HOG1 has an essential role in the stress response, virulence and pathogenicity of *Cryptococcus gattii*

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Abstract. *Cryptococcus gattii* (*C. gattii*) is a lethal pathogen that causes the majority of cryptococcosis cases in previously healthy individuals. This pathogen poses an increasing threat to global public health, but the mechanisms underlying the pathogenesis have remained to be fully elucidated. In the present study, the role of high-osmolarity glycerol (HOG)1 in the stress reaction and virulence control of *C. gattii* was characterized by deleting the HOG1 gene using the clinical isolate strain CZ2012, and finally, the virulence and pathogenic traits of the deletion strain were defined. Deletion of the HOG1 gene resulted in notable growth defects under stress conditions (high salt and antifungal drugs), but different traits were observed under oxidative stress conditions (hydrogen peroxide). Similarly, the *C. gattii hog1Δ* strains (deletion of *HOG1*) also displayed decreased capsule production and melanin synthesis. Furthermore, mice infected with the *hog1Δ* strain had longer survival times than those infected with the wild-type strain and the reconstituted strain. The *hog1Δ* strain recovered from infected organs exhibited significant growth defects in terms of decreased colony count and size. The present results suggested that HOG1 has a significant role in the virulence of *C. gattii* and these results may help to elucidate the pathogenesis of *C. gattii*.

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Introduction

As one of most life-threatening fungal diseases, cryptococcosis affects mostly immunocompromised individuals but also several immunocompetent populations. *Cryptococcus* (*C.*) *neoformans* and *C. gattii* are both etiological causes of cryptococcosis. Cases of cryptococcosis caused by *C. neoformans* infection occurred mostly in immunocompromised populations, such as patients with acquired immunodeficiency syndrome or organ transplantation, whereas cryptococcosis caused by *C. gattii* occurs more frequently in immunocompetent individuals (1). *C. neoformans* is the most commonly isolated species from clinical cases, accounting for a large portion of cases of cryptococcosis.

C. neoformans and *C. gattii* exhibit notable differences in phenotype, ecology, epidemiology and resistance to drugs. First, the morphology of yeast cells is different; *C. neoformans* cells are almost globose, while *C. gattii* cells are both globose and oblong (2). Furthermore, soil and pigeon droppings account for the majority of saprophytic sources of *C. neoformans*, while decaying trees are identified as environmental reservoirs of *C. gattii* (3,4). Cases caused by *C. neoformans* are observed worldwide, but cases caused by *C. gattii* are not frequently observed globally. In addition, *C. gattii* exhibits relatively higher resistance to antifungal drugs, while both *C. gattii* and *C. neoformans* have been recognized as having different degrees of resistance to certain antifungal drugs, such as azoles (5,6). The differences in epidemiology and drug resistance exhibited by *C. gattii* may be attributable to different pathogenic mechanisms. Several studies have indicated that *C. gattii* exhibits different virulence management mechanisms from *C. neoformans* (7,8). *C. gattii* employs various virulence factors to survive and disseminate in the host, such as capsule production and melanin synthesis, growth at the host's body temperature and degradation enzymes (9-12). Under hostile conditions, *Cryptococci* may sense and adjust themselves to severe stress stimuli, such as high osmotic pressure, by activating multiple stress conduction pathways. Several studies have identified abundant and distinctive roles of the different signaling pathways by comparing the stress regulation mechanism between *C. gattii* and *C. neoformans* (13-15). Yeast is able to maintain osmotic homeostasis across the cell

membrane by adjusting the internal environment to a steady state. As one of the most important stress regulatory systems, the high-osmolarity glycerol (HOG) signaling pathway has a notable effect on the osmotic stress reaction and is essential for virulence regulation of *C. neoformans* (16); however, in *C. gattii*, this pathway has remained to be investigated.

The HOG pathway is one of the important signaling pathways in *C. neoformans* and is structurally similar to those present in other fungi; this pathway regulates stress, sexual differentiation and virulence (17). External stress stimuli are sensed and transmitted by the HOG pathway, which governs protective reactions against various deleterious stimuli, including osmotic stress, oxidative response, high ion concentration, antifungal drugs, high temperature, ultraviolet irradiation and toxic metabolites (13,16,18,19). HOG1 is one of the most important components of the MAPK cascade. A large number of studies on the HOG pathway have focused on *C. neoformans*. As reported for the North American outbreak comprising numerous cryptococcosis cases, *C. gattii* has emerged as a life-threatening primary pathogen infecting immunocompetent patients (20). The strains were observed to be significantly more virulent *in vivo* than others. Furthermore, central nervous system infection caused by *C. gattii* is usually associated with the development of more cryptococcosis; more severe complications, such as headaches; and a poor survival and recovery rate, and it usually requires more frequent neurosurgical interventions and follow-ups compared with cases caused by *C. neoformans* infection (21). Previous studies suggested that the ecology and pathogenesis of *C. gattii* were changing significantly and merited further research (7). It was hypothesized that HOG1 may also be involved in the pathogenic mechanism of *C. gattii*.

