Had1 Is Required for Cell Wall Integrity and Fungal Virulence in Cryptococcus neoformans

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ABSTRACT Calcineurin modulates environmental stress survival and virulence of the human fungal pathogen Cryptococcus neoformans. Previously, we identified 44 putative calcineurin substrates, and proposed that the calcineurin pathway is branched to regulate targets including Crz1, Pbp1, and Puf4 in C. neoformans. In this study, we characterized Had1, which is one of the putative calcineurin substrates belonging to the ubiquitously conserved haloacid dehalogenase β-phosphoglucomutase protein superfamily. Growth of the had1Δ mutant was found to be compromised at 38°C or higher. In addition, the had1Δ mutant exhibited increased sensitivity to cell wall perturbing agents, including Congo Red and Calcofluor White, and to an endoplasmic reticulum stress inducer dithiothreitol. Virulence studies revealed that the had1Δ mutation results in attenuated virulence compared to the wild-type strain in a murine inhalation infection model. Genetic epistasis analysis revealed that Had1 and the zinc finger transcription factor Crz1 play roles in parallel pathways that orchestrate stress survival and fungal virulence. Overall, our results demonstrate that Had1 is a key regulator of therмотolerance, cell wall integrity, and virulence of C. neoformans.

KEYWORDS Cryptococcus neoformans calcineurin Crz1 fungal virulence Had1

Calcineurin is a highly conserved serine/threonine protein phosphatase, which is activated by Ca²⁺/calmodulin in eukaryotic organisms, and plays diverse roles in controlling gene expression and cellular processes (Stewart et al. 1982; Rusnak and Mertz 2000; Aramburu et al. 2004). Increased intracellular Ca²⁺ levels in response to internal or external cues bind to calmodulin, and, in turn, the Ca²⁺-calmodulin complex interacts with the calcineurin catalytic subunit and activates the phosphatase (Crabtree 2001). Activated calcineurin dephosphorylates target proteins that then orchestrate the response and adaptation to stress

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associated with cell-wall synthesis, ion transport, and vesicle transport (Yoshimoto et al. 2002; Cyclic 2003). In the major pathogenic fungi, including Candida albicans and Aspergillus fumigatus, the calcineurin signaling pathway plays crucial roles in virulence and stress responses (Badger et al. 2003; Blankenship et al. 2003; Steinbach et al. 2006, 2007a,b; Lee et al. 2013). Loss of calcineurin causes increased cell sensitivity to stresses including high temperature, salt, cell wall stress, and also results in attenuated or loss of virulence in human pathogenic fungi. Therefore, calcineurin is considered a target for anti-fungal drug discovery, and, in fact, the immunosuppressants FK506 and CsA show anti-fungal activity (Brizuela et al. 1991; Odom et al. 1997b). A series of studies also demonstrated that the Crz1 orthologs are prominent calcineurin downstream targets in pathogenic fungi (Onyewu et al. 2004; Cramer et al. 2008; Schumacher et al. 2008; Soriani et al. 2008; Miyazaki et al. 2010).

Cryptococcus neoformans is a ubiquitous fungus that is widespread in the environment, including associations with trees, soil, and bird guano (Monari et al. 1999; Idnurm et al. 2005; Chang et al. 2014). This fungus is a basidiomycetous fungus and both its spores and desiccated yeast cells serve as infectious propagules (Hung and Schreiber 1992; Kwon-Chung et al. 2014). C. neoformans is an opportunistic pathogenic fungus that causes meningocerebalitis in patients who have weakened immune systems, including HIV/AIDS and organ transplant patients, leading to high mortality (Kidd et al. 2004; Park et al. 2009). In this human pathogenic fungus, calcineurin is a key virulence factor that is required for adaptation to stressful host environments, including elevated temperature (Odom et al. 1997b; Fox et al. 2001). In addition, calcineurin is essential for stress adaptation, sexual reproduction, and virulence of C. neoformans (Fox et al. 2001; Danielsen et al. 2013). During thermal and other stress conditions, calcineurin colocalizes with components of P-bodies/stress granules (PBs/SGs) (Kozubowski et al. 2011a,b), which consist of aggregates of RNA binding proteins, mRNA decay machinery, and translation initiation factors (Buchan and Parker 2009; Mitchell et al. 2013; Buchan 2014).

