNAG_00162| -0.82                                | -0.64                                  | Alternative oxidase            |
| CNAG_00664| 0.91                                 | 1.16                                   | Unknown                        |
| CNAG_02392| 0.98                                 | 1.33                                   | Unknown                        |
| CNAG_06375| 2.14                                 | 2.25                                   | Unknown                        |
| CNAG_05010| 2.80                                 | 3.23                                   | Unknown                        |

^aRNA-seq analysis was performed and differential gene expression was compared between the wild-type and GAL7-MGT2 strains. Differential gene expression is represented by binary log of the fold change. Three biological replicates were performed. The full gene list is available in Table S2.

^bRNA-seq analysis was performed and differential gene expression was compared between the wild-type and GAL7-MGT2 strains (supplemented with 50 mM MgSO4). Differential gene expression is represented by binary log of the fold change. Three biological replicates were performed. The full gene list is available in Table S3.

transport, single-organism localization, and organic substance transport (Figure 4b). Two processes were differentially regulated upon the Mg^{2+} treatment, and these GO terms were enriched in monocarboxylic acid catabolic process and small molecule catabolic process. The expression of 19 genes remained constant in GAL7p-MGT2 cells in YPD in the presence or absence of exogenous Mg (Figure 4b and Table 1), and this included MGT2 itself. Real-time PCR was performed for five randomly selected putative target genes of the 19-gene cohort to evaluate the RNA-seq data, and expression regulation patterns resembled those obtained in the RNA-seq analysis (Figure 4c).

### 3.4 Mgt2 influences *C. neoformans* survival in the lungs

Because Mg^{2+} is an essential metal and the mgt2 mutant showed striking growth defects, we hypothesized that Mgt2 participates in regulating fungal virulence in mice. C57BL/6J mice were infected with mgt1Δ mgt3Δ or GAL7p-MGT2 strains (precultured in YPD medium), and the survival rates were recorded (Figure 5a). Mice infected with mgt1Δ mgt3Δ had similar death rates as the wild-type. Surprisingly, mice infected with two independent GAL7p-MGT2 strains had earlier death rates, but these data displayed poor reproducibility (Figure 5b). In the colony-forming unit (CFU) analysis, we detected increased levels of fungal proliferation in two independent GAL7p-MGT2 strains in the lungs (Figure 5c). This was subsequently recapitulated by the lung tissue mucicarmine stain in which more GAL7p-MGT2 cells were observed in lung tissues compared to the lung tissue infected with the wild-type strain (Figure 5f), with no significant changes observed for lung mass (data not shown). Furthermore, using the murine macrophage cell line RAW264.7, we showed that the mgt2 mutant inhibited the rate of phagocytosis, compared to the wild-type cells (Figure 5e), suggesting that Mgt2 may participate in capsule formation (discussed below). A comparison of the CFUs from brain tissues between H99 and GAL7p-MGT2 cells showed no significant difference (Figure 5d).

To gain further insights into the mechanism by which Mgt2 influences fungal proliferation in the lung, we analyzed melanin and capsule formation in the GAL7p-MGT2 strains. As shown in Figure 6a, melanin formation in the wild-type cells required Mg^{2+}. The GAL7p-MGT2 strains demonstrated severe melanin production defects. The addition of Mg^{2+} only partially rescued the pigmentation defects. We also tested the mgt1Δ mgt3Δ strain, and it showed similar melanin production deficits when compared with the wild-type strain (Figure 6a). Our data demonstrated that Mg^{2+} is a critical element for melanin formation in *C. neoformans*, and Mgt2 is required.

Next, we examined capsule formation in Mg^{2+} transporter mutants in DMEM (Dulbecco’s Modified Eagle Medium, high glucose) supplemented with 10% fetal calf serum (FCS) (Figure 6b). We observed a modest increase in capsule thickness in the GAL7p-MGT2 strain.
SUO et al. compared to the wild-type strain (Figure 6c). The addition of Mg$^{2+}$ reduced the size of the capsule structure. To extend these observations, we constructed an Mgt2 overexpression strain in which $MGT2$ expression is under the control of the constitutive promoter, $TEF1$ (Figure 6d). To avoid random integration in the genome, the construct was designed to target the metallothionein 2 locus ($CMT2$) because previous studies demonstrated that the disruption of $CMT2$ had no effect on the expression of virulence factors (Ding et al., 2013). The expression of $MGT2$ was measured using real-time PCR, which demonstrated a 15-fold increase in gene expression compared to that of the wild-type strain (Figure 6e).