In the present study, the HOG1 gene in *C. gattii* was characterized. To functionally characterize HOG1, a mutant strain was obtained by deleting targeted genes for HOG1 by using the clinical strain CZ2012 as a model, and a series of phenotypic strains were compared with the wild-type (WT) and reconstitution strains. In *C. gattii*, the present results suggested that HOG1 has an essential role in regulating the stress response, antifungal drug susceptibility and virulence factor production, including processes such as capsule production and melanin synthesis. Deletion mutation of the HOG1 gene in *C. gattii* resulted in notable growth weakness, not only under stressful conditions but also under normal conditions, and was associated with attenuated virulence in infected mice. The *C. gattii hog1Δ* mutant exhibited reduced capsule production and only a small amount of melanin synthesis, contrary to results obtained with *C. neoformans*, indicating that HOG1 has developed a distinctive virulence regulatory mechanism in the two *Cryptococcus* species. In summary, the present study demonstrated certain convergent and divergent functions of HOG1 in *C. gattii* compared with those in *C. neoformans*, which provides a more detailed understanding of the pathogenic mechanisms of *C. gattii*.

Materials and methods

Strains and media. Cryptococcus isolates exhibit various mechanisms for enhancing virulence, such as growth at 39˚C, adaption to stress and capsule production and marked amplification of ergosterol (22). The strain used in the present study was the clinical strain CZ2012 [*C. gattii (Cg)*, serotype B, mate-α; purchased from the Cryptococcus Laboratory of China Medical Fungi preservation and Management Center], which was isolated from a patient with cryptococcal meningitis in China. Furthermore, the clinical isolate strain of *C. neoformans (Cn)* from a Chinese patient with cryptococcal meningitis was used (23). Yeast extract peptone dextrose (YPD) agar media (1% yeast extract, 2% peptone, 2% dextrose and 2% agar; Invitrogen; Thermo Fisher Scientific, Inc.) was utilized for culture.

Complementary (c)DNA synthesis and cloning of HOG1. Yeast cells were incubated overnight at 30˚C in fresh YPD medium and stimulated with a high concentration of glycerol, and total RNA was isolated with a yeast RNA extraction kit and TRIzol (Invitrogen Inc.; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The HOG1 gene was amplified by Nested PCR. Regarding primers for the construction of the HOG1 gene knockout fragment, H1 and H2 were outer primers, located respectively at both ends of the target sequence, and H3 and H4 were inner nested primers, which were designed for the encoding region. Restriction enzyme cutting sites (*EcoR*I and *Not*I) were respectively added to the 5' end of H3 and H4. The primers were as follows: Outer primers H1 (primer up), 5'-GTATACCAACCGGTCTCA AC-3' and H2 (primer down), 5'-CAGGCTCCTGAATACAAC AC-3'; inner primers H3 (primer up), 5'-GAATTCATGGCC GATTTTGTCAAGCTC-3' (*EcoR*I) and H4 (primer down), 5'-GCGGCCGCCTAGCTAGCAGGAGCAGCCGA-3' (*Not*I). The underlined sites represent the restriction sites (EcoRI and NotI).

The HOG1 cDNA sequence was 1,098 bp in length. The complete HOG1 cDNA sequence was generated using reverse transcription PCR using first-strand cDNA (24). The full-length HOG1 cDNA was cloned into a plasmid vector by the pCR-Blunt Kit (Invitrogen; Thermo Fisher Scientific, Inc.), creating the recombinant clone vector pCR-Blunt-HOG1, which was transformed into competent DH5a cells (Takara Bio, Inc.) for enveloping, the positive clones were then subjected to screening using PCR analysis and DNA sequencing (25).

Disruption and reconstitution of HOG1. Using restriction enzyme identification, PCR and sequence analysis (25), the recombinant plasmid pGAPza-HOG1 containing the intact HOG1 gene was successfully constructed. The HOG1 gene knockout expression vector pGAPza-dHOG1 was constructed by knocking out 400-bp fragment of pGAPza-HOG1 using a single restriction, Sac I (Invitrogen, Thermo Fisher Scientific, Inc.). The knockout product was transformed into Cryptococcus gattii CZ2012 cells by electroporation (electric shock condition: 1,500 V, 400 Ω, 25 μF, 5 mS; twice, internal: 5 min). Stable transformants were obtained through screening on YPD medium containing zeocin and subsequently confirmed by diagnostic PCR, DNA sequencing and Southern blot analysis (25).

To construct the *hog1Δ*+HOG1 reconstituted strain, pGAPza-HOG1 was linearized and transformed into the mutant (deletion of HOG1) by electroporation (26). Stably transfected colonies were selected on medium containing

ampicillin and zeocin. The reconstitution of the HOG1 gene was confirmed by diagnostic PCR and Southern blotting (25).

Assay for capsule and melanin production. For capsule production, three strains were incubated for 24 h in YPD medium, spotted onto DMEM (agar plates; Gibco; Thermo Fisher Scientific, Inc.) at a concentration of $5x10^6$/ml and cultured for 48 h at 30 or 37˚C. Subsequently, the cell capsule was stained with India ink at room temperature for 10-15 min and images were acquired under the microscope. The relative capsule size was determined by measuring the diameter of the capsule and the cell by using rod tool within the Photoshop software (Adobe Photoshop CS6; Adobe Systems Europe, Ltd.). The relative capsule size was expressed as the mean ± standard deviation. All tests above were repeated three times. Three independent experiments with technical triplicates were performed in parallel.