Our recent phosphoproteomic analysis identified 44 putative calcineurin targets in C. neoformans (Park et al. 2016). We demonstrated that the Crz1 ortholog is a bona fide calcineurin target in C. neoformans. Under thermal stress conditions, calcineurin controls the transcriptional activity and nuclear translocation of Crz1 through dephosphorylation (Park et al. 2016). In addition, we proposed that several RNA binding proteins, including Pbp1 (PAB1-binding protein 1), Pu4 (PUMilio-homology domain Family 4), and Lhp1, are potential calcineurin targets. Pbp1 is involved in fungal virulence and sexual reproduction (Park et al. 2016). Both Pu4 and Lhp1 are required for heat stress survival (Park et al. 2016). Employing epistasis analyses, we demonstrated that two downstream branches of the calcineurin pathway govern cell viability at high temperature, sexual reproduction, and fungal virulence (Park et al. 2016).

Although we characterized several calcineurin targets, including Crz1 and RNA binding proteins, in our previous study (Park et al. 2016), many putative targets await characterization. To further characterize the remaining calcineurin targets revealed by the above study, we examined stress responses of 13 putative calcineurin-target mutants from a systematic deletion mutant library of C. neoformans (Liu et al. 2008). Among them, the CNAG_01744Δ mutant showed increased sensitivity to cell wall stress in comparison to the WT. Based on BLAST analysis, CNAG_01744 shares considerable homology with the S. cerevisae Had1 protein. Had1 contains a halocacid dehalogenase (HAD) domain, and is a member of the β-phosphoglucomutase family of proteins that is highly conserved in most organisms (Burroughs et al. 2006; Kuznetsova et al. 2015). In yeast, several β-phosphoglucomutase family proteins, including two 2-deoxyglucose-6-phosphatase (Dog1 and Dog2) and two glycerol-1-phosphate phosphohydrolases (Rhr2/Gpp1 and Hor2/Gpp2), are well characterized and are required for cellular responses to environmental stresses such as osmotic and oxidative stresses (Randez-Gil et al. 1995a,b; Nurbeck et al. 1996; Kuznetsova et al. 2015). Given the relevance of HAD in stress responses, we were prompted to characterize the role of Had1 in the calcineurin pathway functions in C. neoformans. Our results demonstrate that loss of HAD1 causes increased sensitivity to thermal or cell wall stresses and attenuated virulence. To dissect the link between Had1 and Crz1 in calcineurin-related functions, we tested the impact of the had1Δ crz1Δ double mutation. The results show that Crz1 and Had1 play additive roles in thermostolerance and virulence. However, whether Had1 is a direct calcineurin substrate remains to be established.

**MATERIALS AND METHODS**

**Strains, media, and culture conditions**

Fungal strains used in this study are listed in Table 1. Liquid and solid yeast extract-peptone-dextrose (YPD; Difco, Sparks, MD) media were used for general cultures of C. neoformans. To assay thermo-tolerance, fungal cells grown overnight at 30°C were 10-fold diluted, spotted on YPD plates, and then cultured at distinct temperatures (30, 37, 38, and 39°C). To examine susceptibility to other stresses, 2.5–5 µl of cultured cells grown in liquid YPD medium overnight were ten-fold serially diluted and spotted on YPD medium containing the indicated concentration of the following compounds; Congo red (CR; Sigma, St. Louis, MO) and sodium dodecyl sulfate (SDS; Fisher, Fair Lawn, NJ) for membrane destabilizing stress; dithiothreitol (DTT, Sigma) for reducing stress; calciofluor white (CFW; Sigma) for chitin synthesis inhibition, which results in cell wall stress, NaCl (Fisher); and KCl (Fisher) for salt stresses. Fungal cells were incubated at 30°C and photographed post-treatment from d2 to d3.

