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The Cch1-Mid1 High-Affinity Calcium Channel Contributes to the Virulence of *Cryptococcus neoformans* by Mitigating Oxidative Stress

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Pathogenic fungi have developed mechanisms to cope with stresses imposed by hosts. For *Cryptococcus* spp., this implies active defense mechanisms that attenuate and ultimately overcome the onslaught of oxidative stresses in macrophages. Among cellular pathways within *Cryptococcus neoformans*' arsenal is the plasma membrane high-affinity Cch1-Mid1 calcium (Ca^{2+}) channel (CMC). Here we show that CMC has an unexpectedly complex and disparate role in mitigating oxidative stress. Upon inhibiting the Ccp1-mediated oxidative response pathway with antimycin, strains of *C. neoformans* expressing only Mid1 displayed enhanced growth, but this was significantly attenuated upon H_2O_2 exposure in the absence of Mid1, suggesting a regulatory role for Mid1 acting through the Ccp1-mediated oxidative stress response. This notion is further supported by the interaction detected between Mid1 and Ccp1 (cytochrome *c* peroxidase). In contrast, Cch1 appears to have a more general role in promoting cryptococci survival during oxidative stress. A strain lacking Cch1 displayed a growth defect in the presence of H_2O_2 without BAPTA [(1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, cesium salt] or additional stressors such as antimycin. Consistent with a greater contribution of Cch1 to oxidative stress tolerance, an intracellular growth defect was observed for the *cch1*Δ strain in the macrophage cell line J774A.1. Interestingly, while the absence of either Mid1 or Cch1 significantly compromises the ability of *C. neoformans* to tolerate oxidative stress, the absence of both Mid1 and Cch1 has a negligible effect on *C. neoformans* growth during H_2O_2 stress, suggesting the existence of a compensatory mechanism that becomes active in the absence of CMC.

It is well established that Ca^{2+} is a critical secondary messenger that initiates and regulates a plethora of signaling events. For this reason, cytosolic Ca^{2+} levels are exquisitely controlled by regulating the movement of calcium ions into and out of cells via ion channels and transporters (1–3). Fluctuations of Ca^{2+} in the cytosol are transduced via calcium sensors like calmodulin, which, upon calcium binding, activates calcineurin and CaMK (Ca^{2+}/calmodulin-dependent protein kinases). Calcineurin is a Ca^{2+}/calmodulin-activated serine/threonine protein phosphatase highly conserved among eukaryotes. In fungi such as *Cryptococcus neoformans*, *Candida albicans*, and *Saccharomyces cerevisiae*, calcineurin regulates the transcription of genes involved in mating, cell viability, and response to cell stress (4–6). The improper regulation of Ca^{2+} can produce significant cell damage and ultimately lead to cell death (7).

In fungal cells, the Cch1-Mid1 channel complex (CMC) represents the only high-affinity Ca^{2+} channel in the plasma membrane that mediates the specific influx of Ca^{2+} (2). While Cch1 functions as the pore of the channel, Mid1 associates with Cch1 and, in a manner that is not completely understood, facilitates the movement of Ca^{2+} from the extracellular milieu to the cytosol (8, 9). Under conditions of endoplasmic reticulum (ER) stress or low ER Ca^{2+}, the Cch1-Mid1 channel becomes activated and functions to replenish Ca^{2+} stores (1, 2, 10, 11).

It is not known if CMC is required for survival of cryptococci within the macrophage environment. *Cryptococcus neoformans* is a facultative intracellular pathogen capable of replicating within the phagolysosome and promoting host cell lysis (12, 13). Given the oxidative stresses that can promote fluctuating levels of cytosolic Ca^{2+} within active macrophages (14) and the specific role of CMC as the only high-affinity Ca^{2+} channel in the plasma membrane of *C. neoformans*, we sought to resolve whether CMC played a role in promoting survival of *C. neoformans* within an intracellular environment like the macrophage (15). Here we show that both Mid1 and Cch1 promote *C. neoformans* survival upon exposure to oxidative stress *in vitro*, with Cch1 making an additional contribution to cryptococci survival in macrophages.

A yeast two-hybrid screen using the C-terminal region of Mid1 spanning two cysteine-proline (CP) dipeptides and predicted heme-binding sites revealed an interaction with cytochrome C peroxidase (Ccp1), a known heme-binding protein (16). Ccp1 is a mitochondrial antioxidant protein that reduces hydrogen peroxide to water and is released into the cytosol during stress (16). Upon inhibition of the Ccp1-mediated oxidative response pathway with antimycin, we found that strains expressing Mid1 in the absence of Cch1 shows significant resistance to antimycin toxicity, consistent with the interaction observed for Mid1 and Ccp1. Surprisingly, a strain lacking both Mid1 and Cch1 showed significant resistance to oxidative stress *in vitro*, suggesting a compensatory mechanism that is active in the absence of CMC. Together, our work revealed that CMC plays a complex role in the mitigation of oxidative stress in *C. neoformans* with Mid1 and Cch1 having disparate contributions to oxidative stress resistance.

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TABLE 1 Primers used to generate Mid1 CP mutant expression constructs

Target	Primer	Sequence
Mid1 CP^{1x2x}	Mid1-CP1x-R	CCATTGATACCGCGGCTGAGCGAT
	Mid1-CP2x-F	GCATCAGGGCCGCGAAACGC
Mid1 CP^{1x}	Mid1-CP1x-R	CCATTGATACCGCGGCTGAGCGAT
	Mid1-CP2-F	GCATCAGGTGCCCGAAAC
Mid1 CP^{2x}	Mid1-CP1-R	CCATTGATACCGGGCATGAG
	Mid1-CP2x-F	GCATCAGGGCCGCGAAACGC
Mid1 CP^{wt}	Mid1_NotI_F	ATAGCGGCCGCATGCCAGCGAGAGAGGTGTATTTCAAAAGG
	Mid1_MfeI_R	GCTCAATTGCTATCCGTTACACCATCTATTTCCCCAGCGAT

MATERIALS AND METHODS

***C. neoformans* strains and reagents.** Frozen stocks of yeast strains used in this study—H99 (wild type) and the *cch1*Δ, *cch1*Δ::*CCH1*, *cch1*Δ *mid1*Δ, *mid1*Δ, *mid1*Δ::*MID1*, *MID1-CP*^{wt} (CP^1 and CP^2 wild-type allele), *MID1-*$C^{648A}P^{649A}$ (*CP*^{1x} mutant), *MID1-*$C^{656A}P^{657A}$ (*CP*^{2x} *mutant*), *MID1-*$C^{648A}P^{649A}C^{656A}P^{657A}$ (*CP*^{1x2x} double mutant) strains—were streaked on yeast-peptone-dextrose (YPD) plates and incubated overnight at 30°C. Colonies were then grown in liquid YPD overnight at 30°C until log phase (approximately 16 h). Cultures were regrown a second time (to log phase) by adding 100 μl of liquid culture to fresh YPD to ensure that yeast colonies were healthy and robust. Ten milliliters of each cultured strain was washed with sterile 1× phosphate-buffered saline (PBS) (4,000 rpm, 4 min, 25°C) and resuspended in YPD. The concentration of the original culture was determined by hemacytometer count (Brite-Line) and diluted in 1× PBS to appropriate concentrations. Where indicated, a cell-impermeant calcium chelator {BAPTA [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, cesium salt]} (Invitrogen/Molecular Probes, Carlsbad, CA) was added to YPD medium (2). In the presence of BAPTA, the final free [Ca^{2+}] was approximately 100 nm (2). Antimycin A (A8674; Sigma), FCCP [carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone- and paraquat)] (C2920; Sigma), and paraquat (36541; Sigma) were prepared according to the manufacturer's instructions.

