

# Mon1 Is Essential for Fungal Virulence and Stress Survival in *Cryptococcus neoformans*

Ye-Eun Son<sup>a</sup>, Won-Hee Jung<sup>a</sup>, Sang-Hun Oh<sup>b</sup>, Jin-Hwan Kwak<sup>b</sup>, Maria E. Cardenas<sup>c</sup> and Hee-Soo Park<sup>a</sup>

<sup>a</sup>School of Food Science and Biotechnology, Institute of Agricultural Science and Technology, Kyungpook National University, Daegu, Republic of Korea; <sup>b</sup>School of Life Science, Handong Global University, Pohang, Republic of Korea; <sup>c</sup>Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC, USA

## ABSTRACT

Mon1 is a guanine nucleotide exchange factor subunit that activates the Ypt7 Rab GTPase and is essential for vacuole trafficking and autophagy in eukaryotic organisms. Here, we identified and characterized the function of Mon1, an ortholog of *Saccharomyces cerevisiae* Mon1, in a human fungal pathogen, *Cryptococcus neoformans*. Mutation in *mon1* resulted in hypersensitivity to thermal stress. The *mon1* deletion mutant exhibited increased sensitivity to cell wall and endoplasmic reticulum stress. However, the *mon1* deletion mutant showed more resistance to the antifungal agent fluconazole. *In vivo* studies demonstrated that compared to the wild-type strain, the *mon1* deletion mutant attenuated virulence in the *Galleria mellonella* insect model. Moreover, the *mon1* deletion mutant was avirulent in the murine inhalation model. These results demonstrate that Mon1 plays a crucial role in stress survival and pathogenicity in *C. neoformans*.

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## 1. Introduction

Vacuoles, the fungal equivalent of lysosomes, are key organelles essential for the vesicular transport of molecules between the different membrane organelles in fungi [1–3]. These organelles contain various intrinsic vacuolar hydrolases that degrade cellular components and recycle cytoplasmic organelles [4]. Vacuolar hydrolases are transported from donor organelles (vacuoles) to acceptor organelles via selective or non-selective pathways [5–7]. Intracellular membrane fusion, including vacuole fusion, is a key step in these pathways that involves multiple membrane proteins such as the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complex, homotypic fusion and vacuolar protein sorting (C-Vps/HOPS) complex, and Rab GTPase Ypt7p [8–12].


Mon1 is a subunit of the Mon1-Ccz1 hetero-complex that acts as a guanine nucleotide exchange factor (GEF) in *Saccharomyces cerevisiae* [13–15]. This heterodimeric complex activates Ypt7p, thereby regulating endosomal maturation and vacuole fusion in autophagosomes [16–19]. Recently, a detailed molecular mechanism underlying the Mon1-Ccz1 hetero-complex was discovered via structural and biochemical analyses [20]. In many fungi, Mon1 orthologs play a role in vacuole targeting (Cvt) and autophagy pathways in the cytoplasm [1,20–23].

Moreover, Mon1 also plays an essential role in fungal development and pathogenicity in two plant pathogens, *Fusarium graminearum* and *Magnaporthe oryzae* [21,22,24]. However, the pathogenic role of Mon1 in human pathogenic fungi remains unclear.

*Cryptococcus neoformans* is a basidiomycetous yeast that is used as a model organism for studying fungal pathogenesis and antifungal drug discovery [25,26]. This ubiquitous fungus is widespread in the environment and can be inhaled by humans in the form of spores or desiccated yeast cells [27,28]. The inhaled fungal pathogen colonizes in the lung and results in pulmonary infectious diseases or meningo-encephalitis once it crosses the blood-brain-barrier (BBB) in immunocompromised hosts such as organ transplant and AIDS patients [29–32]. Recently, various comprehensive analyses have been conducted that have provided insights into the virulence mechanisms of *C. neoformans* [33–38].