**Generation of mutant strains**

The oligonucleotides used in this study are listed in Table 2. To generate a deletion mutant, gene deletion cassettes were generated using a double-joint PCR (DJ-PCR) as described (Yu et al. 2004). The 5’ and 3’-flanking region of the HAD1 gene were amplified using primer pairs JOHE42780/JOHE42782 and JOHE42781/JOHE42783, respectively, from the C. neoformans serotype A H99 (Perfect et al. 1993; Janbon et al. 2014) genomic DNA as a template. The selectable markers, NAT or NEO (Fraser et al. 2003), were amplified with the primer pair JOHE40706/JOHE40707 using pAI3 and pJA1, respectively. The final deletion cassettes were generated by means of DJ-PCR performed using primer pair JOHE42784/JOHE42785 and the 5’ and 3’-flanking regions and markers as templates. The amplified gene deletion cassettes were purified, combined with 0.6 µm gold microcarrier beads (Bio-Rad) using the QAQuick Gel Extraction kit (Qiagen), and then introduced into the wild type (H99 or KN99a) mutant strains using biolistic transformation methods (Davidson et al. 2002).

To generate the double deletion mutants, 5’ and 3’-flanking regions for CRZ1 (JOHE41391/JOHE41393 and JOHE41392/JOHE41394) were amplified. The NAT marker was used for the disruption cassettes. After fusion by DJ-PCR, crz1 disruption constructs were amplified using JOHE41395/JOHE41396, and introduced into HPC24 (had1Δ mutant). Multiple stable transformants were isolated from independent experiments, and were selected on YPD medium containing nourseothricin sulfate or G418, and then confirmed by diagnostic PCR for the 5’ and 3’ junctions, followed by restriction enzyme digestion.
To express the Had1-FLAG fusion protein, the HAD1 gene region, including its predicted promoter but lacking its termination codon, was amplified using the primers JOHE42786 and JOHE42787. The PCR product was then digested with NotI and cloned into pH2 (Park et al. 2016), which contains a 4× FLAG tag, the HOG1 terminator, and the hygromycin B-resistance gene. The resulting plasmid pHSP1 was then introduced into the recipient had1Δ strains. Multiple transformants were selected on YPD medium containing hygromycin B (Sigma), and then confirmed by PCR and Western blot analyses.

### Had1 mobility assay

Had1 mobility assay was conducted as described previously (Park et al. 2016). Strains expressing Had1-FLAG were grown in YPD at 25°C to an optical density OD600 of 0.6–0.8, and cultures were grown at 25°C or shifted from 25 to 37°C for 1 hr with or without FK506 (2 μg/ml). Cells were collected and disrupted in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% Triton X-100) supplemented with a protease inhibitor tablet (Roche) and phosphatase inhibitor cocktail (Thermo) using a bead beater for 10 cycles (60 sec homogenization with a 60 sec rest). Cell lysates were centrifuged for 15 min at 15,000 × g, the supernatant was recovered, and protein concentration was determined by employing the Bio-Rad Bradford reagent. The supernatant was subjected to SDS-PAGE and transferred to PVDF membranes (Bio-Rad). For western blot analysis, we employed mouse monoclonal anti-FLAG M2 antibodies (Sigma), anti-mouse antibody conjugated to horseradish peroxidase (Thermo), and ECL western blotting detection reagent (GE healthcare).

#### Virulence assay

Cryptococcus strains were cultured overnight in liquid YPD medium at 30°C. The resulting fungal cells were collected, washed with sterile PBS, counted with a hemocytometer, and the final density was adjusted to 1 × 10^7 colony forming units (CFU)/ml. Six- to 7-wk-old Female BALB/c mice were purchased from the Daehan BioLink Co., Ltd., Korea, and used for infection and fungal burden assays. Intranasal infection was performed as previously described (Cox et al. 2000). Fourteen mice were anesthetized, and infected intranasally with 5 × 10^5 CFU in a volume of 50 μl as previously described (Cox et al. 2000).