Generation of Mid1 expression constructs. Mid1 cDNA was cloned into the *C. neoformans* expression plasmid Jmm180 under the control of a constitutive actin promoter using standard methods (17). Briefly, Mid1 cDNA was amplified via PCR from *C. neoformans* cDNA using the primers Mid1_NotI_F and Mid1_MfeI_R (Table 1). The cDNA was then digested with NotI and MfeI, ligated into the Jmm180 plasmid using T4 DNA ligase, and transformed into *Escherichia coli*. Positive bacterial transformants were selected on LB plates containing ampicillin and plasmids were isolated using the Qiagen miniprep kit. The *C. neoformans* transformant expressing the wild-type copy of Mid1 under the control of a constitutive actin promoter was designated Mid1 CP^{wt}.

Site-directed mutagenesis of Mid1 cysteine dipeptide (CP) motif 1 and 2. Mutations in the Mid1 CP motifs 1 and 2 (CP^1 and CP^2) were generated via an inverse PCR method, using the Mid1 expression construct as a PCR template. A high-fidelity DNA polymerase (Thermo Scientific Phusion Hot Start II; Thermoscientific) was used to generate mutations in Mid1 cDNA using the primer pairs listed in Table 1. The cysteine and proline residues in the CP dipeptides were either replaced with alanine or left unchanged, yielding three possible mutations: (i) Mid1 harboring the CP^1 mutation ($C^{648A}P^{649A}$ [CP^{1x}]), the CP^2 mutation ($C^{656A}P^{657A}$ [CP^{2x}]) or both CP^1 and CP^2 mutations ($C^{648A}P^{649A}$ $C^{656A}P^{657A}$ [CP^{1x2x}]). The Mid1 site-directed mutagenesis constructs and the construct harboring the wild-type Mid1 cDNA allele (CP^{wt}) were then introduced into the *mid1*Δ strain of *C. neoformans* using biolistic transformations (18). Positive transformants were then screened by colony PCR for genomic DNA integration. The expression of Mid1 in the biolistic transformants was then confirmed by RT-PCR and by growth sensitivity assay on YPD containing 2 mM BAPTA.

Macrophage infection assay. The J774A.1 (ATCC TIB-67; kindly provided by R. Tsolis) cell line is a lung macrophage-like cell line from a BALB/c, haplotype H-2^d reticulum sarcoma and commonly used as an intracellular survival assay for *C. neoformans*. Macrophages were grown at 37°C with 10% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, 50 μg/ml penicillin-streptomycin (P/S), and 10% NCTC-109 medium. J774A.1 cells were grown in 25-cm^2 or 75-cm^2 flasks and used between passages 4 and 15. For the assay, 2.5×10^5 J774A.1 cells were counted using a hemacytometer and grown in 96-well culture plates overnight. An H99 strain of *C. neoformans* was cultured overnight in YPD at 30°C with agitation. Cultures were then synchronized/normalized based on the optical density at 600 nm (OD_{600}) and allowed to grow overnight. By using a hemacytometer, 1×10^6 cells of *C. neoformans* were added to the macrophages supplemented with 100 U/ml mouse gamma interferon (IFN-γ), 0.3 μg/ml lipopolysaccharide (LPS), and 1 μg/ml monoclonal antibody (MAb) 18B7 (a gift from A. Casadevall) for 1 h. Cells were washed three times with 1× PBS and either incubated overnight with fresh DMEM plus supplemental medium or lysed with 100 μl of ice cold 0.05% SDS for CFU analysis. The following day, the medium was removed from wells and set aside. Approximately 100 μl of ice-cold 0.05% SDS was added to lyse macrophages. The mixture was combined with medium for serial dilutions and plated on plates containing YPD plus P/S. The plates were incubated for 48 h at 30°C, and the number of CFU was determined.

Sensitivity spot assay. Frozen strains of *C. neoformans* were maintained in 15% glycerol stocks at −80°C were plated on YPD agar plates and incubated at 30°C for 2 days. A single colony was placed in a 10-ml glass test tube with fresh liquid YPD and grown overnight in a roller drum apparatus (14 to 16 h) until cells reached mid-log phase. Cells were diluted to 10^7 to 10^2, and 7 μl was spotted on freshly made YPD with or without H_2O_2. H_2O_2 was added to YPD agar once the agar had cooled to at least 60°C. Plates were incubated at 30°C for 2 days.

Protein analysis. A 50-ml culture of KN99α (congenic strain of H99) cells was grown overnight in YPD. Cells were pelleted and resuspended in 0.3 ml lysis buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 5 mM NaF, 5 mM EDTA [pH 8.0], 0.1% Nonidet-P40 detergent; stored at 4°C). Fresh dithiothreitol (DTT) (1 mM) and Sigma yeast protease inhibitor cocktail, which was used following the manufacturer's instructions, were added. Samples were split into two 1.7-ml locking microcentrifuge tubes, and 425 to 600 μm acid-washed glass beads was added until there was approximately 1 to 2 mm between liquid and beads. Samples were vortexed at maximum speed for 1 min and placed on ice for 1 min for 5 cycles. Following cell lysis, the supernatant was collected and centrifuged at 4,000 rpm. The supernatant was collected, and total protein content was measured with the Bio-Rad QuickStart bovine serum albumin (BSA) standard set. Protein lysates were separated on a 6.5% SDS-PAGE gel and semidry transferred onto a polyvinylidene difluoride (PVDF) membrane. Purified mouse anti-Sos1 (BD Biosciences) at 1:500 and secondary horseradish