Several studies demonstrated the importance of the vacuolar trafficking pathway in *C. neoformans* pathogenesis [39]. Deletion of genes encoding proteins associated with vacuole sorting reduced or eliminated virulence [34,40–42]. Although the function of several vacuole-sorting proteins has been studied, the role of Mon1 in the virulence of *C. neoformans* has not yet been elucidated. In this study,

CONTACT Hee-Soo Park  phsoo97@knu.ac.kr

 Supplemental data for this article can be accessed [here](#).

we investigated the roles of the Mon1 ortholog in *C. neoformans* (*Cnmon1*) and found that the deletion of *mon1* (*Cnmon1Δ*) caused growth defects at high temperatures and hypersensitivity to dithiothreitol (DTT; an ER stress inducer) and sodium dodecyl sulfate (SDS; a membrane destabilizing agent). Moreover, loss of *Cnmon1* led to significant defects in fungal pathogenicity *in vivo*, suggesting its role in the response against environmental stresses and virulence in *C. neoformans*.

## 2. Material and methods

### 2.1. Fungal strains and culture conditions

*Cryptococcus neoformans* strains used in this study are listed in Table 1. For general cultures, fungal strains were grown in yeast extract-peptone-dextrose (YPD; Difco, Sparks, MD, USA) broth or agar supplemented with relevant antibiotics and incubated at 30 °C. To test thermotolerance, 5 μL of cells cultured in YPD broth overnight were serially diluted 10-fold and spotted onto YPD agar. The cells were then incubated at different temperatures (30, 37, 38, and 39 °C). To examine stress tolerance, each strain was serially diluted 10-fold and spotted onto YPD agar supplemented with the following compounds: dithiothreitol (DTT; Sigma), sodium dodecyl sulfate (SDS; Fisher, Fair Lawn, NJ, USA), and fluconazole (Sigma). The strains were incubated at 30 °C and the plates were photographed from 48 or 72 hours post-treatment.

### 2.2. Generation of mon1 mutant strains

The oligonucleotides used in this study are listed in Table 2. To generate *mon1* deletion mutants (*Cnmon1Δ*), *Cnmon1* deletion cassettes were

**Table 1.** *Cryptococcus neoformans* strains used in this study.

Strains	Relevant genotype	References
H99	MATalpha, Wild type	[60]
KK1	MATalpha <i>Cncna1Δ</i> ::NAT	[61]
HP242	MATalpha <i>Cncna1Δ</i> ::NEO	[47]
HP55	MATalpha <i>Cnmon1Δ</i> ::NEO	This study
HP56	MATalpha <i>Cnmon1Δ</i> ::NEO	This study
HPC3	MATalpha <i>Cnmon1Δ</i> ::NEO <i>CnMON1_4xFLAG</i> ::HYG	This study
HPC4	MATalpha <i>Cnmon1Δ</i> ::NEO <i>CnMON1_4xFLAG</i> ::HYG	This study

constructed using double-joint PCR as described previously [43]. The 5'- and 3'-flanking regions of *CnMON1* were amplified using primer pairs JOHE41125–JOHE41127 and JOHE41126–JOHE41128, respectively, with the *C. neoformans* serotype A H99 [44,45] genomic DNA as the template (Table 2). The selectable marker *NEO* [46] was amplified with the primer pair JOHE40706–JOHE40707 using pJAF1. The final deletion cassettes were generated using the primer pair JOHE41129–JOHE41130 and the 5'- and 3'-flanking regions and markers as templates. The *mon1* deletion cassettes were purified using the QIAquick Gel Extraction kit (Qiagen) and combined with gold microcarrier beads (Bio-Rad). The gold bead/DNA particles were then introduced into H99 employing the biolistic transformation method. Multiple stable transformants were isolated in independent experiments, selected on YPD agar containing G418, and then confirmed for the 5'- and 3'-junctions via diagnostic PCR followed by restriction enzyme digestion.

To generate the complementary *Cnmon1* strain, the *CnMON1* region, including its predicted promoter, was amplified using the primers JOHE41306 and JOHE41307. Next, the PCR product was digested with NotI and cloned into pHP1 [47]. The resulting plasmid pHSP2 was then introduced into the recipient *Cnmon1Δ* strain (HP55). Multiple transformants were selected on YPD agar containing hygromycin (Sigma) and confirmed using PCR analysis.

