Role of clathrin-mediated endocytosis in the use of heme and hemoglobin by the fungal pathogen Cryptococcus neoformans

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Abstract
Heme is a major source of iron for pathogens of humans, and its use is critical in determining the outcome of infection and disease. Cryptococcus neoformans is an encapsulated fungal pathogen that causes life-threatening infections in immunocompromised individuals. C. neoformans effectively uses heme as an iron source, but the underlying mechanisms are poorly defined. Non-iron metalloporphyrins (MPPs) are toxic analogues of heme and are thought to enter microbial cells via endogenous heme acquisition systems. We therefore carried out a mutant screen for susceptibility against manganese MPP (MnMPP) to identify new components for heme uptake in C. neoformans. We identified several genes involved in signalling, DNA repair, sugar metabolism, and trafficking that play important roles in susceptibility to MnMPP and in the use of heme as an iron source. We focused on investigating the role of clathrin-mediated endocytosis (CME) and found that several components of CME including Chc1, Las17, Rvs161, and Rvs167 are required for growth on heme and hemoglobin and for endocytosis and intracellular trafficking of these molecules. We show that the hemoglobin uptake process in C. neoformans involves clathrin heavy chain, Chc1, which appears to colocalise with hemoglobin-containing vesicles and to potentially assist in proper delivery of hemoglobin to the vacuole. Additionally, C. neoformans strains lacking Chc1, Las17, Rvs161, or Rvs167 were defective in the elaboration of several key virulence factors, and a las17 mutant was avirulent in a mouse model of cryptococcosis. Overall, this study unveils crucial functions of CME in the use of heme iron by C. neoformans and reveals a role for CME in fungal pathogenesis.

KEYWORDS
CHC1, clathrin, Cryptococcus, endocytosis, heme, hemoglobin, iron

1 | INTRODUCTION

Cryptococcus neoformans is an encapsulated fungal pathogen that causes life-threatening infections in immunocompromised individuals, especially HIV/AIDS patients (Kronstad et al., 2011; Kwon-Chung et al., 2014). A recent study estimated that 223,100 individuals acquired cryptococcal meningitis globally in 2014, with a very high mortality rate of nearly 81% (Rajasingham et al., 2017). The common virulence traits of C. neoformans include cell surface elaboration of capsule and melanin, production of extracellular enzymes such as urease and phospholipase B, and adaptation to the host environment including growth at 37°C, sensing nutritional cues and metal-ion availability and evading the host immune system (Almeida, Wolf, & Casadevall, 2015; Campuzano & Wormley, 2018; Djordjevic, 2010; Djordjevic, Del Poeta, Sorrell, Turner, & Wright, 2005; Doering, 2009; Leopold Wager, Hole, Wozniak, & Wormley, 2016).
Sensing and adapting to the availability of metal ions such as iron and copper in the host environment are pivotal for any pathogen to survive and proliferate (Bairwa, Jung, & Kronstad, 2017; Hood & Skaar, 2012; Palmer & Skaar, 2016; Skaar, 2010). Iron is an essential metal required for several enzymatic and biochemical processes in eukaryotic cells, and the metal plays a crucial role in the virulence of microbial pathogens including C. neoformans (Bairwa et al., 2017; Jung et al., 2008; Jung & Kronstad, 2008; Skaar, 2010; Weinberg, 2009). The ability to use host iron sources, such as heme/hemoglobin and transferrin, is advantageous for the growth and survival of C. neoformans inside a vertebrate host (Jung et al., 2008). Previous studies have shown key roles for the ESCRT proteins Vps23, Vps22, and Snf7 in the use of heme iron and in the virulence of C. neoformans (Hu et al., 2013; Godinho et al., 2014; Hu et al., 2015). Additionally, a putative hemophore, Cig1, was discovered to be involved in heme binding and uptake and in virulence (Cadieux et al., 2013). Despite some knowledge of the machinery for heme uptake and use in C. neoformans, the detailed mechanism of heme-iron uptake, trafficking, metabolism, storage, and regulation in this pathogen remain unknown.

Non-iron metalloporphyrins (MPPs) possess strong antibacterial, antifungal, and antiparasitic activities (Stojilkovic, Evavold, & Kumar, 2001; Stojilkovic, Kumar, & Srinivasan, 1999). The uptake of these compounds in bacteria is thought to occur via high-affinity heme transport (Stojilkovic et al., 1999). Previous studies have shown that non-iron MPPs having metal ions such as gallium (Ga), manganese (Mn), zinc (Zn) ruthenium (Ru), or indium (In) at their coordination centre can have antibacterial activity against both gram-positive and gram-negative bacteria to varying degrees, with GaMPP as the most potent (Stojilkovic et al., 1999; Stojilkovic et al., 2001). Because the transport of non-iron MPPs occurs through heme uptake systems in bacteria, it may be possible to identify new components of the machinery for heme use in C. neoformans by characterising mutants with altered susceptibility to non-iron MPPs.

Clathrin-mediated endocytosis (CME) is employed by eukaryotic cells to acquire receptor-bound cargo and proteins from the cell surface. A role for endocytosis in heme uptake has been previously described for the pathogenic yeast Candida albicans (Weissman, Shemer, Conibear, & Kornitzer, 2008). Therefore, it is plausible that the CME may play a similar role in heme uptake in C. neoformans. The process of CME, beginning from cargo identification and coat assembly to coat scission and disassembly, is evolutionary conserved in eukaryotic cells with most of the proteins having well-characterised homologues in yeast and mammalian cells (Kaksonen & Roux, 2018; Lu, Drubin, & Sun, 2016). However, some differences have been reported for CME in yeast compared with higher eukaryotes (Yeung, Phan, & Payne, 1999). CME involves the ordered temporal and spatial recruitment and organisation of approximately 60 conserved proteins at the plasma membrane to internalise receptor-bound cargo in a clathrin-coated vesicle (CCV) (Ferguson et al., 2016; Kaksonen, Toret, & Drubin, 2005; Mettlen & Danuser, 2014). One of the first proteins to be recruited at the internalisation site is clathrin, and this protein is the polymeric assembly unit of a vesicular coat formed by three heavy chains and three light chains encoded by CHC1 and CLC1 in yeast, respectively (Edeling, Smith, & Owen, 2006; Kirchhausen, 2009; McMahon & Boucrot, 2011). The clathrin assembly units have a triskelion shape and interact with each other to form a polyhedral lattice surrounding the vesicle. Clathrin adaptor proteins are involved in linking the clathrin coat with the phospholipids of vesicle membranes and/or cargo transmembrane proteins (Edeling et al., 2006). The adaptor protein AP2 participates in the internalisation of several cargo proteins in mammalian cells, including the well-characterised transferrin receptor, but no such functions have been assigned to AP2 in yeast (Yeung et al., 1999). Following the early stage of assembly of a clathrin coat at the cytoplasmic face of the plasma membrane, several additional proteins including Sla2, Ent1/Ent2 and Lsb3/4/5, Pan1, End3, and Sla1 are recruited at distinct times as the process moves into intermediate and late stages of coat assembly (Feliciano & Pietro, 2012; Mettlen & Danuser, 2014; Sun, Leong, Wong, & Drubin, 2015). At the late stage of CCV formation, a group of proteins (Wiskott–Aldrich syndrome protein [WASP] and myosin) arrive to initiate CCV internalisation (Tyler, Allwood, & Ayscough, 2016). The Las17 protein, which is a yeast homologue of WASP in mammals, acts as a nucleation-promoting factor for actin assembly near the neck region to initiate the internalisation of the CCV with Myosin-3/5 (Robertson et al., 2009; Sun, Martin, & Drubin, 2006). In the final stages of yeast endocytosis, scission of the vesicle from the plasma membrane is achieved by the BAR (Bin Amphilphysin Rvs) domain proteins, Rvs161 and Rvs167, along with the yeast dynamin-related protein Vps1 (Nannapaneni et al., 2010; Rooij et al., 2010; Rooij et al., 2012). As with clathrin, other proteins associated with CCV formation are recycled into the cytosol to participate in subsequent rounds of clathrin-coated vesicle formation.

We report here the identification of several novel players in the machinery for heme use by C. neoformans through a screen for mutants with altered susceptibility to a non-iron MPP. The screen identified genes for various processes, including vesicular trafficking, transcriptional regulation, and DNA repair and signalling, that are required for the robust use of heme iron by C. neoformans. In particular, we discovered a previously unidentified role of CME for heme use and found that multiple components of CME, namely, Chc1, Las17, Rvs161, and Rvs167 are required for the endocytosis, intracellular trafficking, and use of hemin and hemoglobin. Importantly, we show that the clathrin heavy chain protein Chc1 is involved in internalisation of hemoglobin and proper intracellular trafficking. Further characterisation of the C. neoformans mutants lacking CHC1 and LAS17 revealed altered capsule and melanin production and defects in several physiological growth conditions. Additionally, loss of Las17 resulted in attenuated virulence in a mouse inhalation model of cryptococcosis. Overall, this study provides important insights into the contributions of CME in heme use and more broadly in the pathogenesis of C. neoformans and suggests the presence of a heme/hemoglobin receptor in this fungal pathogen.