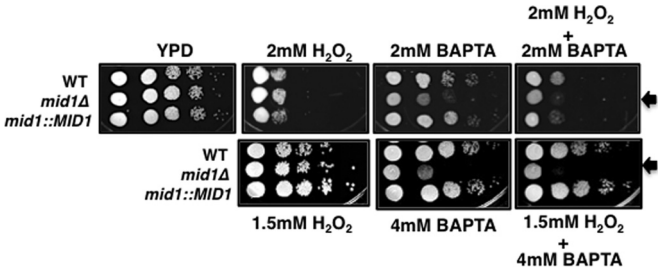

FIG 1 Mid1 plays a calcium-dependent role in mitigating oxidative stress induced by H_2O_2. Sensitivity spot assays were used to evaluate the requirement for Mid1 during oxidative stress. A strain of *C. neoformans* lacking Mid1 exhibited a significant growth defect upon exposure to H_2O_2 under conditions of limiting extracellular free [Ca^{2+}] in the presence of the calcium-specific chelator BAPTA (2), suggesting a requirement for Mid1 during oxidative stress (arrows). Serially diluted cells (10^5, 10^4, 10^3, 10^2, and 10^1) from wild-type H99, the *mid1*Δ mutant, and the Mid1-reconstituted (*mid1*Δ::*MID1*) strain of *C. neoformans* were spotted onto YPD plates with either 1.5 mM H_2O_2 or 2 mM H_2O_2 in combination with either 2 mM or 4 mM BAPTA. Plates were incubated at 30°C overnight.

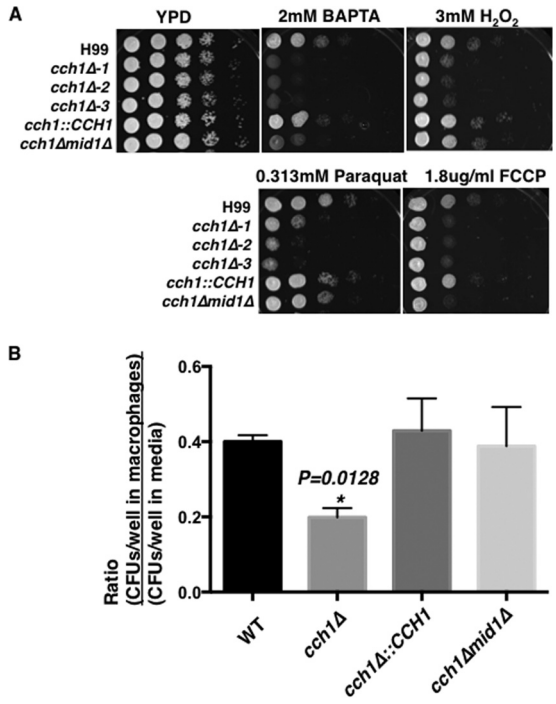

FIG 2 Cch1 contributes to oxidative stress tolerance and intracellular macrophage survival. (A) Tenfold serial dilutions (10^5, 10^4, 10^3, 10^2, and 10^1) of wild-type H99, the *cch1*Δ mutant, the Cch1-reconstituted (*cch1*Δ::Cch1) strain, and the *cch1*Δ *mid1*Δ mutant of *C. neoformans* were spotted on YPD alone or YPD with BAPTA (2 mM), H_2O_2 (3 mM), paraquat (0.313 mM), or FCCP (1.8 μg/ml). (B) The *cch1*Δ strain displayed a modest but significant growth defect following 24 h of incubation within macrophages, suggesting that Cch1 played a role in promoting intracellular survival of *C. neoformans*. Statistical significance was determined by unpaired *t* test with Welch's correction (GraphPad Prism5).

peroxidase (HRP)-conjugated goat anti-mouse Ig (BD Biosciences) at 1:1,000 were used for Western blotting.

CytoTrap yeast two-hybrid assay. The cytosolic C terminus of Mid1 (270 amino acids [aa], corresponding to 813 bp) lacking the last 23 aa (corresponding to 72 bp) (required for localization to the plasmid membrane) was cloned into the multiple-cloning site (MCS) of a pSos plasmid to create a Mid1C-hSos fusion "bait" plasmid. The forward primer was Y2H_Mid1Cterm_BamHF2 (5′-ACCGGATCCATGACAA CTCTATATGCGCCATAAATGCTGC-3′), and the reverse primer was Y2H_Mid1R4_NotIR (5′-ACCGCGGCCGCACTATGGTCGTCT CCCACAAACGC-3′). A *C. neoformans* cDNA library was made using the CytoTrap XR library construction kit. The library "prey" was cloned into the pMyr plasmid containing a myristylation signal to the plasma membrane. Both Mid1C pSos bait and prey plasmids were cotransformed into a temperature-sensitive *cdc25 Saccharomyces cerevisiae* strain. Potential positives were selected by growth at the restrictive temperature of 37°C on synthetic defined (SD) medium containing galactose but lacking leucine and no growth at 37°C on SD medium with glucose but without leucine. Potential positive pMyr plasmids were isolated from the *cdc25* strain, transformed into Invitrogen ElectroMAX DH5α-E electrocompetent *E. coli*, prepared using a midikit (Qiagen), and sequenced. Potential positives were identified by a BLAST search in the Broad Institute *C. neoformans* H99 var. *grubii* (serotype A) database.

***In silico* bioinformatic analysis.** The amino acid comparison of Mid1 was performed by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalo/) and T-Coffee multiple-sequence alignment (http://www.ebi.ac.uk/Tools/msa/tcoffee/).

Statistical analysis. Statistical significance was determined by an unpaired *t* test with Welch's correction (unequal variance) or by one-way analysis of variance (ANOVA) (GraphPad Prism 5.0).

RESULTS

Mid1 plays a role in mitigating oxidative stress in *C. neoformans*. As a facultative intracellular pathogen, *C. neoformans* can replicate within the phagolysosome and promote host cell lysis (12, 13). It is known that oxidative stress can lead to fluctuating levels of cytosolic Ca^{2+} within cells, including active macrophages (14); thus, we sought to resolve whether CMC played a role in promoting survival of *C. neoformans* during oxidative stress.

We have previously shown that strains of *C. neoformans* lacking the genes encoding the Cch1-Mid1 channel complex (CMC) display a significant growth defect in environments that are low in [Ca^{2+}] (~100 nM) (2, 8, 10, 19). This is expected, since CMC represents the only high-affinity calcium channel in the plasma membrane of *C. neoformans*. In low-[Ca^{2+}] environments, a strain lacking either Mid1 or Cch1 is incapable of mediating the influx of calcium from the extracellular milieu to the cytosol. This is consistent with the role of CMC in other fungal species (1, 5, 20, 21).

As predicted, the *mid1*Δ strain displayed a growth defect in the presence of 2 mM and 4 mM BAPTA (a Ca^{2+}-selective chelator) in contrast to the wild-type strain (Fig. 1). Interestingly, the growth defect of the *mid1*Δ strain was exacerbated upon exposure to the oxidative stress-inducing agent H_2O_2 under conditions of limited extracellular [Ca^{2+}] (Fig. 1). This suggested that Mid1 contributed to the survival of *C. neoformans* during oxidative stress likely in a calcium-dependent manner. In contrast, the *cch1*Δ mutants exhibit a strong growth defect during H_2O_2 stress under high-calcium conditions (in the absence of BAPTA), suggesting a significant role for Cch1 in oxidative resistance (Fig. 2A).