### 2.3. The insect-based virulence assay

For the insect-based virulence assay, *Galleria mellonella* was purchased from Vanderhorst Inc. (St. Marys, OH, USA) and infected as previously described [48]. Briefly, the wild-type (WT) and mutant fungal strains were grown overnight in YPD broth at 30 °C, washed thrice, and resuspended in PBS. Cell density was quantified using a hemocytometer and cell suspensions were prepared at a density of  $2 \times 10^6$  cells/mL. Groups containing 12 *G. mellonella* larvae were injected with 4 μL of the cell suspension ( $8 \times 10^4$  cells/larvae) from the second to the last

**Table 2.** Oligonucleotides used in this study.

Name	Sequence (5'-3') <sup>a,b,c</sup>	Purpose
JOHE40706	GTA AACGACGGCCAG	<i>NEO</i> markers (M13F)
JOHE40707	CAGGAAACAGCTATGAC	<i>NEO</i> markers (M13R)
JOHE41125	CGTTCGGATGAGCGTAACAATC	<i>CnMON1</i> disruption (5F)
JOHE41126	GGGCGTGAGGTTCTGATATCTC	<i>CnMON1</i> disruption (3R)
JOHE41127	TCACTGGCCGTCGTTTAC TTTGAACGTCGTA AATTGTGAATTG	<i>CnMON1</i> disruption with marker (5R)
JOHE41128	CATGGTCATAGCTGTTTCTG TCTCACAACGGTGACAAAATGCAG	<i>CnMON1</i> disruption with marker (3F)
JOHE41129	TGAGTAAGAGGGCAGGACGTG	<i>CnMON1</i> disruption (NF_Nested)
JOHE41130	TCAGCAGCCTTCTCTCTGCCG	<i>CnMON1</i> disruption (NR_Nested)
JOHE41306	aatt GCGGCCGC CATGATAGGCTCTGGGTGAGTGG	5' <i>CnMON1</i> with promoter and NotI
JOHE41307	aatt GCGGCCGC GAATACAGGGGCATCTTTGAGA	3' <i>CnMON1</i> with NotI

<sup>a</sup>Underlined portion is homologous to the vector or cassette sequence.

<sup>b</sup>Lowercase portion indicates the linker sequence.

<sup>c</sup>Bold portion denotes the restriction site sequence.

prolegs of each larva using a syringe (PB600-1; Hamilton). For non-infection controls, a larval group was injected with PBS only. The larvae were maintained at 37 °C and monitored daily for 15 days. Larvae that pupated during the experiments were censored. Survival curves were analyzed using the Prism 5 software (GraphPad, San Diego, CA, USA) and statistically analyzed using the log-rank (Mantel-Cox) test.

#### 2.4. Mouse virulence assay

For the survival test, groups containing 10 female A/J mice (16–20 g; Jackson Labs or NCI/Charles River Laboratories) per strain were used. The fungal strains were grown overnight in YPD broth at 30 °C. Cultured cells were collected, washed twice with PBS, and the final concentration was adjusted to  $1 \times 10^7$  CFU/mL. Mice were anesthetized with pentobarbital (Lundbeck Inc. Deerfield, IL, USA) and inoculated at a density of  $5 \times 10^5$  CFU in 50  $\mu$ L via intranasal inhalation as previously described [49]. Survival was monitored daily, and survival curves were generated based on the Kaplan-Meier method using the Prism 5 software (GraphPad). Statistical significance ( $p$  values) was assessed using the log-rank test.

#### 2.5. Ethics statement

Animal care and all the experiments were conducted in accordance with the ethical guidelines of the Institutional Animal Care and Use Committee (IACUC) of Duke University Medical Center (DUMC). Studies on mice were conducted at the Division of Laboratory Animal Resources (DLAR) of

DUMC. The animals were handled according to the guidelines defined by the United States Animal Welfare Act and in full compliance with the DUMC IACUC. The DUMC IACUC approved all the vertebrate studies under the protocol number A245-13-09.