To assess melanin synthesis, cells were spotted onto caffeic acid agar medium (Oxid Corporation.) separately for 30 and 37˚C and observed at 48 and 72 h. The depth of the color of the spots was observed and images were acquired using a light microscope (Canon, Inc.; ESO 200D; x10 magnification). A darker color represented a higher level of melanin. All tests above were repeated three times. Three independent experiments with technical triplicates were performed in parallel.

Assay for urease activity. Cell suspension (5 μl at the same concentration as above) was spotted onto Christensen urea agar medium (Oxid corporation.), followed by culture for 2 days at 30 or 37˚C. Urease turns the medium red and the color change was monitored daily and images were captured. All tests above were repeated three times. Three independent experiments with technical triplicates were performed in parallel.

Sensitivity test for stress. Each strain was incubated overnight at 30˚C in solid YPD medium and subcultured in fresh YPD medium to an optical density at 600 nm (OD600 nm) of 0.7-0.9. The cells were centrifuged, washed with PBS and serially diluted ($1-10^4$). To test the osmotic stress response, cell suspensions were spotted (5 μl per spot) onto solid YPD medium containing 1 or 1.5 M KCl and 1 or 1.5 M NaCl. To test for oxidation stress, media containing 2.5 or 3.0 mmol/ml hydrogen peroxide were prepared. To test the sensitivity of strains to antifungal drugs, cells were spotted on solid YPD medium containing antifungal drugs at the indicated concentrations [16 μl/ml fluconazole (FLC), 0.2 μl/ml itraconazole (ITCZ) or 1.0 μg/ml amphotericin B (AMB)]. All plates were incubated for 3 days at 30˚C and images were acquired. All tests above were repeated three time. Three independent experiments with technical triplicates were performed in parallel.

In addition, the three strains were analyzed to determine their sensitivities to common antifungal drugs, such as AMB, ITCZ, FLC and 5-flucytosine (5-FC). Tests were conducted according to the National Committee for Clinical Laboratory Standards protocol. M-27A (27) and *Candida parapsilosis* ATCC22019 (Microbiologics, Inc.) was employed as a quality control strain. The minimal inhibitory concentration (MIC_{50}) was determined to compare the antifungal activity among different strains. All tests above were repeated three times. Three independent experiments with technical triplicates were performed in parallel.

Virulence assays. In total, 24 female C57BL/6 mice (Fudan University Animal Laboratories; body weight, 20-24 g; age, 4-6 weeks) were used for the present study. The mice were housed at 18-22˚C under 50-60% humidity in a quiet room with dim light, with free access to food and water provided.

The three *Crytococcus gattii* yeast strains (WT, *hog1Δ* and *hog1Δ*+HOG1) were grown in solid YPD medium at 30˚C for 16 h and subsequently subcultured on fresh YPD medium to an OD600 nm of 0.7-0.9. Cell suspensions were centrifuged and washed three times with sterile PBS and the final concentration was adjusted to $5x10^6$ CFU/ml with sterile PBS. Female C57BL mice in each test group (8 mice per group) were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium at a dose of 50 mg/kg and were then inoculated with $2.5x10^5$ CFU in a suspension of 50 μl via intravenous injection (28). Mice were sacrificed using CO_2 inhalation at a displacement rate equivalent to 20% of the chamber volume per minute when they appeared to be in pain, miserable, moribund and rapidly losing weight (>15%), the mice were observed daily. Survival analysis for the different groups was performed using Kaplan-Meier curves and for comparison between groups, the log-rank test was employed with PRISM software version 7.0 (GraphPad Software, Inc.).

Statistical analysis. Statistical analysis of the relative capsule size among the three groups was performed using one-way ANOVAs and post hoc LSD tests using PRISM software version 7.0 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Knockout of HOG1 gene. The complete HOG1 cDNA was generated by RT-PCR and then cloned into pCR-Blunt vector, transformed. The positive clones were screen using PCR analysis and DNA sequencing. The sequence was consistent with GeneBank (Fig. S1). To identify the recombinant clones of pCR-Blunt-HOG1 and pGAPza-HOG1, enzyme digestion using NotI/EcoRI was performed, and the vectors and fragments were obtained. A successful expressor vector was constructed (Fig. S2). Similarly, the pGAPza-dHOG1 was generated by knocking out the 400-bp fragment of pGAPza-HOG1 (Fig. S3). The pGAPza-HOG1 was transformed into *hog1Δ* mutant strain and the successful reconstitution strains were constructed and confirmed using PCR (Fig. S4)

HOG1 has an essential role in the regulation of the stress response in C. gattii in vitro. First, to evaluate the role of HOG1 in the regulation of the stress response in *C. gattii*, various plate tests were performed. The density represents the tolerance to stress with a higher density indicating a higher tolerance to stress.