An interaction between Mid1 and a novel binding partner, cytochrome *c* peroxidase (Ccp1), was detected. To further resolve whether Mid1 played a direct role in an oxidative stress response, we sought to identify the protein-binding partners of Mid1 through the use of a modified version of the yeast two-hybrid system (Fig. 3A) (22, 23). Unlike traditional two-hybrid screens, where the interaction between the bait and the target (also

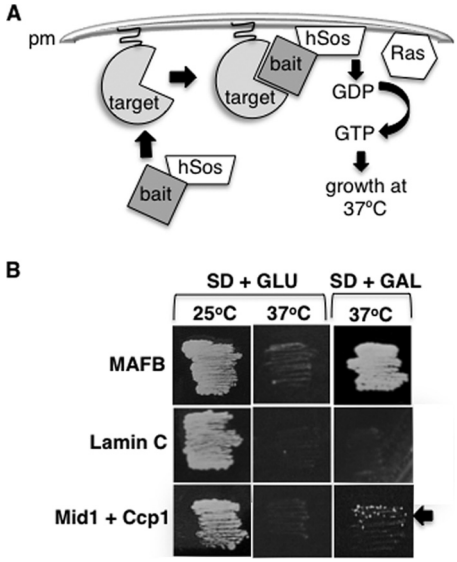

FIG 3 A yeast-two hybrid screen revealed an interaction between Mid1 and Ccp1. (A) Schematic representation of a modified cytosolic version of the traditional yeast two-hybrid system that was used to identify binding partners of Mid1. During a physical interaction between bait (C-terminal region of Mid1) and target (cDNA library of *C. neoformans* with myristoylation signal), hSos is recruited to the plasma membrane, Ras signaling is activated, and growth of the *cdc25-2* strain at the restrictive temperature (37°C) is restored. (B) Approximately 5×10^5 colonies were screened, and colonies that grew at the restrictive temperature (37°C) and in the presence of galactose were determined to be candidate colonies. Plasmids of candidate cDNAs were sequenced and cytochrome *c* peroxidase (Ccp1) was identified. In the presence of galactose at 37°C, the interaction between MAFB and pMyrSB served as a positive control and the lack of an interaction between pSos-MAFB and pl Lamin C served as a negative control for the genetic screen.

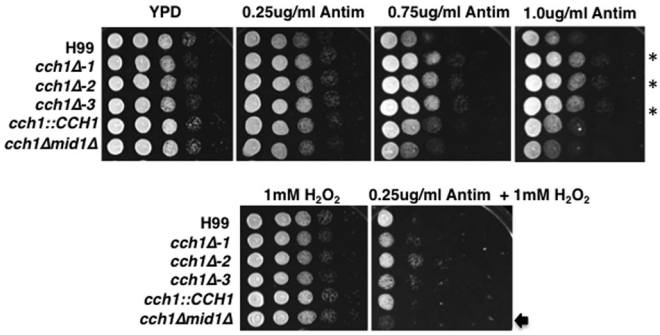

FIG 4 Mid1 is protective and promotes cell survival under conditions that block the cytochrome *c* pathway. Spot sensitivity assays were used to further explore the connection between Mid1 and Ccp1. Strains of *C. neoformans* expressing Mid1 but lacking Cch1 were increasingly resistant to antimycin (Antim) (an inhibitor of the cytochrome *c* peroxidase [Ccp1] pathway), but the addition of H_2O_2 damped the resistance and increased the growth sensitivity. A strain lacking Mid1 and Cch1 was no longer viable under similar conditions suggesting that Mid1 is protective while the Ccp1 pathway is blocked (arrow). Serially diluted cells (10^5, 10^4, 10^3, 10^2, and 10^1) from wild-type H99, the *cch1*Δ mutant (three independent *cch1*Δ isogenic strains), a *CCH1*-reconstituted (*cch1*Δ::*CCH1*) strain, and a *cch1*Δ *mid1*Δ strain of *C. neoformans* were spotted onto YPD plates with 1 mM H_2O_2 either alone or in combination with increasing concentrations of antimycin. Plates were incubated at 30°C overnight.

referred to as prey) occurs in the nucleus, the genetic interaction here takes places in the cytosol by exploiting the Sos-Ras recruitment system. Since Mid1 is a plasma membrane-resident protein, we reasoned that this two-hybrid method would increase the probability of identifying targets of Mid1 that were physiologically relevant.

Our two-hybrid approach takes advantage of the Ras pathway in *S. cerevisiae* in that when it is localized to the plasma membrane, the Ras guanyl nucleotides exchange factor Cdc25 stimulates GDP/GTP exchange on Ras (Fig. 3A) (24). The screen relies on a yeast mutant strain expressing a *cdc25-2* allele that prevents growth at 37°C. Upon the physical interaction between the bait and target, hSos is recruited to the plasma membrane, Ras signaling gets activated and growth of the *cdc25-2* strain at 37°C is restored (Fig. 3A).

In the case of Mid1, a cytosolic C-terminal portion of Mid1 (813 bp, 271 aa) lacking the last 23 amino acids was cloned into a pSos plasmid in order to construct the hSos-Mid1 fusion protein. We chose to use only a specific region of the C-terminal cytosolic portion of Mid1 from *C. neoformans* for several reasons. First, we found that the expression of the full-length Mid1 activated the Sos/Ras recruitment system on its own; therefore, this was not a viable choice for the screen. Second, we recently reported that the last 23 C-terminal amino acids are required for trafficking Mid1 to the plasma membrane; this would likely complicate the readout of the screen (8). Third, the predicted protein structure of Mid1 contains 3 or 4 putative transmembrane regions, indicating that these integral stretches of Mid1 protein would likely not bind to relevant cytosolic or membrane-bound targets. Lastly, as seen with other channel complex proteins, the C-terminal cytosolic regions are often involved in significant protein-protein interactions (19).

The hSos-Mid1 fusion protein was used as bait to screen a myristoylated, galactose-inducible cDNA library of *C. neoformans*. Expression of the hSos-Mid1 fusion protein was confirmed by Western analysis (data not shown). The temperature-sensitive *cdc25-2* yeast strain expressing the cDNA library of *C. neoformans* and a C-terminal region of Mid1 (lacking the last 23 amino acids) predicted to be cytosolic was grown on glucose or galactose plates. Both a positive and negative control were included in the screen to ensure that the *cdc25-2* strain had not reverted. Following the screening of approximately 5×10^5 colonies, only colonies that grew at the restrictive temperature (37°C) and in the presence of galactose were determined to be candidate colonies. Once the plasmids harboring the candidate cDNAs were sequenced, we found that one candidate encoded a cytochrome *c* peroxidase (Ccp1) (Fig. 3B).

