Two cation transporters Ena1 and Nha1 cooperatively modulate ion homeostasis, antifungal drug resistance, and virulence of *Cryptococcus neoformans* via the HOG pathway

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Abstract

Maintenance of cation homeostasis is essential for survival of all living organisms in their biological niches. It is also important for the survival of human pathogenic fungi in the host, where cation concentrations and pH will vary depending on different anatomical sites. However, the exact role of diverse cation transporters and ion channels in virulence of fungal pathogens remains elusive. In this study we functionally characterized *ENA1* and *NHA1,* encoding a putative Na$^+$/ATPase and Na$^+$/H$^+$ antiporter, respectively, in *Cryptococcus neoformans,* a basidiomycete fungal pathogen which causes fatal meningoencephalitis. Expression of *NHA1* and *ENA1* is induced in response to salt and osmotic shock mainly in a Hog1-dependent manner. Phenotypic analysis of the *ena1, nha1*, and *ena1 nha1* mutants revealed that Ena1 controls cellular levels of toxic cations, such as Na$^+$ and Li$^+$ whereas both Ena1 and Nha1 are important for controlling less toxic K$^+$ ions. Under alkaline conditions, Ena1 was highly induced and required for growth in the presence of low levels of Na$^+$ or K$^+$ salt and Nha1 played a role in survival under K$^+$ stress. In contrast, Nha1, but not Ena1, was essential for survival at acidic conditions (pH 4.5) under high K$^+$ stress. In addition, Ena1 and Nha1 were required for maintenance of plasma membrane potential and stability, which appeared to modulate antifungal drug susceptibility. Perturbation of *ENA1* and *NHA1* enhanced capsule production and melanin synthesis. However, Nha1 was dispensable for virulence of *C. neoformans* although Ena1 was essential. In conclusion, Ena1 and Nha1 play redundant and discrete roles in cation homeostasis, pH regulation, membrane potential, and virulence in *C. neoformans,* suggesting that these transporters could be novel antifungal drug targets for treatment of cryptococcosis.

Keywords

C. neoformans; Ena1; Nha1; cation transporters; antifungal drug

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1. Introduction

Cation influx and efflux systems play critical roles in maintenance of plasma membrane potential, cellular ion homeostasis, and intracellular pH and nutrient uptake in all living organisms (Serrano et al., 1986). Potassium ion (K^+) exists at higher concentration inside the cell (mM ranges) than in most natural environments (μM ranges) using diverse types of transporters to electrically neutralize negative charges generated from various cellular proteins and inorganic/organic acids and to regulate many physiological functions, such as enzyme activation and regulation of cell volume and intracellular pH (Rodriguez-Navarro, 2000). When intracellular K^+ is limited, proton (H^+) can be a substitute for electrical neutrality, which may increase intracellular pH and affect normal cell physiology. Alternatively, the sodium ion (Na^+) could replace K^+ under K^+-deficiency and yet is generally more toxic for cells than K^+(Arino, 2010; Sychrova, 2004).

To maintain optimal cation homeostasis, cells employ diverse high affinity cation transporters and ion channels (Sychrova, 2004). *Saccharomyces cerevisiae* has been used as a model organism to understand the regulatory mechanism of cation homeostasis in fungi. For K^+-transport, two active transporters, Trk1p and Trk2p, and two channel proteins, Tok1p and Nsc1p, are involved in uptake of K^+ into the cells (Bihler et al., 1998; Fairman et al., 1999; Gaber et al., 1988; Ko et al., 1990). For Na^+-transport, two active sodium efflux pumps, Ena1 and Nha1, exist. Ena1, a putative Na^+/ATPase plasma membrane transporter, mediates an efflux of toxic cations, such as Na^+ and Li^+, by hydrolyzing ATP (Benito et al., 2002). Ena1 is also involved in K^+ transport. Nha1, a Na^+/H^+ antiporter, not only controls toxic Na^+ and Li^+ levels, but also modulates K^+ and Rb^+ levels (Banuelos et al., 1998; Kinclova et al., 2001; Prior et al., 1996). The proton motive force required for activating Nha1 is generated through the action of the Pma1 H^+-ATPase. Both ion transporters appear to play complementary roles in regulating cation homeostasis because the *ena1 nha1* mutant exhibits more severe cation susceptibility than each single mutant (Banuelos et al., 1998).