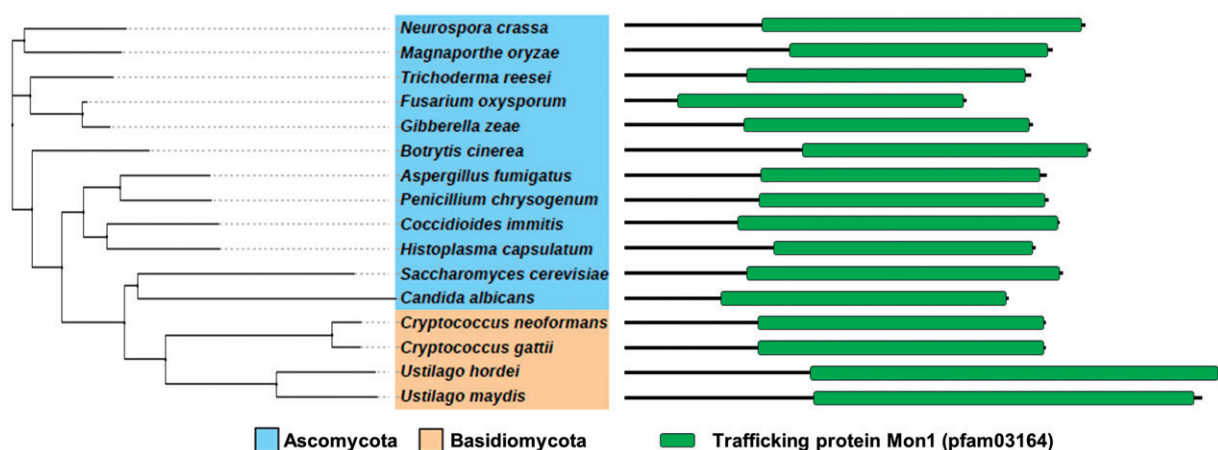
### 3. Results

#### 3.1. Identification of CnMon1 in *C. neoformans*

To identify the gene encoding the *C. neoformans* Mon1 protein (CnMon1), the *S. cerevisiae* Mon1 (ScMon1) protein sequence was blasted against the *C. neoformans* serotype A H99 genome database, where CNAG\_00971 (XP\_012049217) was identified as the most similar protein (31% identity). This gene encodes a 620-amino acid protein featuring a domain related to the CnMon1 trafficking protein on the C-terminus region. Mon1 is highly conserved in all eukaryotic organisms, including fungal species such as ascomycetes and basidiomycetes (Figure 1). To further study the function of CnMon1 in *C. neoformans*, multiple *Cnmon1* $\Delta$  mutants and complementary strains were generated from independent experiments.

#### 3.2. Mutation in *Cnmon1* confers stress sensitivity

Since the *Scmon1* $\Delta$  mutant *S. cerevisiae* strain was hypersensitive to both brefeldin A and monensin (compounds known to affect intracellular transport) [50], we tested the sensitivity of the *Cnmon1* $\Delta$  mutant against brefeldin A and monensin. Unlike the *Scmon1* $\Delta$  mutant, the *Cnmon1* $\Delta$  mutant strain was