Ccp1 and oxidative stress response in *C. neoformans*. Next we sought to resolve the physiological implication of the Mid1-Ccp1 genetic interaction. In *C. neoformans*, cytochrome *c* peroxidase (Ccp1) is known to protect against external oxidative stress-inducing agents (25, 26). It has been shown that treating cells with antimycin A inhibits the cytochrome *c* pathway and leads to the production of free radicals (26). We examined the effect of antimycin in strains of *C. neoformans* lacking either Mid1 or Cch1.

Interestingly, whereas the H99 wild-type strain of *C. neoformans* displayed significant growth sensitivity to antimycin, strains that retained *MID1* expression but lacked *CCH1* (*cch1*Δ mutants) were significantly more resistant to antimycin stress (Fig. 4). This phenotype was observed in three separate isogenic *cch1*Δ strains (Fig. 4). Since the H99 wild-type (WT) strain (which has a func-

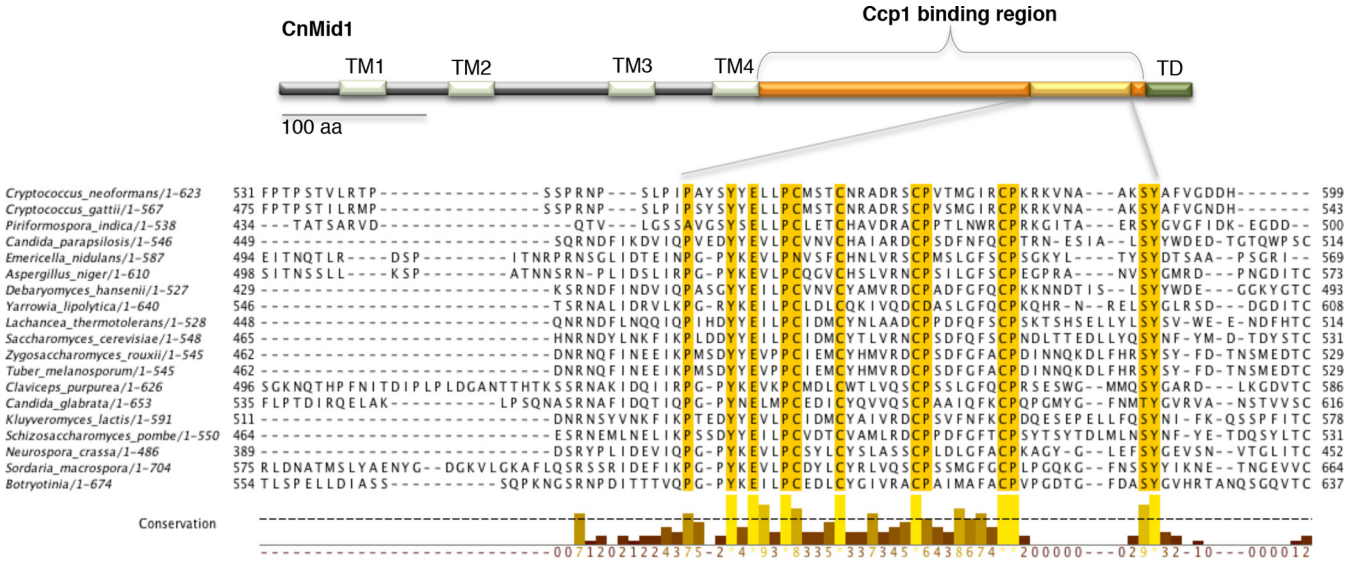

FIG 5 Cysteine-proline dipeptide residues in the C-terminal region of Mid1 are conserved among fungal species. A schematic diagram of the predicted protein structure of Mid1 illustrates the region of Mid1 that associated with Ccp1 in the genetic screen. TD, trafficking domain (8). TM, predicted transmembrane domains according to topology prediction software. It should be noted that the number of TM domains in CnMid1 has not been experimentally defined. The amino acid comparison of Mid1 was performed by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalo/) and T-Coffee multiple-sequence alignment (http://www.ebi.ac.uk/Tools/msa/tcoffee/). Both alignment software programs produced similar results; therefore, only the alignment produced by ClustalW is shown. The two cysteine-proline (CP) dipeptide motifs highlighted in yellow are conserved in fungi and appear within the Ccp1-binding region.

tional Mid1 that forms a complex with Cch1) is sensitive to antimycin, while all three *cch1*Δ strains with functional Mid1 proteins show strong resistance to antimycin toxicity, it is likely that the absence of Cch1 frees up Mid1 to promote its antimycin-detoxifying activities. The contribution of Mid1 in the H99 WT strain during oxidative stress was not apparent until this strain was tested with both antimycin and H_2O_2 (Fig. 4, arrow). Under this more stringent oxidative stress condition, the WT strain was still viable, while the *cch1*Δ *mid1*Δ double mutant failed to grow, consistent with the protective role for Mid1 during Ccp1-mediated oxidative stress response. The results suggested that under conditions of oxidative stress imposed by a combination of stressors like H_2O_2 and antimycin, Mid1 is protective and prevents cell death by attenuating oxidative stress. In addition, when Mid1 is no longer associated with Cch1 (as in the *cch1*Δ strain), the resistance of *C. neoformans* against antimycin becomes much stronger.

The protective role of Mid1 in oxidative stress conditions is independent of the conserved CP (cysteine and proline) dipeptide residues. It has been shown that Ccp1 can bind heme, and this association is mediated by CP dipeptide motifs. Upon analyzing the amino acid sequence of the Mid1 cytosolic C-terminal stretch that bound Ccp1, we identified two cysteine-proline (CP) dipeptide motifs (Fig. 5). The CP dipeptides are highly conserved among fungal species (Fig. 5, highlighting). We and others showed previously that Mid1 has 12 highly conserved cysteine residues near its C-terminal region, several of which are required for the functional activity of Mid1 (8). The two cysteine residues within the CP dipeptides belong to this group of 12 previously reported cysteines (27).

A graphical representation of the Mid1 amino acid multiple-sequence alignment for the region containing the two CP motifs further demonstrated the sequence conservation at the CP dipeptide positions $C^{648}P^{649}$ (referred to here as CP^1) and $C^{656}P^{657}$ (CP^2) and the fairly high frequency of the CP amino acids at this position (Fig. 6A, arrows).