The *C. gattii* (Cg)-*hog1Δ* strain exhibited increased susceptibility to various stress factors (Fig. 1). The experimental data indicated that the Cg-*hog1*Δ strain was more sensitive to high

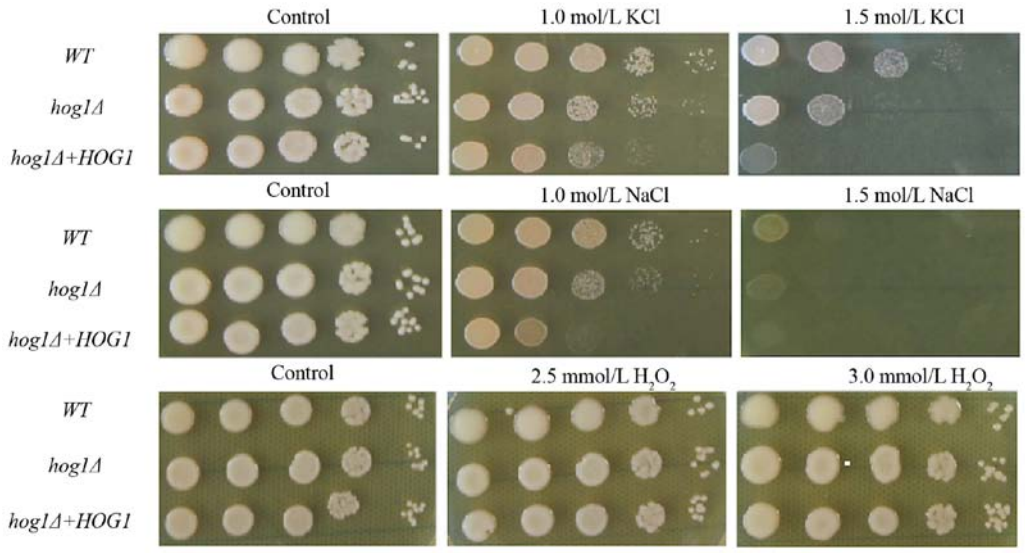

Figure 1. HOG1 is required for growth under certain stress conditions. Each strain (WT strain, *hog1Δ* strain or *hog1Δ*+HOG1 reconstitution strain) was grown to the midlogarithmic phase in YPD medium, 10-fold serially diluted (1-10^4 dilutions) and 2 μl of each diluted cell suspension was spotted on YPD medium containing 1 or 1.5 mol/l NaCl, 1 or 1.5 mol/l KCl for hyperosmotic stress and 2.5 or 3 mM H_2O_2 for oxidative stress. Following incubation for 3 days, images were acquired. All tests are for C.gattii, the top panel is shows the KCl stress, the middle shows the NaCl stress, the bottom shows the H_2O_2 stress. All tests were repeated three times, with representative images being shown. WT, wild-type; HOG, high-osmolarity glycerol; YPD, yeast extract peptone dextrose.

osmotic stress induced by high concentrations of Na^+ and K^+. In terms of oxidative stress, the Cg-*hog1Δ* strain exhibited no enhanced sensitivity, suggesting that HOG1 may not be directly involved in the oxidative stress resistance of *C. gattii*. (29).

In addition, the HOG1 gene was disrupted to identify its effect on the susceptibility of *C. gattii* to several antifungal drugs, including AMB, FLC and ITCZ. Compared to that of the WT strain, the Cg-*hog1Δ* mutant strain displayed a twofold decrease in the MIC of FLC and AMB, a more than fourfold decrease in the MIC of ITCZ and a twofold increase in the MIC of FC-5 (Table I). Of note, reconstitution of the *C. gattii* HOG1 gene did not restore the growth of *C. gattii* at high concentrations of K^+ and in the presence of ITCZ, although tolerance to the other stressors (FLC) was observed *in vitro*. This difference may be attributable to damage caused by repeated biolistic transformations and/or ectopic integration. Also, this could be due to transfection with a relatively large amount of restoration. Taken together, these results demonstrated that HOG1 positively controls the stress response *in vitro*, although with slight differences, in both *C. neoformans* and *C. gattii*.

Table I. HOG1 has a positive effect on antifungal susceptibility of *C. gattii*.

	MIC (μg/ml)			
Strain	AMB	FLC	ITCZ	5-FC
WT	0.50	2.00	0.13	1.00
hog1Δ	0.25	1.00	0.03	2.00
hog1Δ+HOG1	0.25	4.00	0.25	1.00
ATCC22019	2.00	2.00	0.25	0.25

The antifungal susceptibility test was performed. All tests were repeated three times, with representative images being shown. MICs were determined for AMB, FLC, ITCZ and 5-FC in each strain (WT strain, *hog1Δ* strain and the *hog1Δ*+HOG1 reconstitution strain). The hog1Δ strains exhibited an obvious reduction in the MIC. WT, wild-type; HOG, high-osmolarity glycerol; MIC, minimum inhibitory concentration; FLC, fluconazole; ITCZ, itraconazole; AMB, amphotericin B; 5-FC, 5-flucytosine.