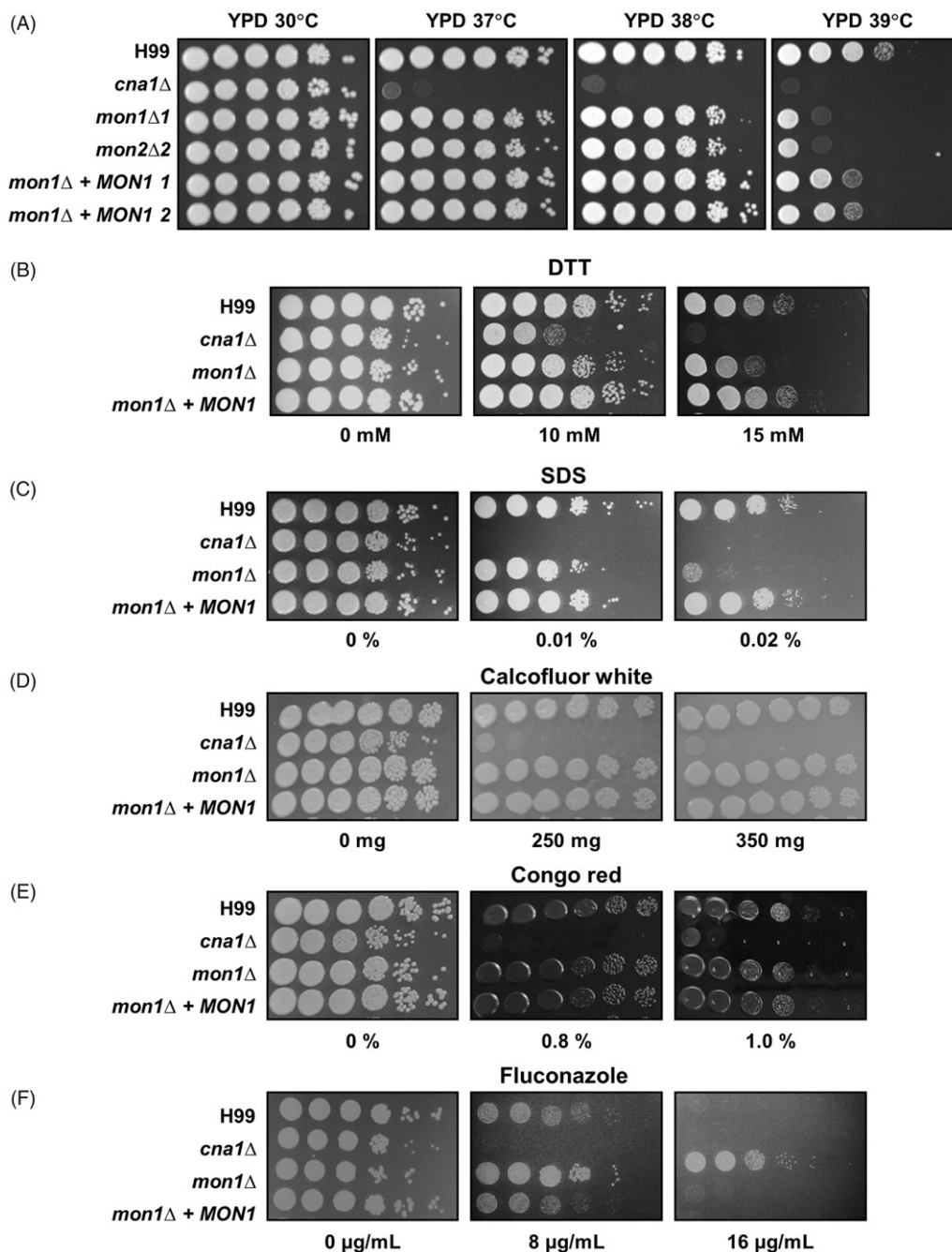
**Figure 1.** Phylogenetic tree depicting Mon1 in various fungal species. Sequences from *Neurospora crassa* OR74A (XP\_959164.3), *Magnaporthe oryzae* 70-15 (XP\_003710676.1), *Trichoderma reesei* QM6a (XP\_006963764.1), *Fusarium oxysporum* Fo47 (EWZ45038.1), *Gibberella zeae* PH-1 (XP\_011326784.1), *Botrytis cinerea* T4 (CCD56988.1), *Aspergillus fumigatus* AF293 (Q4WHL1.2), *Penicillium chrysogenum* (KZN85230.1), *Coccidioides immitis* RS (XP\_001247287.2), *Histoplasma capsulatum* H88 (EGC46448.1), *Candida albicans* SC5314 (KHC78337.1), *Saccharomyces cerevisiae* S288c (NP\_011391), *Cryptococcus neoformans* H99 (XP\_012049217.1), *Cryptococcus gattii* Ru294 (KIR57394.1), *Ustilago hordei* (CCF52623.1), and *Ustilago maydis* 521 (XP\_011389404.1) were compared and a phylogenetic tree of the Mon1 orthologs was generated via the MEGA 5 software (<http://www.megasoftware.net/>) using the alignment data from ClustalW2. The tree results were submitted to iTOL (<http://itol.embl.de/>) to generate the figure (left). Domain architecture of the Mon1 orthologs in fungi (right).

not sensitive to brefeldin A and monensin (Figure S1). Next, phenotypic analysis was conducted under various stress conditions using multiple *Cnmon1Δ* mutants and complementary strains. The *Cnmon1Δ* mutant exhibited growth defects at a high temperature of 39 °C (Figure 2(A)). Deletion of *CnMON1* also caused increased sensitivity towards the ER stress inducer DTT (Figure 2(B)). As shown in Figure 2(C), the *Cnmon1Δ* mutant was more sensitive to SDS than the WT and complemented strains. However, this mutant exhibited growth characteristics similar to those of the WT and could be stained with Congo red

and calcofluor-white (Figure 2(D–E)). Interestingly, the *Cnmon1Δ* mutant also showed an increased resistance to fluconazole (Figure 2(F)). Importantly, normal sensitivity to temperature, SDS, DTT, and fluconazole was restored by reintroducing the WT *CnMON1* gene. Overall, these results indicated that Mon1 is essential for appropriate stress survival.