We sought to determine whether the two highly conserved CP dipeptides in Mid1 were involved in mitigating oxidative stress through its association with Ccp1. To accomplish this goal, site-directed mutagenesis was carried out on the full-length Mid1 cDNA, which was cloned into a *C. neoformans* expression plasmid (Jmm180) under the control of a constitutive actin promoter (Table 1). Three different mutant strains were constructed with the following amino acid substitutions: Mid1 CP mutant strain 1 has the Mid1 $C^{648}P^{649}$ dipeptide replaced with two alanines (Mid1 $C^{648A}P^{648A}$ [referred to here as CP^{1x}]), Mid1 CP mutant strain 2 has the Mid1 $C^{656}P^{657}$ dipeptide substituted with alanine residues (Mid1 $C^{656A}P^{657A}$ [CP^{2x}]), and Mid1 CP mutant strain 3 has both CP dipeptides replaced with alanine residues (Mid1 $C^{648A}P^{649A}$-$C^{656A}P^{657A}$ [CP^{1x2x}]). As a control, the *mid1*Δ strain expressing the full-length wild-type Mid1 under the control of the constitutive actin promoter was also constructed (designated Mid1 CP^{wt}). We then used the BAPTA (low-[Ca^{2+}] medium) assay to monitor whether the CP mutants were required for Mid1 functional activity and/or whether they mediated an oxidative stress response (Fig. 6B; also, see Fig. S1 in the supplemental material).

We found that in the presence of H_2O_2 or low extracellular [Ca^{2+}], the *mid1*Δ strain expressing either the Mid1 $C^{648A}P^{649A}$ dipeptide mutant (CP^{1x}) or the strain expressing the Mid1 $C^{648A}P^{649A}$-$C^{656A}P^{657A}$ dipeptide mutant (CP^{1x2x}) displayed a growth defect similar to that observed for the *mid1*Δ background strain (Fig. 1 and 5B). In addition, a similar phenotype was observed for these strains upon exposure to both H_2O_2 and limited extracellular [Ca^{2+}] (Fig. 1 and 5B). Taken together, these results suggested that the $C^{648}P^{649}$ dipeptide (CP^1) was likely crucial to the overall folding and structure of the Mid1 polypeptide, since its substitution produced a strain with a phenotype similar to that of

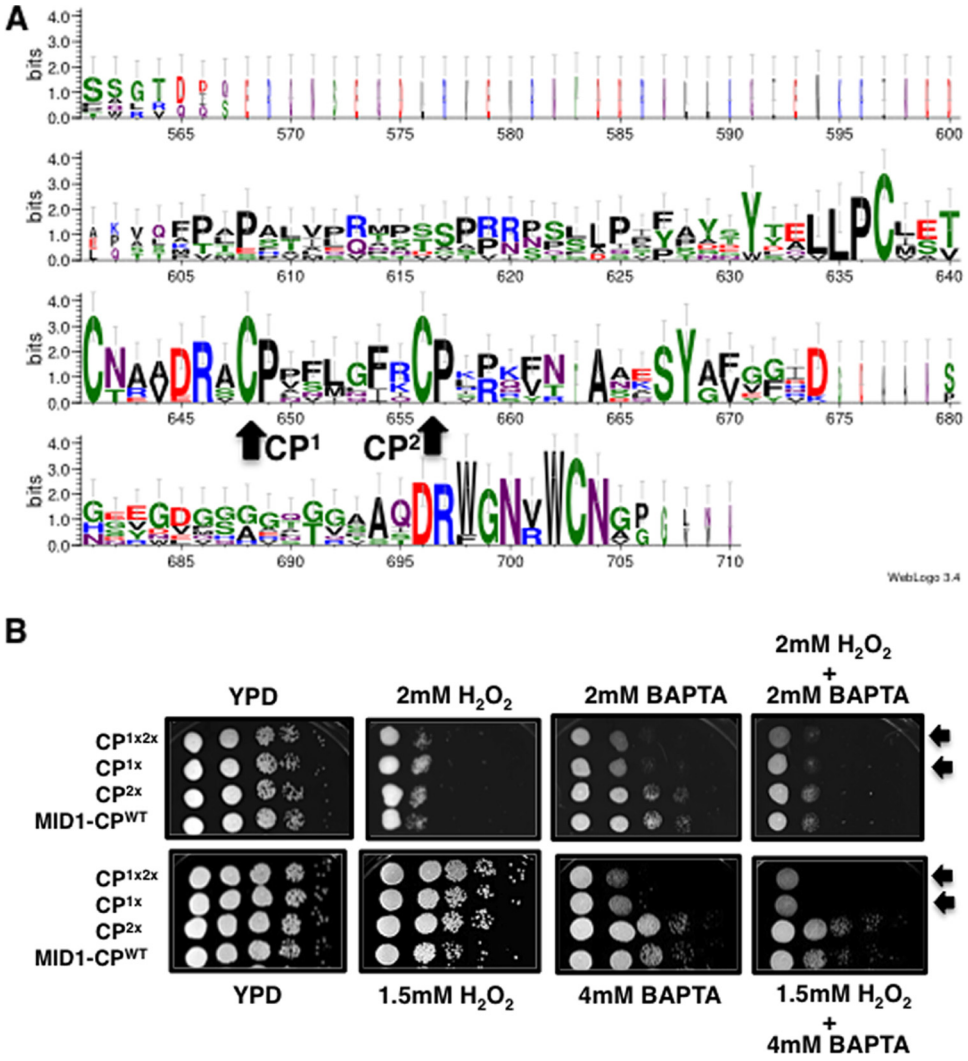

FIG 6 The regulatory role of Mid1 in the Ccp1-mediated oxidative stress response is independent of the conserved CP dipeptides. (A) Graphical representation of the C-terminal region of the Mid1 protein containing the cysteine-proline (CP) dipeptides demonstrating the sequence conservation at the CP dipeptide positions CP^1 ($C^{648}P^{649}$) and CP^2 ($C^{656}P^{657}$) and the fairly high frequency of the CP amino acids at this position (arrows). (B) Site-directed mutagenesis was used to replace CP^1, CP^2, or CP^1 and CP^2 with alanine. The *mid1*Δ strains of *C. neoformans* expressing the Mid1 CP^1 mutant, CP^2 mutant, CP^1-CP^2 mutant, and wild-type CP were designated CP^{1x}, CP^{2x}, CP^{1x2x}, and CP^{wt} strains, respectively. CP^2 was not required for mediating oxidative stress, but CP^1 was required for the functional activity of Mid1, suggested by the significant growth defect of the CP^{1x} mutant strain in limited-[Ca^{2+}] environments (2 mM and 4 mM BAPTA, a Ca^{2+}-specific chelator). This is consistent with the growth defect of the *mid1*Δ strain under similar conditions (Fig. 1).

the strain lacking Mid1 (*mid1*Δ null strain) in limited extracellular [Ca^{2+}]. This is consistent with a recent report that demonstrated that C^{648} is required for Mid1 function in *Saccharomyces cerevisiae* (27).