For survival tests, groups of 10 mice were used. Survival was monitored daily, and moribund mice were killed with CO2. Survival curves were generated using the Kaplan-Meier method using Prism 4.0 (GraphPad software), and statistical significance (p values) were assessed with the log-rank test.

For fungal burden assays, infected mice (three to four) were killed by exposure to CO2. Lung and brain tissues were isolated, placed in saline, and then homogenized. The resulting fungal cells were collected, washed with sterile PBS, counted with a hemocytometer, and the final density was adjusted to 10^7 colony forming units (CFU)/ml of PBS. Six- to 7-wk-old Female BALB/c mice were purchased from the Daehan BioLink Co., Ltd., Korea, and used for infection and fungal burden assays. Intranasal infection was performed as previously described (Cox et al. 2000). Fourteen mice were anesthetized, and infected intranasally with 5 × 10^5 CFU in a volume of 50 μl as previously described (Cox et al. 2000).

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Figure 1 Phenotypes of the potential calcineurin target mutants exposed to various stresses. (A) Targets in the Venn diagram labeled red were characterized in the previous study (Park et al. 2016). Targets labeled blue were either deleted in our laboratory or obtained from a C. neoformans deletion mutant library (Liu et al. 2008) and tested in this study. Cna1 (green) is the calcineurin A catalytic subunit. (B) Functional categories ascribed to the potential calcineurin targets tested in this study. (C) Spot dilution assays with WT (H99), cna1Δ (HP242), and 13 mutants from a systematic gene deletion library were performed under several stress conditions as indicated. Strain cultures were incubated overnight, serially diluted 10-fold, and plated on YPD medium without or with CR and SDS. Cells were incubated for 2–3 d at 30, or 37°C as indicated, and all cultures containing stressor compounds (SDS and CR) were incubated at 30°C.
and then homogenized. The suspension was serially diluted with saline, plated onto YPD agar supplemented with antibiotics (kanamycin, ampicillin, and spectromycin), and incubated at 30°C for 3 days. Colony counts were performed and adjusted to reflect the total lung or brain CFU. Statistical analysis was performed using the Student’s t-test to assess statistically significant differences between the samples.

Figure 2 Deletion of had1 results in hypersensitivity to various stresses. Spot dilution assays with WT (H99), cna1Δ (HP242), and had1Δ mutants (HPC23 and HPC24), and had1Δ + HAD1 complemented strains (HPC27 and HPC28), were performed under several stress conditions as indicated. (A) Cell cultures in liquid YPD medium were grown overnight at 30°C and serially diluted 10-fold. Equal aliquots were spotted on solid YPD medium supplemented, or not, with the stressor compounds as indicated in (B). (A) For thermotolerance, cells were incubated on solid medium for 2 days at 30, 37, or 38°C as indicated. (B) For cell wall, endoplasmic reticulum, or osmolarity stress, the solid YPD culture medium was supplemented with CR, CFW, DTT, SDS, or KCl at the indicated concentrations, and the cultures were incubated at 30°C. Results shown are representative of two independent experimental replicates.

Figure 3 Had1 and Crz1 orchestrate stress response. Stress tolerances of WT (H99), the single cna1Δ (HP242), crz1Δ (HP235), and had1Δ (HPC24) mutants, or double had1Δ crz1Δ (HPC29) deletion mutant strains. Cells were grown overnight at 30°C, serially diluted 10-fold, and plated on YPD medium. Plates were incubated for 2 days at 30, 37, 38, or 39°C as indicated.
Ethics statement
Animal care and all experiments were conducted in accordance with the ethical guidelines of the Ethics Review Committee for Animal Experimentation (ERCAE) of Handong Global University (HGU). The HGU ERCAE approved the entire vertebrate animal protocol (protocol #HGU-20160616-009).