### 3.3. *CnMon1* is required for fungal pathogenicity

Since Mon1 is crucial for the virulence of plant pathogenic fungi [21,22], we hypothesized that



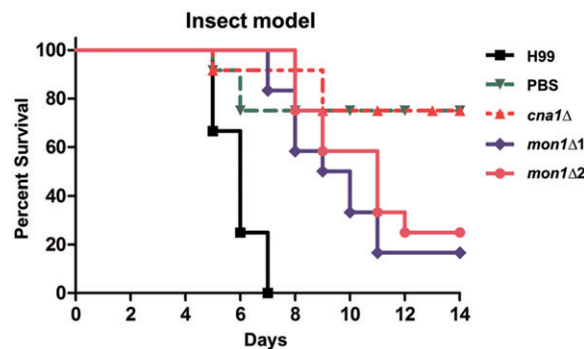
**Figure 2.** Phenotypes of the *Cnmon1Δ* mutant following exposure to various stresses. Spot dilution assays with WT (H99), *Cncna1Δ* (KK1), *Cnmon1Δ* (HP55 and HP56), and *Cnmon1* + *CnMON1* (HPC3 and HPC4). Cells were incubated overnight, diluted 10-fold, and plated on YPD agar. Plated cells were incubated for 2 days at 30, 37, 38, and 39 °C. (A) Serially diluted cells were also plated on YPD agar without or with (B) dithiothreitol (DTT), (C) sodium dodecylsulfate (SDS), (D) calcofluor white, (E) congo red, and (F) fluconazole at the indicated concentrations. Results shown are representative of two independent experiments.

CnMon1 is also involved in *C. neoformans* virulence. To demonstrate the role of CnMon1 in fungal infection, 2 *in vivo* models (insect- and murine inhalation-based) were employed. In the *Galleria mellonella* model, 2 independent *Cnmon1* $\Delta$  mutants were used, which were observed to attenuate virulence when compared to the virulence exhibited by the WT strain (Figure 3). We also found that the *Cnmon1* $\Delta$  mutant was avirulent up to 60 days post-infection in the murine model (Figure 4). Importantly, the virulence of the *Cnmon1* $\Delta$  strain was restored upon reintroducing the CnMON1 WT allele. Overall, these results demonstrated that Mon1 is essential for fungal virulence in both the insect and murine models.

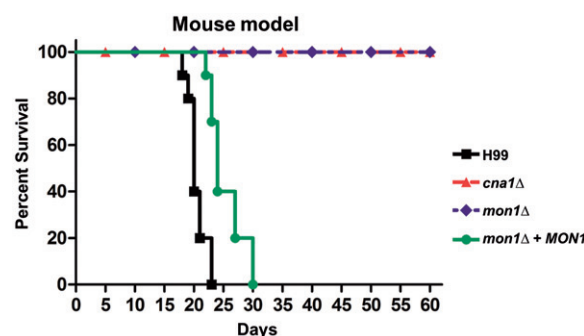
#### 4. Discussion

Mon1 is a highly conserved protein that plays a crucial role in membrane trafficking in all eukaryotic organisms ranging from yeast to mammals. The Mon1/SAND-1 protein activates Rab7 during endosome-controlling membrane fusion in the mammalian system [51,52]. In *Drosophila*, Dmon1 (the *Drosophila* ortholog of Mon1/SAND-1) recruits Rab7 to the maturing endosomes, thereby facilitating phagosome maturation and the trans-synaptic mechanism [53–55]. In plants, Mon1 is conserved in Rab7 activation, vacuolar traffic control, and plant growth [56]. In yeast, ScMon1 is essential for vacuole fusion, autophagy, and the CVT pathway [15]. In filamentous fungi, the Mon1 ortholog proteins are essential for proper vacuole formation, autophagy, and asexual spore formation [21,22]. Importantly, Mon1 is a key factor for fungal virulence, which is demonstrated by the inability of *mon1* deletion mutants in 2 plant pathogenic fungi to infect plants [21,22]. Although the virulent functions of Mon1 have been studied in plant pathogenic fungi, the pathogenic role of the Mon1 ortholog in animal/human pathogenic fungi has not yet been investigated. In this study, we demonstrated that *Cnmon1* deletion results in attenuated virulence in the insect model and avirulence in the murine model (Figures 3 and 4), suggesting that CnMon1 is required for fungal pathogenicity in the opportunistic human pathogenic fungus, *C. neoformans*. These results indicate that the pathogenic role of Mon1 is conserved in plant and human pathogenic fungi.