## RESULTS

### 2.1 Identification of mutants with altered susceptibility to manganese MPP

Non-iron MPPs are toxic for microbes and their uptake is thought to occur via heme transport systems (Mitra, Speer, Lin, Ehrt, &
To establish conditions for screening mutants on MPs, we initially evaluated the susceptibility of a wild-type (WT) C. neoformans strain to two potent antibacterial MPs, manganese and gallium MPP (MnMPP and GaMPP, respectively; Figure 1a). Additionally, we examined the growth of the WT strain with non-iron MPPs after iron starvation. For these experiments, we employed the minimal medium yeast nitrogen base (YNB), YNB with the iron chelator BPS (iron-starved condition), YNB + BPS supplemented with hemin (iron-rescued condition), and YNB + BPS supplemented with hemin and either MnMPP or GaMPP (MPP condition). As shown in Figure 1a, the growth of nonstarved WT cells in different concentrations of MnMPP or GaMPP was comparable with the condition with hemin, suggesting that the concentrations evaluated were not toxic to WT cells containing sufficient iron. In contrast, prior starvation of WT cells for iron resulted in sensitivity to increasing concentrations of both MnMPP and GaMPP, with growth significantly reduced in the highest concentrations tested (Figure 1a). We also observed that GaMPP was toxic for the iron-starved cells at lower concentrations compared with MnMPP. These results indicated that prestarvation was necessary for mutant screening and that MnMPP at 10 μM in the presence of hemin provided a condition to identify susceptible and (potentially) resistant mutants.

We screened three independent libraries of C. neoformans strains to identify mutants with altered susceptibility to MnMPP. Two libraries of gene deletion mutants were generated by Dr. Hiten Madhani’s group at the University of California, San Francisco (designated the UCSF-2008 and UCSF-2015 collections, arranged in 96-well plates), and obtained from the Fungal Genetics Stock Center (FGSC, USA; Liu et al., 2008). A third collection designated the TFKO library consists of mutants deleted for 155 putative transcription factors of C. neoformans (Jung et al., 2015). The two UCSF library collections of ~3,300 mutants were arranged in 36 plates, and the transcription factor deletion strains were arranged in four plates. The strains were screened in a 96-well format using liquid YNB medium containing BPS and hemin, with and without MnMPP, as described in Section 4. The screen was initiated by starving the mutants for iron for 2 days in YNB with BPS in the 96-well format, followed by inoculating the starved cells into test media and measuring their growth by absorbance at OD600 after 72 hr (Figure 1b and described in Section 4). Figure 1c depicts the relative growth difference (in percentage) of individual strains (each denoted with a circle) in medium with hemin and MnMPP versus medium with only hemin for all three libraries. An average of relative growth difference values of all the strains in a library was determined. This average relative growth (ARG) value is denoted by the solid blue line, whereas the green and red dotted lines represent the cut-off for relative growth difference values that are 150% higher or 50% lower than the ARG. The strains that grew ≥150% more than the ARG were selected as resistant candidates, whereas strains growing ≤50% of the ARG values were selected as MnMPP sensitive mutants (Figure 1c and Table S1). Overall, the screens yielded both resistant and susceptible mutants, and we focused on the strains showing resistant to MnMPP toxicity for subsequent analysis. Our rationale was that these mutants might identify functions necessary for the uptake or use of hemin, as well as targets of MnMPP toxicity.

As shown in Figure 1c, four mutants were identified in the TFKO library as resistant strains (pip2Δ, grf1Δ, bzp5Δ, and fzc42Δ) and hap2Δ was identified as a sensitive mutant. Pip2 (CNAG_05170) is a fungal specific Zn(2)-Cys(6) binuclear cluster domain transcription factor and Grf1 (CNAG_05186) is a GRF zinc finger protein. Bzp5 (CNAG_07940) is a basic leucine zipper transcription factor and Fzc42 (CNAG_05112) is a hypothetical protein with a Zn(2)-Cys(6) domain (Jung et al., 2015). Hap2 (CNAG_07435) is a CCAAT-binding transcription factor and is an essential component of Hap2/Hap3/Hap4/Cap5 CCAAT-binding complex involved in global regulation of respiratory gene expression in Saccharomyces cerevisiae (Olesen & Guarente, 1990). In the fungal pathogen C. albicans, Hap2 has been found to be involved in regulation of low-iron induction of the FPR1 gene, which encodes a ferric reductase (Baek, Li, & Davis, 2008). Hap2 in C. neoformans is thought to interact with the Hap3, Hap5, and HapX proteins that were previously shown to be required for robust growth on heme (Jung et al., 2010).

The screens for the two UCSF libraries yielded a large number of mutants that were either sensitive or resistant to MnMPP, and a number of these are highlighted in Figure 1c. We did detect common mutants that were present in all three libraries in support of the fidelity of the screening approach. For example, we found the bzp5Δ and hap2Δ mutants in both the TFKO and UCSF-2015 libraries. Furthermore, two strains deleted for the genes CNAG_06601 or CNAG_00264 were identified as resistant in both the UCSF-2008 and UCSF-2015 libraries. Other than these examples, the candidate mutants that we identified were exclusive to one of the libraries. Notably the screens identified factors already known to be involved in iron use by C. neoformans. These included mutants for the genes STR1 (encoding a putative siderophore transporter), CFO2 (encoding a ferroxidase), and HAP2 mentioned above (Jung et al., 2008, 2010; Jung, Hu, Kuo, & Kronstad, 2009). These results support the idea that the screens captured factors relevant to iron acquisition and homeostasis. Furthermore, the mutants highlighted in Figure 1c revealed that the screen was able to capture genes involved in various cellular processes including signalling, transport, cell wall homeostasis, and transcriptional regulation; these functions suggest that C. neoformans showed an elaborate environmental stress response to MnMPP as described below.

2.2 Mutants with resistance to MnMPP represent diverse GO categories and are enriched for functions in sugar metabolism, DNA synthesis and repair, and transcriptional regulation

We concentrated on the resistant collection of mutants in our subsequent analyses in this study. In total, 151 mutants were obtained from the two FGSC libraries and four mutants were identified from the TFKO library; these were analysed for their functional annotations and potential interactions. Of the 155 mutants, two corresponding genes, CNAG_03546 and CNAG_03505, could not be identified in the FungiDB database (http://fungidb.org). To perform Gene
FIGURE 1  Identification of cryptococcal mutants with altered susceptibility to manganese metalloporphyrin (MnMPP). (a) Growth profile at 72 hr for iron-starved or nonstarved WT cells in YNB medium containing different concentrations of either manganese or gallium metalloporphyrin. The results are the means ± standard errors of the mean (SEM) from three independent experiments. (**P < 0.001). (b) Graphical outline of the strategy used to screen the deletion mutant libraries against MnMPP. Plate A; YNB, Plate B; YNB with BPS (150 μM), Plate C; YNB with BPS (150 μM) and hemin (10 μM) [hemin media] and Plate D; YNB with BPS (150 μM), hemin (10 μM) and MnMPP (10 μM) [MnMPP media]. (c) Growth profile of individual strains of the three libraries screened against MnMPP, representing the relative growth differences in percentage between the MnMPP and hemin media conditions at 72 hr. The blue line represents the average relative growth (ARG) value calculated from the relative growth difference values of all the strains in a library. The red line marks the value which is 50% of the ARG, while the green line marks the value at 150% of the ARG. Circles filled with green colour highlight some of the interesting mutants showing resistance to MnMPP. Red filled circles represent some of the interesting mutants that are sensitive to MnMPP.
Ontology (GO) and STRING analyses, we retrieved the orthologous C. neoformans JEC21 gene names for 146 of the remaining 153 genes from the FungiDB database. Unlike the serotype A strain of C. neoformans that was used for our screen, the JEC21 genome information has been organised to support GO and STRING analyses, and the JEC21 gene designations were used for the presentation of the data in Figure 2.

The GO analysis revealed a large representation of the genes involved in metabolic processes (31%), response to stimulus (10%), and biological regulation (5%). Among these, there was enrichment for genes involved in glucose metabolism and especially gluconeogenesis (e.g., CNA00280 encoding glucose-6-phosphate isomerase and CNF00650 encoding pyruvate carboxylase; Figure 2a,b). Furthermore, a large number of genes in the category of response to stress were involved in the cellular response to DNA damage and mismatch repair (e.g., FEN1 encoding Flap endonuclease, MSH2/CNA07480, CNA03100 encoding a single-stranded DNA specific endodeoxyribonuclease, and two uncharacterised genes: CNK01700 and CND04530), suggesting that DNA stress is a substantial component of MnMPP toxicity. One interesting category of genes was enriched in the list of mutants involved in defective vesicle coat and the associated processes of endocytosis and trafficking. This group was enriched in the list of mutants conferring MnMPP resistance and included CNJ03270 encoding the clathrin heavy chain, CNA03420 encoding pyruvate carboxylase; Figure 2a,b).