Regardless of the redundant roles played by the two Na^+/K^+ efflux pumps, the regulatory mechanisms of Ena1 and Nha1 are different. Nha1 is required for short-term adaptation to high salt shock and Ena1 for long-term adaptation (Proft and Struhl, 2004). Nha1 is constitutively expressed at the plasma membrane and relieves initial osmotic shock by extruding Na^+ upon direct activation by the Hog1 mitogen activated protein kinase (MAPK), which results in re-assembling of transcription factors and transcription complex. Next, *ENA1* is transcriptionally induced, which allows cells to achieve long-term adaptation to external osmotic shock. Expression of *ENA1* is induced by high pH as well as Na^+ and Li^+ whereas expression of *NHA1* is not modulated by low pH, salts or osmotic shock (Banuelos et al., 1998; Platara et al., 2006).

The ability to sense and adapt to changes in alkali metal ion concentration with in the host is important for survival of human pathogenic fungi, as cation concentration and pH levels vary depending on anatomical site. Particularly, Na^+/K^+-transport is closely related to maintenance of intracellular pH and establishment of the membrane potential, which appear to be key cellular factors for growth and survival of fungal pathogens. However, knowledge about the role of Na^+/K^+-transporters in virulence of human fungal pathogens is limited. In *Cryptococcus neoformans,* which causes fungal pneumonia and fatal meningoencephalitis in both immunocompromised and immunocompetent individuals (Hoang et al., 2004; Mitchell and Perfect, 1995), a cation transporter was found to be critical in virulence of the pathogen. The *C. neoformans* Ena1 ortholog has been identified as a key virulence determinant through signature-tagged insertional mutagenesis (Idnurm et al., 2009). Deletion of the *ENA1* gene abolishes virulence of *C. neoformans,* indicating the importance of cation homeostasis for fungal pathogens. The *ena1* mutant was also found to be less fit in strain

competition experiments in the lungs of mice and unable to grow in human cerebral spinal fluid (Lee et al., 2010; Liu et al., 2008). In contrast to the *S. cerevisiae ena1* mutant, Ena1 is required for sodium and potassium salt stress response under glucose-starvation conditions, but not under glucose-rich conditions (Idnurm et al., 2009; Ko et al., 2009). Furthermore, Ena1 was found to be essential in survival under high pH condition (Idnurm et al., 2009). However, the regulatory mechanism of the cation transporter remains elusive. Furthermore, other active cation transporters, such as Nha1, have not been functionally characterized in *C. neoformans.*

Our recent transcriptome analysis of the environmental stress response in *C. neoformans* revealed that the HOG pathway controls basal and osmotic stress-induced expression levels of the *ENA1* and *NHA1* genes. To further address the roles of the two cation transpoters in growth, differentiation, stress response, and virulence of *C. neoformans,* here we employed reverse genetics approaches to functionally characterize the role of Nha1 in comparison with Ena1 in the pathogen. In this study we found that Ena1 and Nha1 played discrete and overlapping roles in controlling intracellular concentrations of cations, such as Na^+ and K^+, potentially affecting membrane polarization. Intriguingly, inhibition of both cation transporters dramatically enhanced susceptibility to antifungal drugs, such as amphotericin B. Therefore the present study not only helps us to understand roles of cation transporters in *C. neoformans,* but also provides a unique opportunity to develop novel antifungal drug targets for treatment of fatal fungal meningitis.

2. Materials and Methods

2.1. Strains and media

Strains and primers used in this study are listed in Table 1 and S1 in the supplementary material. Yeast extract-peptone-dextrose (YPD) medium was used for culturing *C. neoformans* strains unless indicated otherwise. The Niger seed medium for melanin production, agar-based Dulbecco modified Eagle (DME) medium for capsule production, and V8 medium (adjusted to pH 5) for mating response were prepared as previously described (Alspaugh et al., 1997; Bahn et al., 2004; Granger et al., 1985; Hicks et al., 2004).