Deletion of HOG1 attenuates capsule production and melanin synthesis but enhances urease excretion in C. gattii. The effect of HOG1 on capsule production and melanin synthesis, two important virulence factors that are essential for virulence regulation, were observed in *C. gattii*. The Cg-*hog1Δ*-mutant strain displayed a smaller capsule size in DMEM than the WT strains and the *hog1Δ*+HOG1 reconstituted strains (Fig. 2A and B). A total of 30 cells from different strains were collected and images were captured that were subjected to measurements, and finally, the relative capsule size was calculated. In contrast to the WT strain and the reconstituted strain, the Cg-*hog1Δ* mutant strain exhibited smaller relative capsule sizes at 30 and 37˚C, whereas the WT strain had capsule sizes similar to those of the reconstituted strain (Fig. 2C). The Cg-*hog1Δ*-mutant strain displayed hypomelanization in the colonies compared with the WT strain and reconstituted strain when incubated on caffeic acid medium at 30 and 37˚C for 3 days. Furthermore, all of the strains produced less melanin at 30˚C than at 37˚C, which demonstrated that temperature may negatively regulate melanin production (Fig. 2D). Taken together, these results indicated that HOG1 has an essential role in capsule production and melanin synthesis, two important virulence factors in *C. gattii*.

As urease is an important virulence factor, it is involved in the dissemination of *C. neoformans* in the host, which promotes the accumulation of immature dendritic cells within lung-associated lymph nodes and may enhance nonprotective T2 immune responses during lung infection and promote invasion of the central nervous system (9,30). In the assay, the color