A STRING network analysis was performed to reveal clusters of interactions between functionally associated gene products. Most notable interactions and clusters were obtained for the genes involved in (a) Hog1-related stress signalling including HOG1, which encodes a mitogen-activated protein (MAP) kinase, CNB1 encoding the calcineurin subunit B, CNA07470/PBS2 encoding a MAP kinase, SCH9 encoding a protein kinase, and CNH01060 encoding a calmodulin-dependent protein kinase-1; (b) functions for gluconeogenesis (e.g., CNA00280 encoding an alcohol dehydrogenase, CNF03900 encoding an aldehyde dehydrogenase, CND03740 encoding aldehyde reductase); and (c) DNA synthesis and mismatch repair (e.g., FEN1, MSH2, CNA03100, CNG03940, CNK01700, and CND04530; Figure 2c). Another small cluster contained genes encoding candidate transporters including CNL06490 (ABC transporter), CNCO0270 (a multidrug resistance protein), and CNCO3290 (tetracycline efflux protein; Figure 2c). Taken together, these results illustrate the diversity of functions targeted by MnMPPs and suggest that the compound provokes stress and metabolic responses.

### 2.3 MnMPP resistant mutants show sensitivity to a diverse set of stress conditions

To better understand the functional importance of the genes involved in the resistance to MnMPP, we characterised 50 MnMPP resistant mutants for growth under diverse environmental conditions including host temperature (37°C), high salt (e.g., NaCl), trafficking inhibition (brefeldin A), cell wall damage (calcifluor white and congo red), challenges to protein folding (tunicamycin and dithiothreitol), and stress from reactive oxygen species (H₂O₂). As shown in Figures 2d and S1, several of the mutants (e.g., hog1Δ, cnb1Δ, cpA2Δ, ade5Δ, chc1Δ, and strains deleted for CNAG_04091, CNAG_05159, and CNAG_06314) showed susceptibility to a variety of stress conditions. For the strains lacking HOG1 or CNB1, the defect at 37°C has been previously observed (Bahn, Kojima, Cox, & Heitman, 2005; Fox et al., 2001). Among the 50 mutants tested, 10 strains showed susceptibility to at least five stress conditions (Figure S1). Importantly, we did not observe any common phenotypic themes for the strains deleted in the same functional classes of genes. That is, the phenotypic analysis of the selected strains did not reveal enrichment for any specific growth defects under any of the conditions tested.

### 2.4 Chc1 is required to use heme-iron sources in C. neoformans

Our screen revealed an important role for genes encoding endocytic and trafficking functions in the susceptibility of C. neoformans to MnMPP. Given that the uptake of non-iron MPPs is thought to require heme transport machinery in bacteria (Stojilkovic et al., 1999), we carried out a detailed phenotypic characterisation of CHC1 (CNAG_04904), which encodes the clathrin heavy chain with a well-established role in endocytosis. CHC1 is an evolutionary conserved gene and is not essential in C. neoformans, unlike the situation in higher eukaryotes. CHC1 in C. neoformans encodes a 1,684 amino acid protein containing seven clathrin heavy chain repeat sequences of 145-amino acid residues each (Figure 3a). These repeats are arranged tandemly in the C terminal portion of the protein and are evolutionary conserved in different kingdoms (Figure 3a). Earlier studies have shown that the clathrin heavy chain repeat is involved in protein–protein interactions or clathrin binding (Ybe et al., 1999; Young, 2007). Notably, our phylogenetic analysis revealed greater similarity between Chc1 of Cryptococcus spp. and mammalian counterparts compared with the Chc1 proteins of the yeasts S. cerevisiae and C. albicans (Figure 3b). As reported for S. cerevisiae, deletion of CHC1 in C. neoformans also resulted in abnormal cell morphology, defects in cell division, and impaired chitin deposition at the mother–daughter bud-neck region (Figure 4a). In comparison with the WT strain, which has nearly 80% of the cell population with chitin at mother–daughter bud-neck region, only 20% of the cell population in the chc1Δ strain showed the chitin deposition at bud neck (Figure 4b). Our phenotypic analysis with the chc1Δ mutant also showed that its growth was inhibited by the antifungal drug fluconazole (Figure 4c). Furthermore, supplementation with hemin in the fluconazole medium did not rescue the growth defect of the chc1Δ mutant. In contrast, growth of the WT strain could be rescued by hemin even at higher fluconazole (10 μg ml⁻¹) concentrations (Figure 4c). This result suggested that the chc1Δ mutant was unable to efficiently take up hemin to rescue the activity of the heme-dependent lanosterol demethylase targeted by fluconazole.

The possible role for Chc1 in hemin uptake prompted us to evaluate the role of the protein in iron acquisition by analysing the growth of the chc1Δ strain in iron-starved conditions and in the presence of different iron sources. We first generated two independent chc1Δ
FIGURE 2  Gene Ontology and interaction analyses of genes involved in MnMPP resistance. (a) Gene Ontology analysis of the genes involved in MnMPP resistance for the categories of biological process and molecular functions. The H99 genes were matched to the Cryptococcus neoformans JEC21 gene IDs retrieved from the FungiDB (http://fungidb.org/fungidb/) database and analysed with the PANTHER gene ontology online tool (http://pantherdb.org) using C. neoformans as the organism. (b) Functional enrichment analysis of genes involved in MnMPP resistance. Genes were analysed for enrichment of the GO biological process, molecular function, and cellular component categories. Enriched groups were scored by comparison with a background list of the C. neoformans H99 genome database using a cut-off of $P < 0.05$ and Bonferroni correction. (c) STRING network analysis of the C. neoformans genes involved in MnMPP resistance. Interaction network details were obtained with JEC21 gene IDs from the STRING online tool (https://string-db.org). The network was extracted and visualised with Cytoscape 3.6.1 software (http://www.cytoscape.org) and clustering was performed with the clusterMaper plugin using the community cluster (GLay) network algorithm. Individual clusters are marked with an elliptical circle. (d) Heatmap depicting the growth behaviour of selected MnMPP resistant mutants in various environmental stress conditions. Growth of the individual strains at 30°C, 37°C, and in presence of hemin (10 μM), NaCl (1.5 M), brefeldin A; BFA (20 μg ml$^{-1}$), tunicamycin (0.15 μg ml$^{-1}$), dithiothreitol; DTT (10 mM), H$_2$O$_2$ (1 mM), calcofluor white (1 mg ml$^{-1}$), and congo red (2.5 mg ml$^{-1}$) was checked and displayed with colour gradients (indicated as percentages of the WT).
(chc1Δ-1 and chc1Δ-2) mutants in the C. neoformans H99 (serotype A) background using homologous recombination. Next, we starved the WT, chc1Δ, and complemented strains for iron by growing them in YNB medium with BPS (YNB-LIM). We then tested growth by spot assays on YNB and YNB-LIM agar plates with and without inorganic iron sources (FeCl₃ or FeSO₄) or hemin. As shown in Figure 4d, the iron-starved WT, chc1Δ, and the complemented strains could grow well on rich medium (YPD) and the minimal medium YNB, but not on medium depleted for iron (YNB-LIM). Supplementing YNB-LIM with FeCl₃, FeSO₄, or hemin allowed the WT and complemented strains to grow similar to the levels seen on YPD and YNB. However, the chc1Δ mutants could only grow on YNB-LIM supplemented with either FeCl₃ or FeSO₄ but not with hemin at any concentration. In fact, the mutants appeared to show even less growth at the highest heme level, perhaps reflecting greater susceptibility to the toxicity of heme relative to the WT or complemented strains. We also performed the growth assays in liquid YNB media supplemented with hemin or hemoglobin. As shown in Figure 5, the chc1Δ mutants could not grow to the level of the WT strain on any of the concentrations of hemin and hemoglobin tested. Reintroduction of CHC1 in chc1Δ strain completely rescued the growth defects on the low-iron media supplemented with hemin or hemoglobin (Figures 4d and 5). Notably, growth of the chc1Δ mutant did not improve even in the presence of high amounts of hemin/hemoglobin in the medium (Figures 4d and 5), suggesting that the Chc1 could be one of the major factors in utilisation of heme-iron in C. neoformans; that is, its absence appears to severely diminish the ability of cells to uptake hemin and hemoglobin. Additionally, we found that the chc1Δ mutants showed increased sensitivity to the drug curcumin, an iron-chelator found in turmeric, a traditional Indian spice and medicine (Figure S2). Addition of the inorganic iron compounds FeCl₃ and FeSO₄ but not hemin could rescue the growth defect of the chc1Δ mutants on curcumin. All together, these findings support a major role for Chc1 in the use of heme iron by C. neoformans. Additional phenotypes of the chc1Δ mutants are presented below, including aspects of growth on carbon and nitrogen sources.