2.2. Disruption of the NHA1 and ENA1 genes

For disruption of the *NHA1* gene (CNAG_01678.2), information regarding the *NHA1* genomic structure was obtained from the *C. neoformans* genomic database (Broad Institute). The *NHA1* gene-disruption cassette was generated by using overlap PCR with primers listed in Table S1 as previously described (Davidson et al., 2002). Briefly, primers B1673 and B1674 for the 5′-flanking region of the *NHA1* gene, primers B1675 and B1676 for the 3′-flanking region of the *NHA1* gene, and primers M13Fe (M13 forward extended) and M13Re (M13 reverse extended) for the dominant selectable *NEO* marker (neomycin/G418-resistant marker) were used in the first round of PCR. Next, the *NEO*-marked *NHA1*-deletion cassette was amplified by overlap PCR with primers B1673 and B1676. The *nha1* mutant and the *ena1 nha1* double mutant were generated by introducing the *NHA1*-deletion cassette into the background of the *C. neoformans* serotype A strains H99, KN99, and the *ena1* mutant [AI167; KN99 background; (Idnurm et al., 2009)] by biolistic transformation as previously described (Davidson et al., 2002). Stable transformants selected on YPD medium containing G418 were initially screened by diagnostic PCR for the 5′-junction with primers B1677 and B79, and their correct genotypes were subsequently verified by Southern blot analysis as described before (Jung et al., 2011).

To verify any phenotypes observed in the *nha1* mutant, we constructed the *nha1/NHA1* complemented strains as follows. First, a genomic fragment of the *NHA1* gene containing

promoter, the whole open reading frame (ORF), and terminator, was amplified via PCR by using primers B2081 and B2082, that include Xhol restriction sites, with the wild-type (WT) genomic DNA as template. The 4.5 kb gene product was cloned into a plasmid pTOP-V2 (Enzynomics) to produce a plasmid pTOP-NHA1. After sequencing to identify a clone with no errors, the *NHA1* gene insert was subcloned with Xhol into a plasmid pJAF13 containing nourseothricin-resistance marker, *NHT* (nourseothricin acetyltransferase), to produce a plasmid pJAF13-NHA1. The Pacl-digested linearized pJAF13-NHA1 was biolistically transformed into the *nha1* mutants (YSB586 and YSB1111). Targeted re-integration of the *NHA1* gene into its native locus was confirmed by diagnostic PCR using primers B1677 and B1698.

To perform comparative phenotypic analysis of the *nha1* mutant with the *ena1* mutant, we independently generated the *ena1* mutant in the H99 strain background. For disruption of *ENA1,* information regarding the *ENA1* genomic structure was obtained from *C. neoformans* genomic database (Broad Institute). We constructed the *ENA1* gene-disruption cassette by using double joint PCR (DJ- PCR) with primers described in Table S1. The *ena1* mutants were generated by introduction of the *ENA1* deletion cassette marked with *NAT or NEO* into the strains H99, KN99 (*MAT***a**), and *nha1* mutant strains (*MAT***a,** YSB1171). Stable transformants selected on YPD medium containing nourseothricin or G418 were screened by diagnostic PCR and their correct genotypes were confirmed by Southern blot analysis.

2.3. Melanin and capsule assay

All *C. neoformans* strains for melanin and capsule assay were cultured on YPD liquid medium at 30°C for 16 hours. Three microliters of cells were spotted on agar-based DME medium for capsule assay and on agar-based niger seed medium, which contains indicated concentration of glucose (0.1%, 0.5%, or 1%). The plates were incubated at 30°C or 37°C, monitored daily, and photographed by using SPOT insight digital camera (Diagnostic Instrument Inc.) for up to 4 days. For quantitation of *LAC1* transcript levels, WT and *ena1 nha1* (YSB1169) double mutant were cultured in YPD liquid medium at 30°C for 16 hours. The overnight culture was inoculated 300 ml of fresh YPD liquid medium adjusted optical density at 600 nm (OD_{600}) of the culture medium to approximately 0.4. Then culture medium was incubated until 1.0 of OD_{600} at 30°C. The 50 ml out of 300 ml culture was sampled and the remaining 250 ml culture was spun down. The supernatant was removed and added YNB liquid medium without glucose. During incubation, a 50 ml culture was sampled at 10 min, 30 min, 60 min, 90 min, and 120 min. Total RNAs from each sample for Northern blot analysis were prepared as previously described (Ko et al., 2009).