In contrast to the growth of the Mid1 CP^{1x} and Mid1 CP^{1x2x} mutant strains, the *mid1*Δ strain expressing the Mid1 $C^{656A}P^{657A}$ dipeptide mutant (CP^{2x}) was viable on low-[Ca^{2+}] medium, similar to the H99 wild-type strain and to the Mid1 CP^{wt}-reconstituted control strain, suggesting that this second set of $C^{656}P^{657}$ residues was not required for the functional activity of Mid1 in mediating Ca^{2+} uptake. In addition, the Mid1 $C^{656A}P^{657A}$ dipeptide mutant (CP^{2x}) showed growth sensitivity similar to that of the wild-type strain when exposed to either H_2O_2, H_2O_2 stress with limited extracellular [Ca^{2+}], paraquat, or FCCP (Fig. 6B; also, see Fig. S1 in the supplemental material), suggesting that the role of Mid1 in attenuating oxidative stress was not dependent on the $C^{656}P^{657}$ dipeptide residues.

Intracellular survival of *C. neoformans* is dependent on Cch1 but not Mid1. Interestingly, the *mid1*Δ strain was viable and did not display any growth sensitivity when tested in an intracellular survival assay using an immortalized murine lung macrophage cell line (J774A.1) (Fig. 7). In contrast, a strain lacking Cch1 (*cch1*Δ) exhibited a modest intracellular growth defect in the same *in vitro* assay (Fig. 2B). This was supported by spot sensitivity assays, which revealed that the *cch1*Δ strains had significant growth defects upon exposure to the oxidative stress-inducing agents H_2O_2 and paraquat and the mitochondrial inhibitor FCCP (Fig. 2A). The growth differences observed among the strains whose results are shown in Fig. 2A were due solely to the absence of Cch1 because the *mid1*Δ strain showed no defect when grown on H_2O_2,

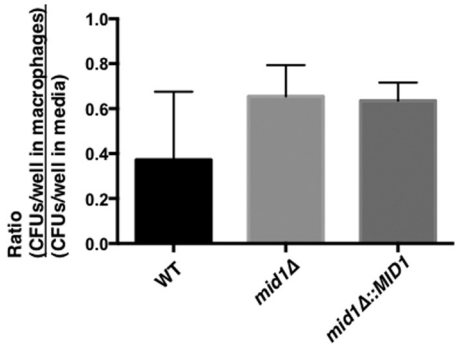

FIG 7 Intracellular survival of *C. neoformans* is independent of Mid1. The J774A.1 macrophage cell line was used to examine whether Mid1 was required for intracellular survival. J774A.1 was infected with wild-type H99, the *mid1*Δ mutant, and the Mid1-reconstituted (*mid1*Δ::*MID1*) strain of *C. neoformans* for 24 h. Lysed macrophages were plated on YPD to determine intracellular growth of *C. neoformans* strains. CFU data were normalized by dividing the CFU counts from wells containing macrophages by the CFU counts from wells lacking macrophages (medium-alone control).

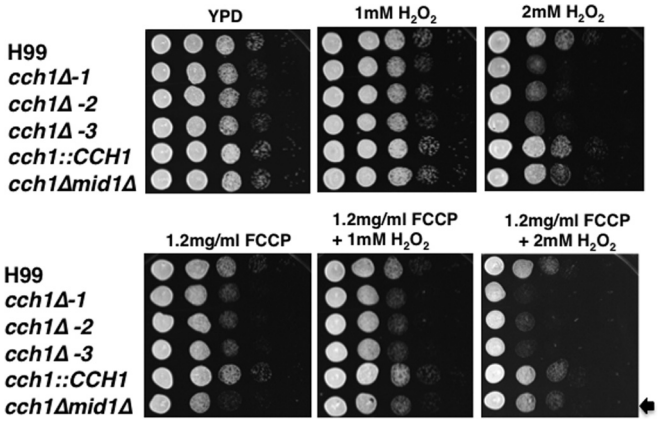

FIG 8 The *cch1*Δ *mid1*Δ strain is resistant to H_2O_2 stress in the presence of FCCP. A sensitivity spot assay was conducted on YPD with or without H_2O_2 in combination with FCCP to determine if a compensatory mechanism in the *cch1*Δ *mid1*Δ double mutant is activated in the presence of H_2O_2. Tenfold serial dilutions (10^5, 10^4, 10^3, 10^2, and 10^1) of wild-type H99, the *cch1*Δ mutant, the Cch1-reconstituted (*cch1*Δ::Cch1) strain, and the *cch1*Δ *mid1*Δ double mutant were assayed on YPD alone or YPD with either 1 mM H_2O_2, 2 mM H_2O_2, 1.2 μg/ml FCCP, or 1.2 μg/ml FCCP in combination with 1 mM H_2O_2 or 2 mM H_2O_2. The arrow indicates a compensatory mechanism in the absence of Cch1-Mid1.

paraquat, and FCCP under similar assay conditions (see Fig. S1 in the supplemental material). The resistance of the *cch1*Δ *mid1*Δ double mutant to H_2O_2 and paraquat was unexpected, because our data showed that both Mid1 and Cch1 played a role in mitigating oxidative stress (Fig. 1, 3, and 7A), and we expected that the loss of both components of the Cch1-Mid1 channel complex would lead to a stronger growth defect phenotype during oxidative stress (Fig. 2A). This result was further supported by the intracellular macrophage assay, where the *cch1*Δ *mid1*Δ mutant did not display a growth defect, in contrast to the growth defect observed for the *cch1*Δ strain (Fig. 2B).

To further confirm the contribution of CMC in oxidative stress resistance, we tested the growth of the *cch1*Δ *mid1*Δ strain in the presence of FCCP, a protonophore known to disrupt mitochondrial functions in other fungi (28) (Fig. 2A and 8). As shown in Fig. 2A, the *cch1*Δ *mid1*Δ double mutant was as defective as the *cch1*Δ strains in the presence of FCCP. This result was consistent with the protective role of Cch1 during oxidative stress, since mitochondrial functions are important for oxidative stress resistance in *C. neoformans* (26). A likely scenario for the resistance of the *cch1*Δ *mid1*Δ strain to oxidative stress would be the existence of a compensatory mechanism that is activated in the absence of both Mid1 and Cch1 under H_2O_2 and paraquat stress but not with FCCP.

Since paraquat is a superoxide (O^{2-}) generator and *C. neoformans* superoxide dismutase (SOD) can convert superoxide to H_2O_2 (25), it is likely that the compensatory response observed for the *cch1*Δ *mid1*Δ mutant is activated in the presence of H_2O_2. To test this idea, the *cch1*Δ *mid1*Δ strain was assayed in the presence of both FCCP and H_2O_2. We reasoned that the presence of H_2O_2 would activate this compensatory mechanism and alleviate the growth defect of the *cch1*Δ *mid1*Δ mutant (Fig. 8). Consistent with the existence of a compensatory response pathway, we found that while the *cch1*Δ *mid1*Δ strain is as sensitive to FCCP as the *cch1*Δ mutants, the addition of 2 mM H_2O_2 to the medium containing FCCP restored the growth of *cch1*Δ *mid1*Δ to the level of the H99 WT strain, while the growth defect of the *cch1*Δ strains became more drastic (Fig. 8).