A significant increased intracellular Mg$^{2+}$ concentration was detected in the $TEF1-MGT2$ strain (Figure 6f). Since previous studies showed that different capsule induction environments result in the variations in capsule thickness (Rathore et al., 2016), we employed ACSF (artificial cerebrospinal fluid) medium to evaluate the capsule structure in the $GAL7p-MGT2$ and $TEF1p-MGT2$ strains. Significant reductions in the size of the capsule structure were observed in the $GAL7p-MGT2$ strain in ACSF medium supplemented with a high concentration of Mg$^{2+}$.
(Figure 6g). These effects were similar to those of DMEM and ACSF media on capsule formation in the GAL7p-MGT2 mutant. In contrast to the GAL7p-MGT2 strain, the TEF1p-MGT2 strain displayed a reduction in capsule thickness, and a greater reduction was observed in the presence of exogenous Mg\(^{2+}\) (Figure 6h and i). These results clearly demonstrated that expression of MGT2 has a negative effect on capsule production through the regulation of intracellular Mg\(^{2+}\) contents, increasing the cell survival of the GAL7-MGT2 mutant strain in the lung tissue.

4 | DISCUSSION

C. neoformans is a deadly human fungal pathogen, yet clinical antifungal therapies are ineffective. Many studies have demonstrated the important function of metal homeostasis in modulating C. neoformans virulence and host defenses (Ding et al., 2014; Samanovic, Ding, Thiele, & Darwin, 2012). Fe has been widely accepted as a key regulator of pathogenicity in major human fungal pathogens (Bairwa et al., 2017; Ding et al., 2014; Jung et al., 2006; Lian et al., 2005; Nyhus et al., 1997; Darwin, 2012). Fe has been accepted as a key regulator of virulence. Indeed, disrupting the C. neoformans metal homeostasis and regulate virulence in the growth medium. For example, gene expression of CTR1 and CTR4 from C. neoformans decreases upon treatment with Cu\(^{+}\) (Ding et al., 2011).

Unlike the response to other metals, the gene expression and protein levels of Mg transporters in C. neoformans are highly stable after supplementation with Mg\(^{2+}\) ions, demonstrating significantly different regulation patterns at the transcriptional and posttranslational level for Mg homeostasis. S. cerevisiae ARI1 and ARI2 are two Mg importers localized to the plasma membrane (Graschopf et al., 2001). Knocking out both genes impairs S. cerevisiae cell growth even at relatively high Mg concentrations (4 mmol/L Mg\(^{2+}\) supplementation in the growth medium), indicating the essential function of these Mg\(^{2+}\) importers in fungal biology (Graschopf et al., 2001; Pisat et al., 2009). Interestingly, the deletion of C. neoformans MGT3 (an ortholog of plasma membrane Mg\(^{2+}\) transporters in S. cerevisiae) causes no Mg-dependent growth phenotypic difference compared to the wild-type strain. Normal growth rates are observed in the mgt1 mutant, which is an ortholog of S. cerevisiae mitochondrial Mg transporters. A double mutant for MGT1 and MGT3 also has a comparable phenotype as the wild-type C. neoformans strain, and no defects in cell growth, melanin, capsule formation, or virulence are observed in the animal model. These results imply that the C. neoformans genome encodes other Mg\(^{2+}\) transport factors that facilitate Mg\(^{2+}\) import into the cytosol and mitochondria.

A C. neoformans MGT2-repressing mutant has a reduced intracellular Mg\(^{2+}\) content and cell growth defects under normal growth conditions, resembling the phenotype of S. cerevisiae (Pisat et al., 2009). Moreover, S. cerevisiae Mnr2 functionally complements C. neoformans Mgt2, and the C. neoformans Mgt2 has vacuolar protein localization. Taken together, our work demonstrates the conserved function of Mnr2 in C. neoformans and S. cerevisiae. Because of the striking cell growth defects in the mgt2 mutant, we speculate that the mgt2 mutant strain would have an impaired virulence and fungal burden in lung tissue. Surprisingly, our animal infection studies showed minor increases in mortality in the animals infected with the mgt2 mutants, in addition to significant inductions in the fungal burden in the lung tissue. Further analysis indicated that the increased fungal proliferation in the host results from an enlarged capsule structure when expression of Mgt2 was repressed, leading to the inhibition of macrophage phagocytosis. In agreement with the observation of capsule enlargement in the mgt2 mutant, the overexpression of Mgt2 resulted in less capsule production. Furthermore, the reduction in the

In our study, three Mg\(^{2+}\) transporter gene orthologs are identified in the genome of C. neoformans, based on BLAST searches using S. cerevisiae Mg transporters. Many key players involved in metal homeostasis are regulated transcriptionally by metal concentrations in the growth medium. For example, gene expression of CTR1 and CTR4 from C. neoformans decreases upon treatment with Cu\(^{+}\) (Ding et al., 2011).