For measurement and visualization of capsule production, cells scraped from the DME medium were resuspended in phosphate buffer saline (PBS) buffer and stained with India ink. For quantitative measurement of capsule production, relative packed cell volume by using hematocrit capillary tubes was measured as previously described (Alspaugh et al., 2002; Jung et al., 2011).

2.4. Mating assay

Mating assay was performed as previously described (Bahn et al., 2004; Hicks et al., 2004). Each strain of opposite mating type (*MAT* and *MAT***a**) for mating assay were grown in YPD liquid medium at 30°C for 16 hours. Both mating type cells (10^7 cells/ml) were mixed with the same concentration and 5 μl of the mixture was spotted onto V8 medium. Then the plates were incubated in the dark at room temperature for 3 weeks. All colonial and cellular images were monitored and photograped by using microscope equiped with Olympus BX51 microscope (Diagnostic Instrument Inc.).

2.5. Stress and antifungal sensitivity test

Each strain was cultured in YPD liquid medium at 30°C for 16 hours, washed, serially diluted (1 to 10^4 dilutions), and spotted in 3 μl of volume onto solid YP or YPD medium containing indicated stress-inducing agents. For cation stress test, YP and YPD media containing indicated concentration of NaCl and KCl (0.5 to 1.5 M), LiCl (0.05 to 0.07 M), or CaCl$_2$ (0.5 to 0.7 M) were used. For pH sensitivity test, cells were spotted onto YPD medium adjusted to pH 4.5 with succinic acid buffer or adjusted to pH 8.5 with Tris-HCl buffer. For measuring antifungal drug susceptibility, YPD media containing fluconazole (FCZ, Sigma), ketoconazole (KCZ, Sigma), and amphotericin B (AMB, Sigma) were used. For measuring thermotolerance, cells were spotted onto solid YPD medium and then incubated at 37°C or 39°C.

2.6. Northern blot analysis

For expression analysis of *ENA1* and *NHA1* under osmotic or salt stress, cells were cultured in 50ml YPD liquid medium for 16 hr at 30°C. Then 15 ml of overnight culture was inoculated into 135 ml of fresh YPD medium and further incubated at 30°C until optical density at 600 nm (OD$_{600}$) of the culture medium reached approximately 1.0. For the zero time sample, 50 ml out of the 150 ml culture was sampled and the remaining 100 ml culture was added with 100 ml of YPD containing 2 M NaCl, 2 M KCl, or 2 M sorbitol. During incubation, a 50 ml culture was sampled at 10 min, 30 min, and 60 min. Total RNA from each sample for Northern blot analysis was prepared as previously described (Ko et al., 2009). For expression analysis of *ENA1* and *NHA1* in response to pH shock, cells were cultured in 50 ml of YPD liquid medium for 16 hr at 30°C. Then the overnight culture was inoculated into fresh YPD medium, of which volume was adjusted to 200 ml and incubated at 30°C until OD$_{600}$ of the culture medium reached approximately 1.0. For the zero time sample, 50 ml out of the 200 ml culture was sampled. The remaining 150 ml culture was pelleted by centrifugation, added with 150 ml of fresh YPD medium adjusted to pH 8.5 with Tris-HCl buffer or pH 4.5 with 50mM succinic acid-NaOH buffer, and incubated at 30°C. During incubation, a 50 ml culture was sampled at 30 min and 60 min and total RNA was isolated.