Previous studies demonstrated that several genes involved in vacuole trafficking played important roles in the regulation of *C. neoformans* growth and virulence at high temperatures [34,39–42]. For example, deletion of *vps15*, which encodes a serine/threonine protein kinase required for the CVT pathway, results in reduced fungal virulence, growth defects at high temperatures, and hypersensitivity to



**Figure 3.** Virulence of the *Cnmon1* $\Delta$  mutant in the insect larvae model. Each *Galleria* larva (12 per group) was infected with approximately 80,000 WT (H99), *Cncna1* $\Delta$  (KK1), or *Cnmon1* $\Delta$  (HP55 and HP56) cells. The infected larvae were maintained at 37 °C and monitored daily for 12 days.



**Figure 4.** Virulence of the *Cnmon1* $\Delta$  mutant in the murine inhalation model. *Cnmon1* $\Delta$  mutant exhibits avirulence when compared to the WT and *Cnmon1* + CnMON1 complement strains. WT (H99), *Cncna1* $\Delta$  (KK1), *Cnmon1* $\Delta$  (HP55), and *Cnmon1* + CnMON1 (HPC3) cells were grown overnight in YPD broth at 30 °C and washed with PBS. Cells ( $5 \times 10^5$ ) were inoculated into A/J mice via intranasal instillation. Animal survival was monitored for 60 days post-infection.

multiple stresses [34]. Similar to the *vps15* or *vps23* null mutants, the *Cnmon1* $\Delta$  mutant was also highly susceptible to multiple stresses and demonstrated growth defects and reduced virulence. These results strongly show vacuolar sorting to play a crucial role in the fungal pathogenicity and stress response of *C. neoformans*.

Owing to its role in vacuole fusion, ScMon1 is required for autophagy, an intracellular process in *S. cerevisiae* that is essential for the degradation of cytoplasmic components [16,57,58]. Autophagy has been reported to be essential in some pathogenic fungi [40]. For example, the *ATG8* knockdown mutant strains of *C. neoformans* showed attenuated virulence in 2 mouse models [40]. Our preliminary data also show that *atg8* or *atg15* deletion results in attenuated virulence (data not shown). Although we did not test the importance of CnMon1 in the autophagy pathway in *C. neoformans*, we speculate that CnMon1 could regulate autophagosome formation, thereby regulating fungal virulence. The relationship between CnMon1 and autophagy in

*C. neoformans* needs to be thoroughly examined. Alternatively, autophagy also affects stress granules and/or P-bodies in yeast [59]. Loss-of-function of components involved in the autophagy process causes an increased accumulation of stress granules and P-bodies in the cytosol [59]. For example, *Cnmon1* deletion increases stress granules and P-bodies. We examined the role of Mon1 in P-body formation and found that it does not affect the formation of P-bodies in *C. neoformans* (data not shown).

In yeast, the *Scmon1Δ* mutant was shown to be hypersensitive to brefeldin A and monensin (inhibitors of intracellular protein transport), suggesting that the *Cnmon1Δ* mutant could also be sensitive to these compounds [50]. Thus, we examined the sensitivity of the *Cnmon1Δ* mutant strain to these 2 compounds and found that it was not sensitive to brefeldin A and monensin (Figure S1). These results propose that Mon1 probably plays a different role in *C. neoformans* and yeast. To further test the role of CnMon1 in endocytosis, we examined the uptake of FM4-64, a fluorescent endocytic marker, in WT and mutants. However, we did not observe any differences in the WT and *Cnmon1Δ* mutant strains. Overall, these results propose that CnMon1 may not be a key factor for intracellular protein transport.

In summary, we identified CnMon1 and demonstrated its crucial role in fungal growth at high temperatures, sensitivity against several stresses, and fungal virulence in *C. neoformans*. The cellular and biochemical roles of CnMon1, which includes vacuolar fusion, autophagy, endocytosis, and GEF, will be the subject of future studies in *C. neoformans*.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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