2.5 | Loss of Chc1 impairs endocytosis and trafficking

We next examined the role of Chc1 in endocytosis and trafficking in C. neoformans. In S. cerevisiae, Chc1 is a key component of CME and plays an important role in internalisation of several factors such as receptor-bound α-factor involved in mating and in the proper localisation of several proteins (Baggett & Wendland, 2001). We initiated a characterisation of Chc1 by tagging the protein at the C-terminus with green fluorescence protein (GFP) to assess its

FIGURE 3 Sequence analysis of the clathrin heavy chain 1 protein in fungi and higher eukaryotes (a) Predicted domain architecture of the Cryptococcus neoformans Chc1 protein and multiple alignment of the first clathrin heavy chain repeat (CHCR) sequences of the Chc1 proteins from selected organisms. The amino acid sequence alignment was performed with Clustal W (http://www.ch.embnet.org); identical residues are boxed in black (indicated with *) and similar residues are shown in grey (indicated with ●). The numbers on the left represent the starting position of the CHCR repeat sequence in the protein from the respective organism. (b) Phylogenetic tree of the Chc1 proteins from selected organisms. The phylogenetic analysis of the Chc1 protein was performed in MEGA7 (https://www.megasoftware.net) by using the Maximum Likelihood method and applying Neighbor-Join and BioNJ algorithms. The tree is drawn to scale, with branch lengths measured in the number of substitution per site. CnA, C. neoformans var. grubii, H99 (serotype A); CnD, C. neoformans var. neoformans JEC21 (serotype D); Cg, C. gattii; Sc, Saccharomyces cerevisiae; Ca, Candida albicans; Hs, Homo sapiens; Mm, Mus musculus
location in cells grown in YNB and low-iron YNB with and without hemin. The strain expressing Chc1-GFP had no discernable phenotypic defects in comparison with the WT strain. Importantly, introduction of the GFP-tagged version of Chc1 in the chc1Δ mutant fully rescued the growth defects of the strain (Figures 4d and 5). As shown in Figure 6a, the Chc1-GFP protein was localised in punctate structures in cells grown in YNB, and we hypothesise that these structures represent the trans-Golgi network (TGN), as has been previously shown for Chc1 in S. cerevisiae. Clathrin is known to mediate cargo selection and vesicle fission at the TGN and treatment with...
brefeldin A, an inhibitor of endoplasmic reticulum to Golgi trafficking, impairs assembly of the clathrin-dependent Golgi apparatus (Radulescu, Siddhanta, & Shields, 2007). Therefore, we treated the cells expressing Chc1-GFP with BFA to determine whether localisation of Chc1-GFP was altered. Incubation of the cells with BFA for an hour caused reorganisation of the Chc1-GFP puncta from the cytosol to the plasma membrane with nearly 40% of the cells showing an increase in plasma membrane accumulation of Chc1-GFP after treatment (Figure S3A,B). This result suggests that Chc1-GFP in C. neoformans localises to the TGN or endocytic vesicles similar to Chc1 in S. cerevisiae. We also examined whether localisation of Chc1-GFP was altered in different conditions of iron availability including iron-starvation and in presence of hemin. Our time course analysis revealed that under both iron-starvation condition or in presence of hemin, no apparent changes in the localisation of Chc1-GFP was observed (Figure S3C). Under both these conditions, Chc1-GFP localised to the endocytic vesicles similar to the observed pattern for the cells grown in YNB medium.

We further examined the role of Chc1 in endocytosis by performing assays with FM4-64, a lipophilic styryl dye that binds to the plasma membrane and is then endocytosed and trafficked to the vacuolar membrane (Vida & Emr, 1995). A defect in endocytosis either prevents or delays the trafficking of FM4-64 to the vacuolar membrane. As shown in Figure 6b, most of the FM4-64 dye was internalised within 10 min of binding to the plasma membrane in WT cells. Furthermore, trafficking of internalised FM4-64 dye to the vacuolar membrane was completed by 30 min in the WT cells. In contrast, the chc1Δ mutant showed a significant delay in FM4-64 endocytosis and trafficking. Specifically, the internalisation of plasma membrane-bound FM4-64 took more than 20 min, and trafficking to the vacuolar membrane was only partially completed by 90 min, a rate that was significantly (~threefold) slower than the WT cells.

**FIGURE 5** The *Cryptococcus neoformans* chc1Δ mutant cannot use hemin and hemoglobin as sole iron sources. Cells of the indicated strains were starved for iron for 24 hr and then transferred to YNB-LIM medium containing either hemin or hemoglobin. Growth at 30°C was monitored by recording OD_{600} over a 96-hr time course at the indicated time intervals. Data are represented as the mean ± SEM of three to four independent experiments.
FIGURE 6  The Cryptococcus neoformans Chc1 protein is required for endocytosis and intracellular trafficking. (a) C. neoformans Chc1 was tagged with GFP at its C-terminus and its localisation was visualised in exponentially growing cells under a fluorescence microscope using the EGFP filter. White arrowheads indicate the Chc1-GFP puncta. Images are the representative of two independent experiments. Scale bar = 2 μm. (b) The absence of CHC1 results in a significant delay in FM4-64 endocytosis and trafficking to the vacuole. YPD-grown cells of the indicated strains were labelled with FM4-64 on ice for 20 min and were visualised under a fluorescence microscope after the indicated times of incubation at room temperature. FM4-64 was visualised using a rhodamine filter. Images are representative of two independent experiments. Scale bar = 5 μm. (c) Chc1 in C. neoformans colocalises with FM4-64 labelled structures. The WT strain expressing Chc1-GFP was labelled with FM4-64 and visualised under a fluorescence microscope after the indicated times of incubation at room temperature. Images are the representative of two independent experiments. Scale bar = 2 μm
Importantly, the defect in endocytosis of FM4-64 in the chc1Δ mutant was completely recovered to the WT level after complementation with the CHC1-GFP gene fusion at its native locus. To further examine the dynamics of Chc1-GFP localisation during endocytosis, we followed the FM4-64 trafficking in the cells expressing Chc1-GFP. We observed that a portion of the Chc1-GFP puncta showed colocalisation with FM4-64-labelled structures at both 0 and 30 min of time-course analysis (Figure 6c). Taken together, these data indicate that Chc1 in C. neoformans plays an important role in endocytosis, as described for the protein in other eukaryotes.

### 2.6 Chc1 is required for the uptake and trafficking of hemoglobin

Hemoglobin- and heme-containing proteins provide a major potential source of iron for pathogens because ~80% of iron resides in heme in vertebrate hosts. Pathogens have therefore developed mechanisms to obtain iron from heme and hemoglobin to support proliferation during infection (Hood & Skaar, 2012; Palmer & Skaar, 2016). We previously demonstrated that C. neoformans can use heme and hemo- globin as sources of iron (Jung et al., 2008). We hypothesised that Chc1 would affect the uptake or endocytosis of hemoglobin given that mutant lacking Chc1 failed to robustly proliferate with this iron source. To examine the role of Chc1 in hemoglobin utilisation, we labelled the human hemoglobin with the fluorescent dye Alexa Fluor 594 and investigated the kinetics of uptake and internalisation by chc1Δ cells. Iron-starved cells from the WT, chc1Δ, and chc1Δ/CHC1-GFP complemented strains were incubated in YNB-LIM containing Alexa Fluor 594-labelled hemoglobin (HbAlexa594) for 20 min on ice. The cells were then shifted to room temperature and observed under a fluorescence microscope to visualise the HbAlexa594 cell-surface binding, uptake, and trafficking at different time intervals. As shown in Figure 7a, labelled HbAlexa594 could be observed on the cell surface of the WT, chc1Δ, and chc1Δ/CHC1-GFP complemented strains after 5 min. By 30 min, we observed that most of the HbAlexa594 was internalised and endocytosed into small endocytic vesicles in both WT and chc1Δ/CHC1-GFP complemented strains. In contrast, internalisation of the cell surface bound HbAlexa594 was significantly impaired in the chc1Δ and most of the labelled hemoglobin remained on the cell surface, at both 5- and 30-min time intervals, as observed by simultaneous staining of the cells with the cell-wall staining dyes, Fluorescent Brightener 28 or Lucifer Yellow (Figures 7a and S4A,B). Incubation for a longer time (60 min) also did not lead to the internalisation of HbAlexa594 in the chc1Δ strain (Figure S4A), suggesting a severe defect in the endocy- tosis of hemoglobin.