2.7. Nha1 localization study

For the Nha1 localization study, the *nha1/NHA1-GFP* strain was constructed as follows. The terminator region of *NHA1* (NHA1t) was amplified via PCR by using B3361 and B3362 containing Xbal restriction site with pTOP-NHA1 as template. The B3358, containing glycine-serine linker (5′-GGCGGTGGCTCT-3′) and B3360 primers were used to amplify the *GFP* gene with pACT-HOG1fGFP as a template. These PCR products were combined and overlap PCR was performed by using B3359 and B3362 containing Sacll and Xbal restriction sites and Sacll restriction site, respectively. The GFP-NHA1t product was cloned into a plasmid pTOP-V2 (Enzynomics) to produce a plasmid pTOP-GFPNHA1T. After sequencing, the Sacll-digested GFP-NHA1t was subcloned into a plasmid pJAF13 containing nourseothricin resistance marker to construct pJAF13-GFPNHA1T. The promoter and ORF region of *NHA1* (NHA1PE) were amplified via PCR by using B3356 and B3357 including Apal or Xbal restriction sites, respectively, with pTOP-NHA1 as a template and cloned into a plasmid pTOP-V2 (Enzynomics) to produce a pTOP-NHA1PE. After sequencing, the Apal-and Xbal digested insert was subcloned into the Apal-and Xbaldigested pJAF13-GFPNHA1T to produce a plasmid pJAF13-NHA1GFP. The Pacl-digested linearized pJAF13-NHA1GFP was transformed into the *nha1 Δ* mutant (YSB586) and targeted re-integration of the *NHA1-GFP* gene into its native locus was confirmed by diagnostic PCR using primers B1677 and B1698. Functionality of the *NHA1-GFP* fusion gene was confirmed by comparative phenotypic analysis of the *nha1/NHA-GFP* and *nha1/NHA1* strains.

For visualization of Nha1, we cultured the *nha1Δ/NHA1-GFP* strain in SC liquid media for 16 hr at 30°C. Then 5ml of overnight culture was inoculated into 45ml of fresh SC liquid media and incubated at 30°C until OD_{600} of the culture medium was approximately 1.0. For the zero time sample, 1 ml out of the 50 ml culture was sampled and the remaining culture was pelleted and added with fresh 50 ml of SC liquid media containing 1 M NaCl or 1 M KCl. During incubation, 1 ml of culture was sampled at 30 min and 60 min. Cells from each sample for GFP visualization were fixed by following the method recommended by Koshland (http://mcb.berkeley.edu/labs/koshland/Protocols/MICROSCOPY/gfpfix.html). Briefly, cells were spun down and supernatant removed. One hundred micro liters of 4% paraformaldehyde containing 3.4% sucrose were added to the sample and incubated at room temperature for 15 min. After incubation, cells were pelleted and washed in 500 μl 0.1 M potassium phosphate buffer adjusted to pH 7.5 containing 1.2 M sorbitol. Next, cells were resuspended in potassium phosphate buffer and stored in refrigerator. Fixed cells were visualized by confocal microscope (Carl Zeiss).

2.8. Virulence study

Each strain [WT (H99), *ena1* (YSB1161), *ena1 nha1* (YSB1169), *nha1* (YSB586), *nha1/NHA1*(YSB1201) strains] was inoculated into 2 ml of YPD and grown overnight at 30°C. After incubation, cultures were pelleted by centrifugation and washed three times in 1ml sterile PBS. Cell suspensions were counted by hemocytometer and concentrations adjusted to 1×10^6 CFU/ml. Seven week old A/J female mice (Jackson Labs, Bar Harbor, MA) were anesthetized with an intraperitoneal dose of pentobarbital and 50 μ l of the prepared inocula (5×10^4 CFUs) were administered to the nares of each animal. Ten animals per group were infected with each strain. The concentration of each initial inoculum was verified by viable cell count with serial dilution on YPD agar plates. Mice were checked visually for the first 13 days of infection, and were weighed and checked for signs of infection beginning on day 14 post infection. Signs of morbidity included weight loss (1g/day for 2 days, or 2g loss in two days), abnormal gait, and extension of the cerebral portion of the cranium. Animals exhibiting these signs were sacrificed by administration of CO_2. Animals who did not exhibit signs of infection by 43 days post infection were euthanized and target tissues (lungs, spleen, and brain) were harvested to determine whether or not the infection had been cleared (data not shown). All animal experiments were done at the University of Minnesota in strict accordance with good animal practice as defined by the National Institutes of Health Office of Animal Welfare (OLAW), and the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experiments were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC) under protocol number 1010A91133.