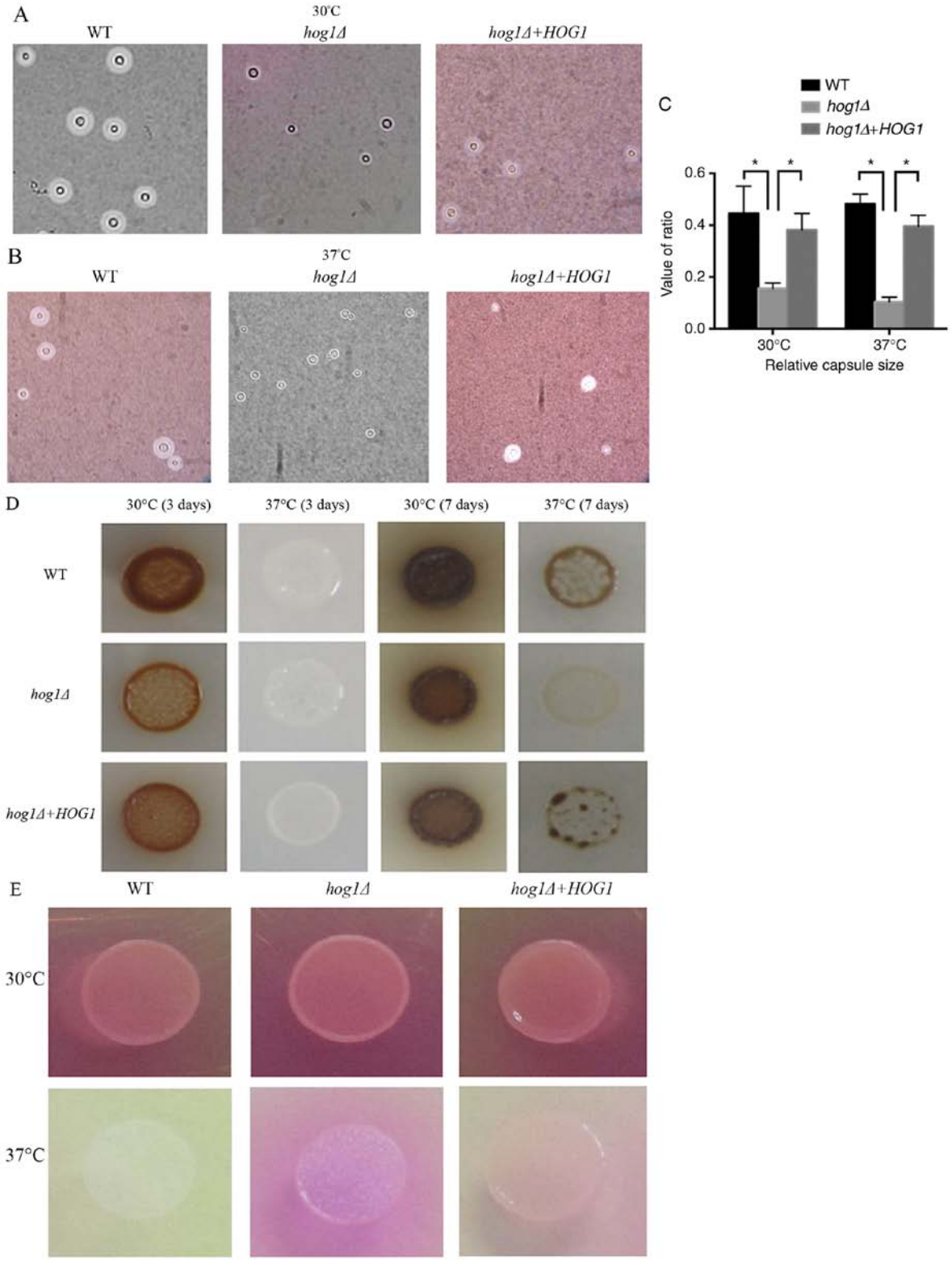

Figure 2. HOG1 mutation reduces capsule production by *C. gattii*. Strains (WT strain, *hog1Δ* strain or *hog1Δ*+HOG1 reconstitution strain) were grown on solid DME medium at (A) 30˚C or (B) 37˚C for 48 h and were stained by India ink and visualized at 40x magnification. (C) The relative capsule size (%) was quantitatively measured by calculating the ratio of the length of the packed cell volume phase per length of total volume phase. All tests were repeated three times. *P<0.01. (D and E) Deletion of HOG1 attenuates melanin synthesis but enhances urease excretion in *C. gattii*. Each strain (WT strain, *hog1Δ* strain or *hog1Δ*+HOG1 reconstitution strain) was grown to the midlogarithmic phase in yeast extract peptone dextrose medium for 24 h, 10-fold serially diluted (1-10^4 dilutions) and 2 μl of each diluted cell suspension was spotted on (D) caffeic acid agar medium for melanin production and (E) Christianson's urea agar medium for urease excretion. Following incubation for 48 h, images were acquired. All tests were repeated three times, with representative images being shown. WT, wild-type; HOG, high-osmolarity glycerol.

represents the activity of urease, with a darker color indicating a higher activity of urease. When the HOG1 mutant strain was incubated on Christianson's urea agar medium, it exhibited an obvious color development, changing the medium from yellow

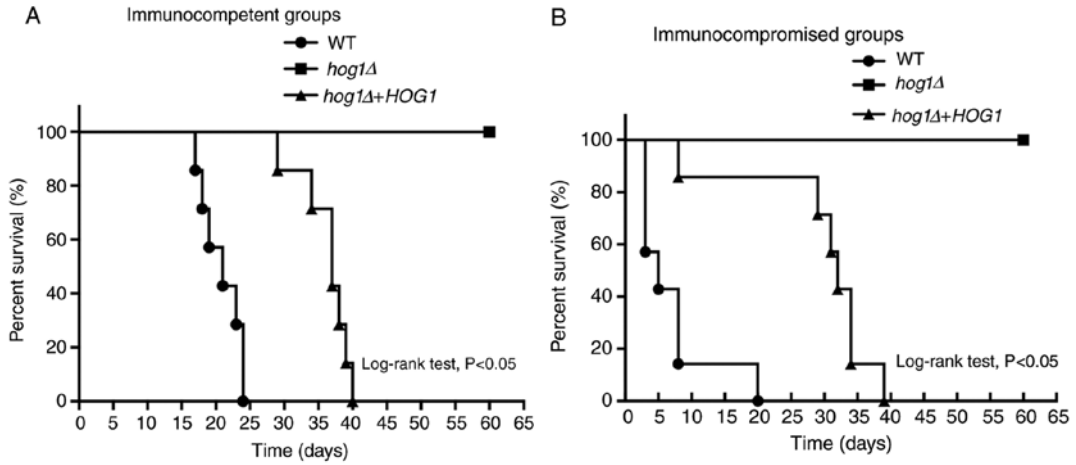

Figure 3. HOG1 is essential for the virulence of *C. gattii* in a murine model. C57bl/6 mice were intravenously inoculated with $2.5x10^5$ cells of each stain (WT strain, *hog1Δ* strain or the *hog1Δ*+HOG1 reconstitution strain). (A) immunocompetent groups and (B) immunocompromised groups. The percentage of survival was monitored for 60 days after inoculation. Mice infected with the *hog1Δ* strain survived the entire period. WT, wild-type; HOG, high-osmolarity glycerol.

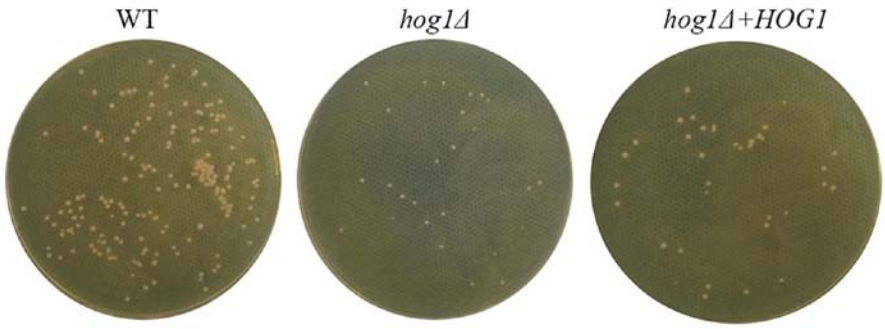

Figure 4. HOG1 has a role in the propagation of *C. gattii*. After inoculation for 3 days, each strain (WT strain, *hog1Δ* strain or the *hog1Δ*+HOG1 reconstitution strain) were isolated from the lungs of infected mice and spread on yeast extract peptone dextrose agar medium. Following incubation for 3 days at 30˚C, images were acquired. The *hog1Δ* strains exhibited an obviously decreased growth. WT, wild-type; HOG, high-osmolarity glycerol.

to a bright color, indicating higher urease activity than that of the WT and the *hog1Δ*+HOG1 reconstituted strains (Fig. 2E). In the present study, HOG1 was observed to negatively regulate the production of urease.

HOG1 is important for virulence in C. gattii. To determine the role that the HOG1 gene has in the virulence of *C. gattii*, a survival analysis was performed through intravenous injection of a murine animal model. The Cg-*hog1Δ* strain and the reconstituted strain Cg-*hog1Δ*+HOG1 were collected and a suspension was prepared for each strain. Finally, groups of immunocompromised and immunocompetent C57BL/6 mice were inoculated intravenously with one of each of the strains (10^5 cells per animal).