DISCUSSION

Oxidative stress implies that the cellular redox status has shifted to an increased oxidized state, and this shift may be a result of cells exposed to environmental oxidants or stresses like heavy metals, ionizing and UV irradiation, and others. Reactive oxygen or nitrogen species can also be endogenously produced under pathological conditions or the aging of cells (29, 30). Either way, once formed, oxidants can interact with cellular components and modify calcium-mediated signaling events (30).

It is well known that oxidative stress alters calcium signaling (31). In the presence of exogenous agents that produce acute oxidative stress, cells respond by altering and sustaining high cytosolic calcium levels via the influx of calcium across the plasma membrane and calcium release from intracellular stores like the ER (32). A rise in cytosolic calcium triggers a rise in mitochondrial calcium, and depending on the strength or type of oxidative stress, mitochondrial calcium accumulation can switch from a positive effect to a cell death signal (32, 33).

The mitochondrial antioxidant protein cytochrome *c* peroxidase (Ccp1) catalyzes the degradation of hydrogen peroxide (25, 34). Ccp1 is a water-soluble heme-containing enzyme of the peroxidase family that takes reducing equivalents from cytochrome *c* and reduces hydrogen peroxide to water (34). In *C. neoformans*, Ccp1 promotes resistance to oxidative stress-inducing agents *in vitro*, consistent with similar observations made with *S. cerevisiae* (25, 26, 35). *In vivo*, the *ccp1*Δ strain of *C. neoformans* was as virulent as the wild-type strain, suggesting that Ccp1 does not act alone and that other compensatory mechanisms are in place in order to mitigate oxidative stress (25).

We detected an interaction between Mid1 and Ccp1, but the regulatory role of Mid1 in the Ccp1-mediated oxidative stress response does not appear to depend on heme binding. Not only does heme serve as a prosthetic group in enzymes like Ccp1, it also functions as a signaling molecule that controls various cellular processes (36). For instance, HapI is a heme-activated transcrip-

tion factor that mediates oxygen sensing in *S. cerevisiae* (37), and cytochrome *c* peroxidase also functions as a heme-based H_2O_2 sensor that can mediate antioxidant stress (34). The association between HapI and heme may be dependent on a series of conserved cysteine-proline (CP) dipeptide residues that we identified in Mid1 as well. The CP dipeptides are known as the heme-regulatory motifs (34). Despite the high level of conservation of both CP dipeptides across fungal species, amino acid substitution analysis revealed that the loss of the second CP dipeptide had no effect on H_2O_2 stress response in *C. neoformans*. We could not assess the role of the first CP dipeptide, since this cysteine residue was critical for Mid1 functional activity, consistent with a previous finding (27).

Antimycin blocks the Ccp1 pathway by binding to Qi site of cytochrome *c* reductase and thus inhibiting oxidation of ubiquinol in the electron transport chain of oxidative phosphorylation (38). Inhibition blocks formation of proton gradients across the inner membrane and inhibits production of ATP, as protons cannot flow through the ATP synthase complex. The depletion of ATP produces a rise in $[Ca^{2+}]$ in both the cytosol and the mitochondria, which could lead to cytochrome *c* release and ultimately cell death (39). In certain cell types, blocking calcium influx by inhibiting L-type calcium channels in the plasma membrane is cytoprotective, likely due to prevention of the uncontrolled accumulation of calcium in mitochondria (33, 39).

In this study, we found that uncoupling the Cch1-Mid1 channel complex so that Mid1 was expressed independently of Cch1 protected cryptococci from antimycin-induced stress. The underlying mechanism may reflect the inability of Mid1 to mediate the influx of extracellular calcium into the cytosol, which would prevent sustained accumulation of mitochondrial calcium and thus curtail cell death. Alternatively, the release of Ccp1 into the cytosol and its subsequent association with Mid1 may promote a complex cellular defense network, possibly one that transmits stress signals to the oxidative-stress response transcriptome (26). In yeast, Ccp1 functions as an antioxidant enzyme; however, it may also play a role as a sensor in conveying oxidative stress in the mitochondria to Pos9, a transcription factor that mediates the oxidative stress response (16).

The "individual" contributions of Mid1 and Cch1 to oxidative stress resistance in *C. neoformans* appear disparate and complex. Mid1 contribution to the mitigation of oxidative stress is likely less significant and more specific than that of Cch1. The growth differences between the *mid1*Δ mutants and the WT H99 strain can be discerned only in low-calcium medium, under antimycin stress in the absence of Cch1 (i.e., growth sensitivity of the *cch1*Δ mutants), and under antimycin in combination with H_2O_2 in the absence of both Mid1 and Cch1 (i.e., growth sensitivity of the *cch1*Δ *mid1*Δ mutant). In contrast, Cch1 appears to have a more general role in promoting cryptococcal survival during oxidative stress. The growth defect of the *cch1*Δ mutants can be easily discerned by the addition of H_2O_2 in the absence of the calcium chelator BAPTA and without the need for additional stressors, such as antimycin. Consistent with a greater contribution of Cch1 to oxidative stress tolerance, an intracellular growth defect was observed for the *cch1*Δ strain, while no growth differences were observed for the *mid1*Δ mutant compared to the wild-type strain in the macrophage cell line J774A.1.

Interestingly, the *cch1*Δ *mid1*Δ double mutant was resistant to oxidative stress induced by both H_2O_2 and paraquat but remained susceptible to oxidative stress induced by FCCP. The resistance of the *cch1*Δ *mid1*Δ strain to H_2O_2 remained strong even in the presence of FCCP. Together, these data suggest that in the absence of both Mid1 and Cch1, a compensatory mechanism is active to promote *C. neoformans* survival in the presence of H_2O_2 and paraquat but not FCCP. This compensatory mechanism is likely to be robust, since it is able to restore *C. neoformans* growth to levels comparable to that of the WT strain in the presence of FCCP, a highly toxic drug that disrupts mitochondrial function. The ability of the *cch1*Δ *mid1*Δ strain to resist oxidative stress was further supported by the macrophage intracellular survival assays, where the *cch1*Δ mutant displayed an intracellular growth defect but the *cch1*Δ *mid1*Δ mutant did not, suggesting the presence of a complex web of factors that can alleviate oxidative stress independently of CMC.

Collectively, this study suggests that Cch1 and Mid1 participate in a multiprotein complex that mediates both Ca^{2+} influx and intracellular Ca^{2+} homeostasis. However, the observed differences in the phenotypic responses of the *cch1*Δ and *mid1*Δ mutant strains to oxidative stress suggest partially overlapping functions in mitigating oxidative stress.

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