To better understand the role of Chc1 in hemoglobin uptake, we followed the trafficking of HbAlexa594 in the WT cells expressing Chc1-GFP to determine whether Chc1 associates with the labelled hemoglobin during endocytosis. Incubation of the strain expressing Chc1-GFP with HbAlexa594 revealed that the Chc1-GFP protein colocalises with a subset of HbAlexa594 puncta after 10 and 30 min of labelling (Figure 7b,c). We carried out a colocalisation analysis on images of whole cells using the coloc 2 test in ImageJ software. Positive correlations for the colocalisation between Chc1-GFP and HbAlexa594 puncta were assessed by Pearson’s R value and Costes P value (Dunn, Kamocka, & McDonald, 2011). Despite the likely transient nature of the colocalisation between Chc1-GFP and HbAlexa594, nearly 11% of the cell population having HbAlexa594 staining also showed colocalisation for a subset of Chc1-GFP and HbAlexa594 puncta at the 10-min interval with a mean Pearson’s R value of 0.256 ± 0.073 (Figure 7b,c). Importantly, by 30 min, the cell population with colocalisation between a subset of Chc1-GFP and HbAlexa594 puncta was significantly increased to 30% with a mean Pearson’s R value of 0.398 ± 0.077, emphasising the dynamic nature of the interaction between Chc1-GFP and HbAlexa594 puncta (Figure 7b,c). It is important to note that not all the puncta of Chc1-GFP colocalises with HbAlexa594, suggesting that there may be additional trafficking pathways for hemoglobin in C. neoformans including the involvement of ESCRT complexes as reported earlier (Hu et al., 2015). Based on these results, we hypothesised that the poor ability of the chc1Δ mutant to proliferate in different heme-iron sources and the severe defect in internalisation of hemoglobin would lead to reduced heme levels in the chc1Δ cells. As expected, the total heme content of the cells of the chc1Δ mutant grown in rich medium was reduced by twofold compared with the WT strain. The heme levels were restored back to the WT levels in the chc1Δ/CHC1-GFP complemented strain (Figure 7d). It is important to note here that the residual heme levels detected in the chc1Δ mutant could be due to functional heme-biosynthesis machin- ery, which we speculate was not altered. Overall, these results high- light a major contribution of Chc1 in hemoglobin uptake, endocytosis, and trafficking.

### 2.7 Loss of Chc1 affects the elaboration of key virulence factors

C. neoformans survival inside the human host depends on elaboration of several key virulence determinants such as capsule and melanin production and the ability to grow at 37°C. We examined these phe- notypes and found that deletion of CHC1 completely abolished growth at 37°C. We characterised the chc1Δ mutant for capsule elaboration by growing cells in capsule inducing medium and observ- ing capsule size by negative staining with India ink. Despite having a comparatively larger cell size, the capsule size of the chc1Δ mutant was reduced by twofold compared with the WT strain (Figure 8a, b). Additionally, melanin production was reduced in the chc1Δ mutant compared with the WT strain on both solid and liquid L- DOPA media (Figure 8c,d). Importantly, all these defects for the chc1Δ mutant were recovered to the WT level in the chc1Δ/CHC1-GFP complemented strain.

To further understand the role of Chc1, we analysed the pheno- types of the chc1Δ mutant under different stress conditions including salt, osmotic, and oxidative stresses, inhibition of secretion, use of alternate carbon and nitrogen sources, stressors of the endoplasmic reticulum/protein folding, and agents that challenged cell wall integ- rity. As shown in Figure 8e, lack of CHC1 caused significant growth inhibition on the salts NaCl, KCl, and LiCl. Sensitivity to LiCl was consistent with the reduced capsule phenotype of the chc1Δ mutant.
FIGURE 7  Chc1 is required for the endocytosis and trafficking of hemoglobin in Cryptococcus neoformans. (a) To assess the uptake of labelled hemoglobin (HbAlexa594), WT, chc1Δ, and chc1Δ/CHC1-GFP complemented strains were incubated with HbAlexa594 and the cell-wall labelling dye Fluorescent Brightener 28 (FB-28) at 4°C and subsequently shifted to room temperature for the indicated times. Representative images of three independent experiments are shown. White triangles indicate the presence of HbAlexa594. Scale bar = 5 μm. (b) Chc1 in C. neoformans colocalises with hemoglobin containing vesicles. The WT strain expressing Chc1-GFP was incubated with HbAlexa594 and visualised under a fluorescence microscope at the indicated time points of incubation at room temperature. Representative images of three independent experiments are shown. The white triangles indicate the colocalisation of Chc1-GFP and HbAlexa594. Scale bar = 2 μm. (c) Quantification of the Chc1-GFP expressing cell population showing HbAlexa594 labelling (HbAlexa594 + ve) and colocalisation of Chc1-GFP and HbAlexa594 (HbAlexa594 + ve and GFP + ve) after 10 and 30 min. Data represent the analysis for at least 50 cells with mean ± SEM of three independent experiments. (**P < 0.01). (d) The chc1Δ strain exhibits reduced intracellular heme levels. Total heme content was measured from the cells grown overnight in rich YPD medium and is represented relative to the WT levels. C. neoformans atm1Δ and atm1Δ/ATM1-GFP complemented strains were used as the controls. Data represent the mean ± SEM of four independent experiments.
because we previously found a connection between capsule and LiCl (Hu et al., 2007). Furthermore, chc1Δ strain was unable to grow in the presence of the secretion inhibitors brefeldin A (BFA) and monensin, which is consistent with a role for Chc1 in intracellular trafficking. We also observed significant growth inhibition of the chc1Δ mutant in the presence of calcofluor white (CFW) and congo red that challenge cell wall integrity. Additionally, the chc1Δ mutant grew poorly in the presence of SDS, suggesting an altered plasma membrane composition (data not shown). In contrast to this, chc1Δ mutant was able to grow on alternate carbon sources including glycerol, ethanol, and...
sodium acetate, in the presence of ER stress agents tunicamycin and DTT, the oxidative stress agent H2O2, and in different pH conditions (Figure 8e and data not shown). We did observe slightly reduced growth on a high concentration (2%) of sodium acetate. This growth defect could be attributed to the increased sodium content in the medium to which the chc1Δ mutant showed sensitivity. Additionally, no defects were observed for the chc1Δ mutant in the use of various nitrogen sources including ammonium sulfate, L-glutamate or urea, and in the presence of copper or zinc chelators with or without metal supplementation (Figure S5). Taken together, these results suggest that in addition to the role in endocytosis and intracellular trafficking of heme-iron sources, Chc1 plays an important role in the response to salt, osmotic, and temperature stress and contributes to cell wall physiology in C. neoformans. Notably, despite its essential roles in surviving stress conditions, Chc1 in C. neoformans does not play any readily apparent role in utilising carbon and nitrogen sources and surviving ER and oxidative stresses.

2.8 Additional components of CME contribute to the use of heme and are required for virulence in mice

We also characterised the role of other components of CME in the use of iron from heme and hemoglobin. Strains deleted for the genes involved in different steps of CME were identified in the UCSF libraries and characterised for their ability to use different iron sources. Specifically, we tested mutants for the genes LAS17, BBC1, YAP1801, SLA1, BZZ1, ARP2, CAP1, CAP2, SAC6, ARK1, PFY1, RVS161, and RVS167. A serial dilution spot assay on different iron sources revealed that strains lacking LAS17 (encoding a homologue of WASP) as well as RVS161 and RVS167 (encoding amphiphysin-like lipid raft proteins) showed impaired growth on low-iron media containing hemin as an iron source, similar to the chc1Δ mutant (Figure 9a). Importantly, as observed for the chc1Δ mutant, the growth defects of the las17Δ, rvs161Δ, and rvs167Δ mutants were also exclusively observed for the hemin and not for the inorganic iron sources, FeCl3 and FeSO4 (Figure 9a). Notably, the inability to utilise iron from heme was even more pronounced in the las17Δ mutant compared with the chc1Δ strain, suggesting a crucial role of Las17 in heme-iron utilisation in C. neoformans. To confirm this phenotype, we reconstructed two independent las17Δ mutants (las17Δ-1 and las17Δ-3) in the H99 strain background and examined their growth on hemin or hemoglobin in liquid media assays. As expected, the las17Δ mutants were unable to grow properly in presence of either hemin or hemoglobin unlike the WT strain (Figure 9b). These results further support a role for CME specifically in the use of iron from heme.

We focused on las17Δ for further phenotypic characterisation. As observed for the chc1Δ mutant, the two independent las17Δ mutants are also required for heme-iron utilisation in Cryptococcus neoformans. (a) 10-fold serial dilutions of iron-starved cells of the indicated strains were spotted on medium supplemented as shown including BPS (100 μM), FeCl3 (100 μM), FeSO4 (100 μM), and hemin (100 or 500 μM). All of the plates were incubated for 3–4 days at 30°C before being photographed. (b) Iron-starved cells of the indicated strains were transferred to YNB-LIM medium containing either hemin or hemoglobin and their growth at 30°C was monitored by recording OD600 over a 96-hr time-course at the indicated time intervals. Data are presented as the mean ± SEM of three to four independent experiments.
also showed reduced growth in the presence of BFA and monensin and the salts NaCl, KCl, and LiCl (Figure 10a). Importantly, unlike the chc1Δ mutant, the las17Δ mutants did not show any growth inhibition at the host temperature of 37°C. Also, no growth defects were observed in utilising alternate carbon sources, or in the presence of oxidative stress, ER stress, and cell wall damaging agents. Finally, we also observed reduced capsule production and melanin secretion at 37°C in the las17Δ mutants (Figure 10b,c). A previous study in the C. neoformans serotype D strain JEC21 revealed a role of LAS17 (CNBE5050) in growth at 37°C, cytokinesis, chitin distribution, endocytosis, and virulence (Shen, Whittington, & Wang, 2011). We also conducted a virulence assay to compare the survival of mice infected with las17Δ mutants with the mice infected with the WT strain in H99 background. As shown in Figure 10d, mice infected with the las17Δ mutants showed significantly increased survival compared with the mice infected with the WT strain, although the mutants eventually caused disease. A preliminary test of the rvs161Δ and rvs167Δ mutants in three mice for each strain also indicated attenuated virulence (data not shown). We did not test the chc1Δ mutant in mice because of the growth defect at 37°C for this strain. Taken together,
these results suggest that CME in C. neoformans not only is important in endocytosis but also plays essential roles in use of heme-iron, virulence, and elaboration of several key virulence determinants including growth at high temperature, capsule and melanin production, and survival in various stress conditions.