In the immunocompetent groups, mice infected by the WT strain had a survival time of up to 24 days and the median survival time was 21 days. The survival pattern of mice inoculated with the reconstituted strain was similar, with the longest survival time among all the mice was 40 days and the median survival time was 37 days, but there was no significant difference. By contrast, mice infected with the *hog1Δ* mutant strain survived until the endpoint at 60 days after infection, suggesting that disruption of the HOG1 gene significantly attenuated the virulence of *C. gattii* ($P<0.05$; Fig. 3A).

In the immunocompromised group, similar results were obtained. Mice infected with the Cg-*hog1Δ* strain survived significantly comparatively longer and had not died at 60 days after infection, whereas the median survival times of the mice infected with the WT strain and WT reconstituted strain were 5 and 32 days, respectively (Fig. 3B). There was a significant difference among the groups.

Taken together, the results stated above demonstrated that HOG1 has a significant role in the virulence regulation of C. *gattii*.

C. gattii requires HOG1 to propagate in the mammal host. Finally, the roles of HOG1 in survival and dissemination in the mammalian host were evaluated. The WT strain, the *hog1Δ* strain and the reconstituted strain *hog1Δ*+HOG1 were isolated from the lung tissues of the infected mice and cultured on YPM medium for 3 days at 30˚C. As presented in Fig. 4, colonies of the *hog1Δ* strain were fewer and their sizes were smaller than those of the WT strain and the reconstituted strain *hog1Δ*+HOG1. These differences in the colonies further verified that the *hog1Δ* strain had poorer growth in the host environment, suggesting that HOG1 is able to prompt the propagation of *C. gattii* and promote the dissemination in the mammalian host.

Discussion

C. gattii is a major pathogenic fungus that is most commonly detected among immunocompetent patients and occurs not only in China but also in other parts of the world (31). However, *C. gattii* is only scarcely distributed in temperate zones of the world. A previous outbreak of *C. gattii* infection in the Pacific Northwest United States and Vancouver Island in Canada indicates that this species is spreading to other geographical areas (32), which has received increased global scientific attention (33). To date, only a small number of experimental studies on the pathogenic mechanisms of *C. gattii* have been performed. In the present study, the effect of HOG1 on virulence regulation of *C. gattii* was functionally determined using the clinical strain CZ2012 and certain distinctive and shared properties with *C. neoformans* were defined.

HOG1 is one of the most important components of the HOG-MAPK signaling pathway and was first characterized in *C. albicans* (34). In *C. neoformans*, HOG1 is also responsible for various cellular processes. Knockout of the HOG1 gene results in numerous phenotypic changes, such as sensitivity to hyperosmotic stimuli and oxidative stress, resistance to azole, UV irradiation, growth at body temperature and increased production of capsules and melanin (29). However, HOG1 exhibits different regulatory mechanisms in different environments and clinical strains (35). A small portion of *C. neoformans* isolates, such as JEC21, the HOG1 gene is mostly dephosphorylated under normal circumstances and is phosphorylated under stress shock, similar to HOG1 homologs in other fungi, such as *Saccharomyces cerevisiae*. Conversely, HOG1 is mostly phosphorylated under normal conditions and is rapidly dephosphorylated under an external stress response, which is common in almost all *C. neoformans* strains, including H99. This type of pattern of HOG1 phosphorylation may contribute to differences in the development of virulence attributes. These differences have all been observed in the *C. neoformans* strains, while the role that the signaling pathway has in *C. gattii* has remained to be elucidated to date.

In the present study, the *hog1Δ* strains had a poor growth performance under stress conditions. Similar to the role exerted by HOG1 in *C. neoformans*, this protein positively regulated the responses of *C. gattii* to various stresses, including high K^+/Na^+. There are numerous possible explanations for the effect of HOG1 in stress control. Intracellular glycerol appeared to be increased after Cryptococcus was exposed to various stresses to adapt to environmental changes, which are controlled by the HOG-MAPK signaling pathway. The HOG-MAPK signaling pathway also has an essential role in stress responses in other fungi, such as *Candida albicans*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (36-38). As a core component of the HOG-MAPK pathway, HOG1 has an essential role in maintaining the cellular balance and responding to various stresses. HOG1 knockout results in reduced accumulation of intracellular glycerol, thereby affecting normal cell growth and eventually causing cell swelling and bursting (39). Therefore, a growth defect of the *hog1Δ* strains was observed in the present experiment.

However, deletion of HOG1 resulted in a difference between *C. gattii* and *C. neoformans* regarding susceptibility to oxidation stress. The *C. gattii hog1Δ* strain and *C. neoformans* had a similar sensitivity to H_2O_2. Multiple signaling pathways and regulatory systems control the response to various stresses. However, HOG1 may act on different substrates to differentially regulate the reaction to oxidation stress.