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capsule is inversely correlated with the addition of exogenous Mg\(^{2+}\) ions, demonstrating a direct connection between Mg\(^{2+}\) homeostasis and capsule production in *C. neoformans*. This highlights the important function of Mgt2 in controlling the capsule structure. Despite our data showing melanin formation defects in the Mgt2-repressing strain, pigment development is not fully complemented when exogenous Mg is supplemented, indicating that both Mg\(^{2+}\) and Mgt2 are required for proper melanin production in *C. neoformans*.

It is interesting that Mgt2 has a positive effect on the cell growth, which is independent of intracellular Mg level. This result indicates the potential novel functions of Mg\(^{2+}\) transporters in *C. neoformans*. In fact, Mg\(^{2+}\) transporters have been demonstrated to be involved in the RNA group II splicing process in *S. cerevisiae* (Gregan et al., 2001; Sponder et al., 2010). While it is still unclear what the regulation mechanism is in, it is possible that the Mgt2-repressing strain dramatically dampens the intracellular Mg levels, and thus impairs the RNA splicing process.
in C. neoformans, thus blocks the gene expression. Using transcriptomic analysis, we show here that C. neoformans Mgt2 differentially regulates the expression of 19 genes, regardless of Mg concentration. We observed genes encoding alternative cyclin Pcl12 and carnitine O-acetyltransferase were depressed in expression when Mgt2 was repressed. Previous studies have demonstrated the critical functions of Pcl12 and carnitine O-acetyltransferase in the regulation of cell cycle progression and cell morphology (Brunner, Kramar, Denhardt, & Hofbauer, 1997; Flor-Parra, Castillo-Lluva, & Perez-Martin, 2007; Perez-Martin & Castillo-Lluva, 2008). Therefore, it is most likely that the growth defects of the GAL7-MGT2 strain after Mg treatment is due to the inhibition of gene expression of cell proliferation regulators.

Despite a previous study using semiquantitative RT-PCR showing that Mg\(^{2+}\) influences the expression of genes involved in capsule production, we do not detect capsule-related RNA expression differences in our RNA-seq analysis. This is probably because different strain isolates were used in each study, causing different responses to metal ions (Sun et al., 2014; Waterman et al., 2012; Zhang et al., 2016). In fact, C. neoformans is a fast-evolving human fungal pathogen. Comparative genomic analyses have demonstrated inconsistent phenotypic variations in stress responses, mating and virulence factor expression among H99 lineages because of these microevolutionary processes (Bahn, Hicks, Giles, Cox, & Heitman, 2004; Geddes et al., 2016; Gong, Grodsky, Zhang, & Wang, 2014; Janbon et al., 2014; Maeng et al., 2010; Pukkila-Worley & Alspaugh, 2004; Shen, Wang, Whittington, Li, & Wang, 2008). Most importantly, the enlargement of capsule structure and increased fungal lung proliferation in MGT2-repressed cells may also be determined by other factors, because C. neoformans Mgt2 differentially regulates the expression of 19 different genes. Previous studies demonstrated that C. neoformans capsule formation is induced by low glucose conditions, via the activation of the cAMP-PKA pathway, which is also an extremely important regulator of C. neoformans virulence. Interestingly, the expression of the glucose transporter (CNAG_03772) is repressed when Mgt2 expression is inhibited. It is likely that the mgt2 mutant activates the cAMP-PKA pathway via intracellular glucose deficiency, which in turns, causes capsule structure induction and increases lung fungal burden. Therefore, we suggest that multiple inputs are needed for the capsule development and the induction of fungal virulence in the mgt2 mutant. Moreover, we also detected that Mgt2 regulates 13 hypothetical genes that may play important roles in C. neoformans capsule formation.

In conclusion, we have demonstrated an evolutionarily conserved role for a vacuolar Mg transporting protein in C. neoformans, and highlighted the important function of Mg\(^{2+}\) in modulating C. neoformans virulence factors.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

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