3 | DISCUSSION

Heme and hemoglobin serve as important iron sources for the proliferation of C. neoformans (Bairwa et al., 2017; Jung et al., 2008). However, very few factors have been identified for heme and hemoglobin uptake and internalisation. Non-iron metalloprotoporphyrins are potential antimicrobial agents as well as useful reagents for characterising heme uptake by microbes. In this study, we screened for mutants with altered susceptibility to MnMPP and focused on the subsequent characterisation of CME because a chc1 mutant displayed enhanced growth on MnMPP and reduced growth on heme as an iron source. In addition to Chc1, we also identified other components of CME, including Las17, Rvs161, and Rvs167 that were necessary for efficient use of heme and hemoglobin as iron sources. Growth of the mutants lacking CHC1 and LAS17 in a medium with hemin or hemoglobin was strongly impaired, and growth was also significantly reduced for mutants lacking RVS161 and RVS167. Importantly, our study showed that Chc1 is specifically involved in the uptake of heme-iron sources and is not required for inorganic iron utilisation. In addition, we also did not observe any role of Chc1 in utilisation of other metal ions such as copper or zinc or other carbon and nitrogen sources. The chc1, las17, rvs161, and rvs167 mutants shared some of the conserved phenotypes seen for their counterparts in S. cerevisiae, C. albicans, and Aspergillus nidulans, including defects in cell morphology, cell division, growth at high temperature, and endocytic processes (Baggett & Wendland, 2001; Bauer, Urdaci, Aigle, & Crouzet, 1993; Douglas, Martin, & Konopka, 2009; Naqvi, Zahn, Mitchell, Stevenson, & Munn, 1998; Schultzhaus, Johnson, & Shaw, 2017; Sivadon, Bauer, Aigle, & Crouzet, 1995; Walther & Wendland, 2004). Furthermore, lack of CHC1, LAS17, RVS161, and RVS167 in C. neoformans had a profound effect on key virulence factors, namely, capsule and melanin production and, for LAS17, the ability to cause disease in a mouse model of cryptococcosis.

A role for CME in heme/hemoglobin utilisation has been described in the intracellular parasite Leishmania donovani and the model worm Caenorhabditis elegans (Agarwal et al., 2013; Yuan et al., 2016). However, to date no role of CME in heme uptake/utilisation has been reported for fungi. Our study revealed that Chc1 in C. neoformans localises to endocytic vesicles and presumed TGN-like structures in the cells, and this is consistent with observations in S. cerevisiae and mammalian cells (Newpher, Smith, Lemmon, & Lemmon, 2005; Puertollano et al., 2003). Importantly, we observed the localisation of Chc1 with intracellular hemoglobin-containing vesicles and this suggests that hemoglobin internalisation in C. neoformans occurs via an endocytic process involving clathrin-coated vesicle formation. Of course, there may also be CME-independent mechanisms of heme or hemoglobin uptake. Furthermore, because CME occurs for receptor bound factors, our study highlights the possibility of cell-surface heme/hemoglobin binding factors in C. neoformans. In the pathogenic fungus C. albicans, proteins containing the common in fungal extracellular membranes (CFEM) domain including Rbt5, Rbt51, Csa2, and Pga7 play important roles in heme and hemoglobin binding, uptake, and delivery into the cell (Kuznets et al., 2014; Nasser et al., 2016; Okamoto-Shibayama, Kikuchi, Kokubu, Sato, & Ishihara, 2014; Weissman & Komitzer, 2004). These cell surface-associated proteins function in a sophisticated relay network system to bind, extract, and shuttle the heme molecule for its subsequent delivery into the cell (Kuznets et al., 2014). In this regard, it is important to highlight that proteins containing the CFEM domain have been identified in C. neoformans (Zhang et al., 2015). Our unpublished work also found that the C. neoformans genome encodes at least four CFEM-domain containing proteins. However, we observed that deletion of these genes individually does not result in any defects in the use of various iron sources including inorganic iron and hemin or hemoglobin, in contrast to the reported roles for the CFEM proteins in C. albicans and C. glabrata (Kuznets et al., 2014; Nasser et al., 2016; Okamoto-Shibayama et al., 2014; Weissman & Komitzer, 2004). Further study is needed to characterise strains lacking multiple CFEM-protein encoding genes to overcome potential redundancy and determine whether or not CFEM proteins are involved in heme or hemoglobin cell surface binding and internalisation in C. neoformans.

Studies in both C. albicans and C. neoformans revealed that components of ESCRT-I, II, and III machinery are required for the use of heme as an iron source (Hu et al., 2013, 2015; Weissman et al., 2008). Specifically, the ESCRT endocytic trafficking machinery proteins Vps23, Vps22, and Snf7 have been implicated in heme-iron use in C. neoformans (Hu et al., 2013, 2015). Based on our findings, we speculate that in C. neoformans, heme or hemoglobin is bound by as yet unknown cell surface-associated protein/s and eventually internalised by a coordinated assembly of clathrin-coated vesicles formation and sequential recruitment of Vps-proteins culminating in endocytosis and trafficking of the heme/hemoglobin-receptor cargo to the vacuole. A major step in internalisation of cell-surface protein by CME involves mono-ubiquitination of cargo proteins (Bertelsen et al., 2011; Egner & Kuchler, 1996; Goh, Huang, Kim, Gygi, & Sorkin, 2010; Hicke & Riezman, 1996). Similarly, the ESCRT machinery mainly targets mono-ubiquitinated cell surface proteins for trafficking to the vacuole (Schuh & Audhya, 2014). Therefore, it is possible that a cell-surface protein involved in heme or hemoglobin binding in C. neoformans is also ubiquitinated and internalised by the CME and ESCRT pathways for its final delivery to vacuole. In this regard, it would be interesting to identify the structural components of clathrin-labelled hemoglobin-containing vesicles to potentially discover the predicted cell surface heme/hemoglobin receptor/s or transporter/s. Certainly, additional studies are required to delineate the molecular interactions between CME and ESCRT pathways to fully understand the intracellular trafficking of heme/hemoglobin. Unexpectedly, our MPP screening did not reveal any potential transporters or cell-surface associated protein/s that could be directly involved in heme transport or binding. However, we did identify several candidate transporter proteins including a multidrug transporter, Pdr5, two uncharacterised drug efflux proteins encoded by CNAG_03474 and CNAG_01844, a siderophore transporter, and sugar transporters.
Further research is necessary to demonstrate any possible role of these transporters in heme-iron binding or transport, or in the efflux of toxic molecules such as MnMPP.

It is interesting that additional components of CME in *C. neoformans*, including the Las17 homologue of WASP, and the amphiphysins, Rvs161 and Rvs167, were also required for efficient use of heme/hemoglobin as iron sources. Individual mutants lacking these genes show significant growth defects in the presence of hemoglobin, and this highlights the essential role of endocytosis in proper sorting and trafficking of heme cargo. We also observed defects in the expression of several key virulence factors in chc1, las17, rvs161, and rvs167 mutants including capsule elaboration and melanin secretion and attenuated virulence for the las17 mutant in mice. In addition to heme-iron utilisation, a contribution of CME to capsule elaboration is interesting because it suggests an important role in the trafficking of proteins and/or polysaccharides for the capsule synthesis. Similarly, impaired trafficking of laccase to the cell surface may account for the dependence of melanin formation on CME. It is important to highlight that other than the major role of CME in heme and hemoglobin uptake and utilisation and elaboration of key virulence factors in *C. neoformans*, we did not observe any role of this machinery in the use of specific carbon or nitrogen sources, or the response to copper and zinc.

Environmental sensing of iron and heme availability is linked to intracellular signalling events leading to transcriptional and translational regulation of multiple factors involved in iron uptake, trafficking, and metabolism, as well as the response to stress. Our MPP screening also identified mutants deleted for the components of the Hog1 and Ca²⁺-calcineurin signalling networks including hog1Δ, pbs2Δ, bem2Δ, sch9Δ, ste20Δ, and cnb1Δ. Notably, both Ca²⁺-calcineurin and Hog1-mediated MAPK signalling in *C. neoformans* play important roles in the response to diverse environmental stresses (e.g., osmotic, oxidative, antifungal drug, heavy metal, and high temperature stress) and in the production of capsule and melanin (Jung & Bahn, 2009; Kozubowski, Lee, & Heitman, 2009; Kronstad, Hu, & Jung, 2013). Additionally, the Hog1-dependent MAPK signalling pathway is involved in iron uptake and homeostasis in *S. cerevisiae* and in the fungal pathogens *C. albicans*, *C. glabrata*, and *A. fumigatus* (de Castro et al., 2016; Kaba, Nimtz, Müller, & Billetewski, 2013; Martins et al., 2018; Srivastava, Suneetha, & Kaur, 2015). It is plausible that a deficiency for iron or heme-iron in *C. neoformans* also acts as an external/internal stress signal that induces activation of major signalling events, and this may eventually result in modification of the iron-uptake machinery or intracellular iron metabolism. However, the functions of different components of the Hog1 and calcineurin signalling pathways in heme-iron uptake and metabolism and their potential connection with the CME and heme-iron trafficking remain to be elucidated.