HOG1 regulates numerous key downstream proteins and governs several virulence traits in *C. neoformans*, such as ergosterol biosynthesis, which is a target antifungal drug to which it binds. After knockout of the HOG1 gene, the expression levels of 545 genes changed significantly, more than two times the normal level, which was confirmed by transcriptomics analysis (40). In the *hog1Δ* mutant, the expression levels of genes involved in the synthesis and content of ergosterol were significantly increased, indicating that HOG1 inhibits the synthesis of ergosterol under normal conditions. There is another finding that supports this possibility. The *hog1Δ* mutant strain had a greater sensitivity to AMB but was resistant to azole antifungal drugs, probably because ergosterol is the binding site of AMB, and the expression level was clearly increased. The effect of the HOG signaling pathway on the synthesis of ergosterol varies significantly among different strains (38). Deletion of HOG1 results MICs of FLC and ITCZ increased (40). However, in the present study, the *hog1Δ* strains exhibited distinct antifungal susceptibility, such as a twofold decrease in the MIC for AMB and FLC, at least a 4-fold decrease in the MICs of ITCZ, but a 2-fold increase in the MIC of 5-FC, which are divergent compared with *C. neoformans*. These results suggested that there may be another pathway that negatively coordinates with HOG-MAPK, affecting ergosterol biosynthesis. Another explanation may be that during evolution, *C. gattii* lost either precise upstream or downstream feedback control of HOG1, resulting in phosphorylation changes in the MAPK pathway and contributing to antifungal drug sensitivity. This phenomenon may provide certain benefits to *C. gattii* regarding host infection, such as high stress resistance.

To further determine the effect of HOG1 on virulence factors, capsule production and melanin synthesis were evaluated, which are essential for protection from oxidants, phagocytosis and dissemination (41). Both capsule production and melanin synthesis are controlled by the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway (13,42-44). HOG-MAPK is involved in crosstalk between these processes and the cAMP/PKA signaling pathway (15). In *C. neoformans*, HOG1 controls capsule production and melanin synthesis differentially in different serotype strains; for instance, HOG1 decreases capsule production and melanin synthesis in the H99 mutant strain but not in other strains. In the present study, HOG1 was evidenced to participate in crosstalk with the cAMP/PKA signaling pathway and knockout of the HOG1 gene resulted in a significant reduction in capsule production and melanin synthesis in *C. gattii*. These results indicated that HOG1 may positively modulate a component of the cAMP/PKA signaling pathway controlling capsule and melanin synthesis in *C. gattii*. Taken together, these findings indicate that in contrast to *C. neoformans*, HOG1 also has an important role in governing virulence factors in *C. gattii*. This result suggests that the phosphorelay system diverges functionally and structurally, which, in turn, differentially regulates the HOG1 cascade in both *Cryptococcus* species. Because of the complexity of

the interactions between cAMP/PKA and the HOG pathway, further study is required to understand the interplay between the two pathways.

Urease activity is essential for the spread of *Cryptococcus* in lung infections, as the enzyme induces a nonprotective T2 immune response by promoting the accumulation of immature dendritic cells (9). Urease may facilitate cell body transmigration to the blood-brain barrier by enhancing sequestration within microvascular beds (45) and promoting central nervous system (CNS) invasion (46). The function of urease activation has been extensively studied in such organisms as plants and bacteria but rarely in fungi, particularly in *C. gattii*. However, prior research demonstrated that after translocation of the pathogen into the CNS, the virulence of urease is attenuated, which results in scarcity or absence of inflammation in brain tissues (30). In the present study, unexpectedly, the *hog1Δ* strain exhibited more viable urease activities than the WT strain and the reconstituted strain at 37˚C, but the difference in urease activities was not as obvious at 37˚C. These results suggest that HOG1 may regulate urease activities in *C. gattii* strains within the host environment.

The *Cryptococcus* species may utilize multiple tools to persist in the host environment and cause damage to the host (47). Numerous virulence factors are considered to contribute to virulence, such as adoption to stress, secreted enzymes and capsule and melanin synthesis (30,48,49). In *C. gattii*, knockout of the HOG1 gene led to a significant reduction in the pathogen's virulence in the mouse model, despite the higher level of urease excretion in the *hog1Δ* strain. The fact that the *hog1Δ* strain isolated from infected mice had a growth defect compared with the WT strain and the reconstituted strain further indicated the role of HOG1 in virulence. All of these results suggested that HOG1 has a significant role in virulence regulation in both *C. neoformans* and *C. gattii*, with several shared and distinctive mechanisms.

In conclusion, the present study demonstrated the role of HOG1 in the regulation of various virulence factors of the *C. gattii* strain CZ2012. HOG1 is essential for propagation in the lung, resistance to stress, capsule production and melanin synthesis, as well as the pathogenicity of *C. gattii* in a mouse model. Furthermore, the commonalities and differences of HOG1 in both *Cryptococcus* species demonstrated that *C. gattii* may have developed certain specific mechanisms to adapt to changes in the environment *in vivo* and *in vitro*. However, the mechanism by which HOG1 affects the immune function of the host during the infection process has remained to be elucidated and warrants further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YMH and YBF confirmed the authenticity of all the raw data. YMH, WQX and YBF designed the present study. YMH and XHT collected clinical samples and performed analysis of data. YMH and YT performed statistical analysis. DFX and YY performed the experiments. YMH wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Zhejiang Provincial People's Hospital (Hangzhou, China; reference no. 2019-180).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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