Data availability
All the strains and plasmids used in this study are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS

CNAG_01744 encodes a HAD-like hydrolase protein (Had1)
Previously, we performed phosphoproteomic analyses and identified 44 putative calcineurin targets. Among these targets, we generated deletion mutants for eight, and characterized these as authentic calcineurin targets (Park et al. 2016). In the present study, 13 additional putative calcineurin targets identified by the above phosphoproteomic analysis mutants were available in a systematic deletion mutant library of C. neoformans (Liu et al. 2008) (Figure 1, A and B), and these were selected and analyzed for calcineurin-related phenotypes. Because calcineurin is essential for responses to stress, these mutants were subjected to phenotypic analyses under several stress conditions. The CNAG_01744Δ and CNAG_03841Δ mutants exhibited increased sensitivity to cell wall stress (SDS and CR) as compared to the wild type (WT) (Figure 1C). CNAG_03841 encodes a hypothetical protein and CNAG_01744 encodes a HAD-like hydrolase protein. In this study, we focused on characterizing the functions of CNAG_01744.

The protein sequence of the CNAG_01744 open reading frame (ORF) was blasted against the S. cerevisiae S288 genome database and was found to bear identity to six HAD β-phosphoglucomutase family proteins (Supplemental Material, Figure S1A). Furthermore, C. neoformans genome blast searches revealed a total of four ORFs that feature HAD domains, including CNAG_01744, CNAG_06122, CNAG_06132, and CNAG_06698 (Figure S1B). CNAG_01744 showed the highest homology (Score 110, identity 32%) to S. cerevisiae Gpp2. However, we found that CNAG_06122 shows much higher homology (Score 119, identity 33%) to S. cerevisiae Gpp2 (a glycerol-1-phosphatase induced in response to osmotic and oxidative stress) than CNAG_01744. Therefore, CNAG_01744 was named Had1 instead of Gpp2.

Had1 is essential for appropriate stress response and may function independently of calcineurin
To test the functions of Had1, we generated a had1 deletion (had1Δ) mutant, and complemented strains, and examined their phenotypes under a variety of temperature conditions. As mentioned above, we confirmed that the had1Δ mutant exhibited increased sensitivity to high temperatures when compared to the WT strain (Figure 2A). To further examine the role of Had1, we tested the growth phenotypes of had1 mutants on solid media containing various cell stressors agents including CR, CFW, SDS, DTT, and KCl. As shown Figure 2B, the had1Δ mutant showed increased sensitivity to cell wall perturbing agents, in particular to SDS, as compared to the WT strain, suggesting that Had1 may be involved in controlling stress response.

Because Had1 is a potential calcineurin target identified from the phosphoproteomics results (Park et al. 2016), we examined the migration of Had1 under both heat or high salt osmotic stress conditions. Cultures of yeast cells expressing FLAG tagged Had1 were cultured at 25°C and shifted (or not) either to 37°C for 1 hr, or to medium containing 1 M NaCl, and incubated at 25°C for 1 hr. For both stress conditions, cultures were treated (or not) with FK560 for 1 hr. Interestingly, The Had1-FLAG protein isolated from cells shifted from 25 to 37°C or from cells exposed to high salt osmotic stress, displayed reduced gel migration as compared to that isolated from cultures grown at 25°C (Figure S2A). However, FK560 did not affect the gel migration of Had1 under either stress condition (Figure S2B). These results suggest that the stress-induced post-translational modification resulting in altered Had1 gel mobility is independent of calcineurin, but do not rule out the possibility that Had1 is a substrate of calcineurin that cannot be detected by gel mobility shift assay.