In conclusion, our study highlights the value of genome-wide screening approaches in a human fungal pathogen for not only identifying new contributions of CME and signalling pathways in heme-iron utilisation but also potential applications of non-iron MPPs in the treatment of cryptococcosis. Importantly, the contributions of CME and MAPK/calcineurin-signalling for heme/hemoglobin uptake and metabolism in *C. neoformans* appear to be evolutionary conserved with yeast and higher eukaryotes. Further exploration of the molecular mechanisms for heme sensing, uptake, endocytosis, signalling, and regulation will provide a deeper understanding of iron metabolism in fungal pathogens and subsequently assist in the development of new therapeutic targets.

4 EXPERIMENTAL PROCEDURES

4.1 Strains, plasmids, media, and growth conditions

*C. neoformans* var. grubii strains H99 (serotype A) and Kn99-alpha were used as WT parental strains. *C. neoformans* strains were routinely maintained on YPD agar medium (1% yeast extract, 2% Bactopeptone, 2% D-glucose, and 2% agar). Overnight cultures were obtained by inoculating a single colony into liquid YPD medium and growing at 30°C with shaking at 200 revolutions per min (rpm). YPD medium plates containing nourseothricin (NAT; 200 μg ml⁻¹) were used to select the transformants deleted for the CHC1 and LAS17 genes. Solid YPD medium containing neomycin (NEO) was used to select the chc1Δ/CHC1-GFP complemented strain. The neomycin resistance cassette and the GFP gene was from the plasmid pWH091 as described earlier (Jung et al., 2009). Low iron YNB medium (YNB with amino acids; YNB-LIM) was prepared by adding the iron-chelator bathophenanthroline disulfonate (BPS; 150 μM) in YNB medium prepared in Chelex 100 resin-treated low iron water. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise. The strains used in this study are listed in Table S2.

4.2 Construction of deletion mutants and complemented strains

The candidate genes CHC1 (clathrin heavy chain) and LAS17 (WASP homologue) were deleted in the *C. neoformans* strain H99 by homologous recombination using gene-specific knockout cassettes containing the resistance marker nourseothricin and the 5' and 3' flanking sequences of the respective genes. These gene-specific knockout cassettes were amplified from genomic DNA of chc1Δ and las17Δ mutants in Kn99α background (mutants obtained in UCSF-libraries) using primers GB-04904-ForKO, GB-04904-RevKO and GB-02029-ForKO, GB-02029-RevKO, respectively. The constructs were introduced into the *C. neoformans* H99 wild type strain by biolistic transformation, as described previously (Davidson et al., 2000). Two independent chc1Δ mutants (chc1Δ-1 and chc1Δ-2) and two las17Δ mutants (las17Δ-1 and las17Δ-3) were selected and evaluated in the experiments. Complementation of the chc1Δ mutation was accomplished with a modified overlapping polymerase chain reaction (PCR) strategy to include the GFP sequence at the C-terminus of Chc1 before the stop codon. Briefly, the left arm (~1 kb amplon of the 3' end of CHC1 ending just before stop codon: amplicon-1) and right arm (~1 kb amplicon starting after the stop codon of CHC1: amplicon-2) from the CHC1 genomic locus were amplified from WT genomic DNA using the primers GB-04904-For and GB-04904-GFP-Rev (containing an 18 base overlap with the GFP sequence at the 5' end) and GB-04904-3’UTR-GFP-For (containing a 20 base overlap
with the GFP sequence at the 3′ end) and GB-04904-3′UTR-Rev, respectively. The GFP gene and the neomycin resistance cassette (amplicon-3) were amplified from the plasmid pGH025 (Hu et al., 2017) using primers GB-CnGFP-Neo-For and GB-CnGFP-Neo-Rev. Overlap PCR was performed using amplicon-1, amplicon-2, and amplicon-3 as templates with primers GB-04904-For and GB-04904-3′UTR-Rev to obtain the CHC1-GFP-Neo-3′UTR fusion product. This fusion product was transformed into the WT strain by biolistic transformation, and the positive transformants were selected for resistance to G418 (Geneticin, Sigma).

For complementation of chc1Δ with the CHC1-GFP sequence, the CHC1 genomic locus from WT DNA containing the CHC1-GFP variant was amplified with primers GB_04904_For_Comp and GB_04904_Rev_Comp and transformed into the chc1Δ strain. The positive transformants were selected for resistance to G418. Proper location and orientation of the gene fusions at the CHC1 locus were determined by PCR. Primers used in this study are listed in Table S3.

### 4.3 MPP screening and identification of mutants with altered MnMPP susceptibility

To identify new factors involved in heme-iron use and susceptibility to MnMPP, we designed the screening strategy outlined in Figure 1b. Two libraries of deletion strains of *C. neoformans* (~3,300 genes; arranged in 96-well plates) constructed by Dr. Hiten Madhani’s group at the University of California, San Francisco, USA, were obtained from the Fungal Genetics Stock Center (http://www.fgsc.net/crypto/crypto.htm). These libraries include a set of 14 plates of deletion strains submitted to FGSC in year 2008 (UCSF-2008) and another set of 22 plates of deletion strains submitted in 2015 (UCSF-2015). Additionally, a library of ~322 signature tagged gene deletion strains (155 putative transcription factors (TF) of *C. neoformans* at least two independent deletion strains per TF; TFKO) was constructed by Dr. Won Hee Jung (Chung–Ang University, Korea). In this study, the three libraries (UCSF-2008, UCSF-2015, and TFKO) were screened for susceptibility to MnMPP. To initiate the screening, deletion mutants were grown in 96-well plates supplemented with different compounds. Plates were incubated at 30°C with shaking at 50 rpm, and the OD600 was measured after 72 hr using a microplate reader (Infinite M200; Tecan). The ability of mutants to grow under different environmental conditions and in the test media conditions including regular YNB, YNB with BPS supplemented with hemin (10 μM; MnMPP) were identified by assessing the 72-hr growth differences between the hemin with MnMPP medium versus hemin-only medium conditions. The OD₆₀₀ values in these two media conditions were normalised with their respective blank media controls, and the relative growth difference (in percentage) for each strain was calculated using the following equation: (OD₆₀₀ of the strain in MnMPP medium at 72 hr/OD₆₀₀ of the strain in hemin medium at 72 hr) × 100. An average of all the percentage relative growth difference values from all the strains in each library was determined (ARG, ARG4 for 4 plates in TFKO library, ARG14 for UCSF-2008 library, ARG22 for UCSF-2015 library) to evaluate the overall growth behaviour of the strains in each individual library. To identify the strains with altered MnMPP susceptibility, we used a cut-off of 1.5-fold difference between the individual percentage growth and the ARG value of the respective library. The strains that grew ≥150% or ≤50% of the ARG values were selected as MnMPP resistant and sensitive strains, respectively.

### 4.4 GO enrichment and STRING network analysis

GO analysis for the genes identified in the screens was performed using the PANTHER gene ontology online tool (http://pantherdb.org). The genes were evaluated for enrichment of GO biological processes, molecular functions, and cellular component categories by comparing with a background genome set of *C. neoformans* present in the PANTHER database with a cut-off of *P* < 0.05. The interaction network for the genes was obtained from the STRING (https://string-db.org) online network analysis with the *C. neoformans* var. *neoformans* JEC21 genome database as a background using all active interaction sources and a medium confidence value of 0.400. The network that was obtained was extracted and visualised using the Cytoscape network analysis tool (http://www.cytoscape.org). The cluster identification in Cytoscape was done using the Community Cluster (GLay) network clustering algorithm in the clusterMaker plugin.