Had1 and Crz1 play an additive role in stress response and virulence
Previously, our study demonstrated that calcineurin regulates high temperature growth and virulence via transcriptional and post-transcriptional processes (Odom et al. 1997b; Cruz et al. 2001). Among the characterized calcineurin targets, Crz1 is the only known target involved in cell wall integrity. To examine whether Crz1 and Had1 function in a single pathway or in parallel pathways within the signaling cascades governing cell wall integrity, we generated had1 crz1Δ double deletion mutants, and examined their phenotypes during stress responses. As shown Figure 3, the had1Δ crz1Δ double mutant strains exhibited increased sensitivity to various stresses compared to either the crz1Δ or had1Δ single deletion...
mutant. These results demonstrate that Had1 and Crz1 play an additive role in thermotolerance and cell wall stresses, and suggest they operate in parallel pathways.

**Had1 is involved in fungal pathogenicity**

Because of hyper-susceptibility to heat and other stress conditions, we hypothesized that Had1 would be required for pathogenicity of *C. neoformans*. To examine virulence of these mutants, we conducted animal virulence studies using a murine inhalation model. As shown in Figure 4A, the had1Δ mutant exhibited attenuated virulence compared to the WT strains. Virulence was restored to nearly WT levels when the had1Δ mutant was complemented by reintroduction of the WT *HAD1* gene in the had1Δ + *HAD1* strain. The had1Δ crz1Δ double mutant strains were more attenuated in virulence as compared with either the had1Δ or the crz1Δ single mutant (Figure 4A). These results demonstrate that Had1 contributes significantly to *C. neoformans* virulence.

To further examine the virulence defect conferred by the had1Δ mutation, fungal burden in the lungs and brain of mice infected with WT and had1Δ mutant strains was analyzed at d 14 post infection (Figure 4B). The cna1Δ mutant exhibited an undetectable fungal burden in both lung and brain tissues. By comparison, the had1Δ and crz1Δ single mutant strains showed reduced fungal burden in the lung compared with the WT strain. Moreover, the had1Δ crz1Δ double mutant strains exhibited significantly reduced fungal burden in the lung compared with either single deletion mutant strain. While we recovered WT and mutant strains from the lung, we did not recover any yeast cells from brain tissue 14 d after infection for both WT and mutant strains (data not shown). Collectively, these findings demonstrate that Had1 is required for full virulence, and that Had1 and Crz1 function in parallel pathways controlling virulence.

**DISCUSSION**

Given that calcineurin is a key pathway regulating stress responses, mating, and fungal virulence, identification and characterization of calcineurin targets is crucial for understanding the calcineurin signaling network (Aramburu et al. 2004; Steinbach et al. 2006; Goldman et al. 2014). In a previous study, we identified Had1 as a potential target of calcineurin in *C. neoformans* (Park et al. 2016). In the present study, although we were unable to confirm that Had1 is a calcineurin target, had1Δ mutation resulted in hypersensitivity to thermal and other stresses, and attenuated virulence, suggesting that Had1 is important for stress responses and virulence. Moreover, double deletion mutant analysis suggests that Had1 and Crz1 function in parallel pathways controlling fungal pathogenicity and cell wall stress responses (Figure 5). However, we could not determine if Had1 functions dependently or independently of calcineurin. The preliminary results of our Had1 gel mobility analysis showed that, while both thermal and osmolarity stresses seem to affect Had1 mobility, in both cases, this is not altered by FK506, and, thus, it seems to be independent of calcineurin (Figure S2).

Our previous phosphoproteomic screen revealed only one calcineurin-dependent phosphorylation site in Had1 (Park et al. 2016). Therefore, it is likely that this single site could not be detected by our mobility shift assay (Figure S2), which is more suited to detecting multiple phosphorylation sites on a protein. Thus, the present study does not rule out the possibility that Had1 function is controlled by calcineurin; testing this model will require more sensitive assays and further analysis.