### 4.5 Serial dilution spotting and liquid growth assays

The ability of mutants to grow under different environmental conditions was evaluated by the spotting of serial dilutions on solid agar plates and by optical density (OD₆₀₀) measurement in liquid growth assays. For serial dilution spotting assays, the cells were grown in YPD at 30°C and 200 rpm for 14–16 hr, washed three times with either PBS or Chelex 100 resin-treated low iron water, and adjusted to 2 × 10⁷ cells ml⁻¹. The cell suspension was diluted 10-fold serially and 5 μl of each dilution was spotted onto YPD and/or YNB plates supplemented with different compounds. Plates were incubated at 30°C or 37°C for 2–5 days before being photographed. Strains were evaluated for growth responses towards temperature (30°C and 37°C), salts (1.5 M NaCl, 1.5 M KCl, 100 mM LiCl), trafficking inhibitors (20 μg ml⁻¹ brefeldin A; BFA and 1.25 mg ml⁻¹ monensin), alternate carbon sources (2% of either glycerol, ethanol or sodium acetate), nitrogen sources (5 g ml⁻¹ ammonium sulfate or L-glutamate, 20 mM urea), antifungal drugs (5, 7.5 or 10 μg ml⁻¹ fluconazole), agents that cause the unfolded protein response in the endoplasmic reticulum (0.15 μg ml⁻¹ tunicamycin and 10 mM dithiothreitol; DTT), alter redox state (1 or 1.5 mM H₂O₂), metal ion availability (100 μM of CuSO₄ or ZnSO₄), and agents that challenge cell wall integrity (1 mg ml⁻¹ calcofluor white and 2.5 mg ml⁻¹ Congo red).
To evaluate the growth on different iron sources, cells were starved for iron before growth evaluation, as described earlier with slight modifications (Hu et al., 2017). Briefly, overnight YPD-grown fungal cells were washed three times in Chelex 100 resin-treated low iron water and inoculated into OD_{500} of 0.2 in YNB with BPS (150 μM, YNB-LIM). The cells were incubated for 24 hr before spotting onto YNB and YNB-LIM solid agar plates with and without supplemented inorganic iron sources (10 or 100 μM of FeCl_3 or FeSO_4) or hemin (10, 100, or 1000 μM). Liquid growth assays were performed in conical flasks with YNB and YNB-LIM media with and without supplementation of hemin and hemoglobin at different concentrations. Iron-starved cells (1 day) were inoculated to OD_{500} of 0.2 in fresh media. The total medium volume was 20 ml. The flasks were incubated in a shaker at 30°C and 200 rpm, and OD_{500} was measured at regular time intervals.

### 4.6 Capsule formation and melanin production

To evaluate capsule formation, cells were grown overnight in YPD, washed with water, and incubated in low-iron capsule induction medium (5 g L\(^{-1}\) glucose, 5 g L\(^{-1}\) L-asparagine, 0.4 g L\(^{-1}\) K\(_2\)HPO\(_4\), 0.25 g L\(^{-1}\) CaCl\(_2\)-2H\(_2\)O, 0.08 g L\(^{-1}\) MgSO\(_4\)-7H\(_2\)O, 4.78 g L\(^{-1}\) HEPES, 1.85 g L\(^{-1}\) NaHCO\(_3\) [dissolved in Chelex 100 resin-treated water], pH 7.4) at 30°C and 200 rpm for 48 hr. To visualise the capsule, cells were stained with India ink and observed by differential interference contrast (DIC) microscopy (Zeiss Axioscop 2) at 100× resolution. Capsule and cell sizes were measured in ZEN lite software (ZEISS Inc.) from at least 50 to 60 cells for each strain.

Melanin production was evaluated on either solid or liquid L-3,4-dihydroxyphenylalanine (L-DOPA; Sigma) media (1 g L\(^{-1}\) L-asparagine, 3 g L\(^{-1}\) KH\(_2\)PO\(_4\), 0.25 g L\(^{-1}\) MgSO\(_4\)-7H\(_2\)O, 0.2 g L\(^{-1}\) L-DOPA, and 0.0001% thiamine hydrochloride [dissolved in distilled water], pH 5.6) containing 0.1% glucose. For melanin production on solid media, serial dilution spotting of the overnight YPD-grown cells was done on L-DOPA solid agar plates as described above. The plates were incubated in the dark at 30°C or 37°C for 48 hr before being photographed. Melanin secretion in the liquid L-DOPA medium was analysed as described earlier with some modifications (Tirado, Peng, Yang, Hang, & Doering, 2015). Briefly, YPD-grown overnight cultures were collected, washed twice in Chelex 100 resin-treated low-iron water, and adjusted to 10^7 cells ml\(^{-1}\) in low-iron water; 300 μl of the cell suspension was added to 3 ml L-DOPA, and samples were incubated in the dark at 30°C and 200 rpm for 24 hr. After incubation, 1 ml culture was centrifuged at 1,000 g for 5 min, and the OD_{405} was measured for the supernatant fractions to analyse the melanin secretion.

### 4.7 FM4-64 and solophenyl flavine labelling and microscopy

To examine the endocytosis process, trafficking of the lipophilic styryl dye, N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl-hexatrienyl)pyridinium dibromide (FM 4-64, Molecular Probes) was examined as described earlier with some modifications (Vida & Emr, 1995). Briefly, YPD-grown overnight cells were collected, washed twice with cold-PBS, and incubated in cold-PBS with FM4-64 (20 μM) at 4°C for 30 min. The cells were harvested at 1,000 g for 3 min at 4°C, washed three times with ice-cold PBS, resuspended in fresh YPD medium, and further incubated at RT. At different time intervals, a small portion of the cells was withdrawn from the YPD suspension and placed on a standard glass slide for visualisation under a fluorescence microscope (Zeiss Axioscop 2) with rhodamine filter.

To visualise chitin, exponentially growing cells were labelled with solophenyl flavine in PBS for 10 min at room temperature as described earlier (Hoch, Galvani, Szarowski, & Turner, 2005). Labelled cells were visualised under a fluorescence microscope using the EGFP filter set. All photomicrographs were captured at 100× resolution using a Plan DIC achromatic objective and analysed with ZEN lite software.

### 4.8 Labelling of hemoglobin with Alexa Fluor 594 and analysis of the kinetics of intracellular trafficking

The uptake and intracellular trafficking of hemoglobin (Hb) in C. neoformans cells was examined with labelled hemoglobin. Human hemoglobin (Sigma) was labelled with Alexa Fluor 594 according to the manufacturer’s protocol (Invitrogen, Molecular Probes). Next, exponentially growing cells (with and without iron starvation) were collected, washed twice with ice-cold PBS, and incubated in PBS containing Alexa Fluor 594-labelled hemoglobin (Hb\(^{Alexa594}\), 50 μg/ml) for 20 min on ice to allow cell surface binding. After 20 min, 0.001% of either Fluorescent Brightener 28 or Lucifer Yellow was added to the cells to label the cell wall (with 5 min of incubation on ice). Following this staining, cells were washed three times with ice-cold PBS to remove unbound Hb\(^{Alexa594}\) and cell-wall labeling dyes and resuspended in PBS prewarmed at 30°C. After incubation at different time points, cells were visualised under a fluorescence microscope (Zeiss Axioscop 2) using Alexa Fluor 594 filter set with exposure time of 5,000–10,000 ms and DAPI or EGFP filter sets for Fluorescent Brightener 28 or Lucifer Yellow visualisation with an exposure time of 1,000 ms. For Chc1-GFP and Hb\(^{Alexa594}\) colocalisation experiments, cells expressing Chc1-GFP were labelled with Hb\(^{Alexa594}\) as described and visualised with Alexa Fluor 594 and EGFP filter sets at exposure times of 5,000 and 1,000 ms, respectively. Colocalisation analyses for Chc1-GFP and Hb\(^{Alexa594}\) were performed on images of whole cells using ImageJ software and the coloc 2 test. A total of 10–20 cells were analysed for each time point. Positive correlations between Chc1-GFP and Hb\(^{Alexa594}\) puncta were determined by Pearson’s R value (0.10–0.65) and Costes P value (0.98–1.00). All photomicrographs were captured at 100× resolution using a Plan DIC achromatic objective and analysed with ZEN lite software.

### 4.9 Total cellular heme level quantification

Total heme content of the cells was determined using the BioVision hemin assay kit (BioVision, Milpitas, California) as described earlier (Do et al., 2017). Briefly, cells were grown in YPD medium overnight.
at 30°C, washed twice with PBS, and lysed using bead beating in buffer containing Tris–HCl (10 mM, pH 8.0) and NaCl (150 mM). After lysis, supernatant was collected by centrifugation at 13,000 rpm for 3 min at 4°C and used to measure the heme content as described by the manufacturer. Total heme level in each strain was normalised to the protein concentration.

4.10  Virulence assay

A mouse inhalation model of cryptococcosis was used to assess the virulence of mutants using 4- to 6-week-old female BALB/c mice, as described earlier (Hu et al., 2017). Briefly, strains were grown overnight in YPD medium at 30°C, washed, and resuspended in PBS. A total of 2 × 10^5 cells in a 50 μl volume of PBS were used for intranasal inoculation of 10 mice per strain. The status of each mouse was monitored daily post-inoculation. Mice reaching the humane endpoint were euthanised by CO₂ anoxia. The protocol for the virulence assay (protocol A17-0117) was approved by the University of British Columbia Committee on Animal Care.

4.11  Statistics

Data were visualised and statistically analysed using GraphPad Prism version 6.0 (Graph-Pad Software, Inc., USA). Statistical tests were performed by one-way analysis of variance followed by a Bonferroni correction for melanin secretion assays, or by Student’s t test for capsule size, or by log-rank tests for in vivo mouse survival assay. P values of 0.05 were considered to be significant.

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AUTHOR CONTRIBUTIONS

G. B. and J. W. K. conceived the study and designed the experiments. G. B. performed the experiments. G. B. and J. W. K. analysed the data and wrote the manuscript. G. H., M. C., and L. H. conducted the virulence assays in mice and contributed to the preparation of the manuscript.

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