HAD-like hydrolases are large superfamilies of enzymes that exhibit phosphatase, ATPase, phosphonatase, and phosphomutase activity, and are conserved in both prokaryotic and eukaryotic organisms (Burroughs et al. 2006; Kuznetsova et al. 2015). Forty-five genes encoding HAD-like hydrolases are found in the yeast genome, and 15 of their protein products have been biochemically characterized for this enzymatic activity (Randez-Gil et al. 1995a,b; Norbeck et al. 1996; Kuznetsova et al. 2015). Among these, the β-phosphoglucomutase family proteins, such as Dog1, Dog2, Gpp1, and Gpp2, are well characterized in yeast. These proteins are required for cellular responses to environmental stresses. The expression of Gpp1, Gpp2, and Dog2 is induced by...
environmental stresses, including osmotic or oxidative stresses, and by glucose starvation (Tsujimoto et al. 2000; Pahlman et al. 2001). Interestingly, the expression of these genes is controlled by either the HOG (High Osmolality Glycerol) or the Snf1 kinase pathways (Tsujimoto et al. 2000; Pahlman et al. 2001). In addition, loss of both the GPP1 and GPP2 genes results in hyper-sensitivity to oxidative stress (Pahlman et al. 2001). However, gpp1Δ, gpp2Δ, and dga2Δ single mutants did not show any increased susceptibility to osmotic or oxidative stresses. Our study establishes that Gad1 is required for proper responses to heat and osmotic stresses, and this finding is in accord with previous studies suggesting this function. Previous microarray analyses shown that the expression of HAD1 (formerly GPPI) was induced in response to osmotic stress, and this microarray data were confirmed by our qRTP-PCR analysis (Figure S3A). Ko et al. (2009) also demonstrated that induction of HAD1 in response to osmotic stress was decreased in hog1 and ssk1 mutants, suggesting that the expression of HAD1 is controlled by the stress-activated Hog1 signaling pathway in C. neoformans (Ko et al. 2009). These results suggest that the regulatory mechanism of HAD1 mRNA expression in response to osmotic stress is conserved in S. cerevisiae and C. neoformans. We demonstrate here, that upon thermal stress, the expression of HAD1 slightly increases (Figure S3B). To examine whether the HAD1 expression is regulated by the calcineurin-Crz1 pathway, we published our published transcriptome analysis (Chow et al. 2017), and found that HAD1 expression is regulated independently of calcineurin or Crz1 under thermal stress.

Previously our phosphopeptidome screen revealed that Had1 is a potential calcineurin substrate in C. neoformans. The single calcineurin-dependent phosphorylated peptide (RRAS_{376}QSGQAGVTLDAFRR) in Had1 was increased more than twofold in abundance in the calcineurin cnip1Δ mutant compared to WT cells (Park et al. 2016). In addition, the Had1 protein contains two predicted calcineurin substrate docking sites PxlxIT motifs ([P]+PG)[IVLF]+PG] (Park et al. 2016). However, as discussed earlier in this section, whether Had1 is a bona fide calcineurin target remains to be characterized. Previous studies identified 13 phosphorylation sites located in the Had1 C-terminal region, and some of these residues were proposed to be phosphorylated by the PKA signaling cascade in C. neoformans (Selvan et al. 2014; Geddes et al. 2016). These results indicate that phosphorylation of Had1 is important for Had1 activation, but the detailed mechanisms await further characterization to better understand Had1 roles in stress responses and environmental adaptation. Based on the transcriptomic (Ko et al. 2009; Chow et al. 2017) and phosphopeptidome (Selvan et al. 2014; Geddes et al. 2016; Park et al. 2016) studies from our and other laboratories, we propose that the expression of HAD1 mRNA is induced by the HOG signaling pathway in response to environmental stresses (Ko et al. 2009), and, in turn, phosphorylation of the Had1 protein may be regulated by the PKA signaling cascade (Geddes et al. 2016), and possibly by the calcineurin pathway (Park et al. 2016). Activated Had1 is required for appropriate stress responses; however, the detailed mechanisms controlling Had1 function should be further studied to better understand the roles of the (HAD)-like hydrolase superfamily in stress responses, environmental adaptation, and virulence.

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