3. Results

3.1. Identification of the NHA1 gene in C. neoformans

Our prior transcriptome analysis of the stress-activated HOG signaling pathway in *C. neoformans* revealed that expression of two cation transporter genes, *ENA1* (CNAG_00531.2) and *NHA1* (CNAG_01678.2), appear to be regulated by the Hog1 MAPK under high salt stress (Ko et al., 2009). In *S. cerevisiae, NHA1* is constitutively expressed but rapidly activated by Hog1-mediated phosphorylation in response to salt (Na^+) shock for very early adaptation, whereas *ENA1* is transcriptionally activated in a Hog1-dependent manner (Proft and Struhl, 2004). Therefore, the potentially discrete regulatory mechanism and a lack of any study for *NHA1* in *C. neoformans* prompted us to investigate the role of the putative Na^+/H^+ antiporter with connection to the Ena1 Na^+/ATPase efflux pump.

C. neoformans NHA1 is predicted to encode a 916 amino acid protein (CnNha1). Based on the hydrophilicity plot, CnNha1 exhibits a typical transporter domain structure, which is widely conserved in Nha1 orthologs in other fungi, such as *C. albicans* (CaCnh1) and *S. cerevisiae* (ScNha1; Fig. 1A). CnNha1 and other Nha1 orthologs contain the hydrophobic membrane spanning regions (11 or 12 membrane spanning regions) in the amino terminus and the hydrophilic cytoplasmic tail in the carboxy terminus (Fig. 1A). Furthermore, CnNha1 showed 42% identity in overall protein sequence to ScNha1 and CaCnh1. Based on the phylogenetic analysis, CnNha1 are grouped with Na^+/H^+ antiporter orthologs in other basidiomycete fungi (Fig. 1B). Taken together, CnNha1 is likely to be an ortholog for the Nha1 Na^+/H^+ antiporter in *C. neoformans.*

3.2. Differential expression of ENA1 and NHA1 in response to osmotic and salt stresses

We monitored the expression pattern of *NHA1* and *ENA1* by Northern blot analysis under high salt shock. In agreement with the previous microarray data, both *ENA1* and *NHA1* were induced in response to 1 M NaCl. *ENA1* was more rapidly (with in 10 min) and strongly induced than *NHA1* (Fig. 2A). In response to 1 M KCl, *ENA1* expression was also induced with in 10 min and peaked at 30 min whereas *NHA1* was weakly induced (Fig. 2B). Extended running of Northern blot gel revealed two alternatively spliced *ENA1* transcriptsin WT, but not in the *ena1* mutant (Fig. 2 and 4A). These data indicate that both Ena1 and Nha1 transporters may be involved in Na^+/K^+ efflux in *C. neoformans.* The salt stress-induced expression of *ENA1* and *NHA1* was significantly blocked by a loss of Hog1 (Fig. 2A and 2B), indicating that the HOG pathway is the major controller of the two cation transporters.

In order to address whether induction of *ENA1* and *NHA1* is triggered by salt stress or osmotic shock, we examined expression patterns of the two cation transporter genes in response to 1 M sorbitol. Interestingly, the expression patterns of *NHA1* and *ENA1* in response to 1 M sorbitol seemed to be different from those in response to 1 M NaCl or KCl. Upon exposure to 1 M sorbitol, *ENA1* expression was transiently induced at 10 min and then rapidly returned to the basal levels in a Hog1-dependent manner (Fig. 2C). In contrast, *NHA1* expression was weakly induced at later time points (30 to 60 min). However, the pattern of *NHA1* expression in WT is indistinguishable from that of *hog1Δ* mutant (Fig. 2C). These data indicated that Ena1 is modulated by HOG pathway in response to cation and osmotic shock whereas Nha1 is mainly governed by Hog1 under cation shock, but not under osmotic shock.

3.3. Ena1 and Nha1 play discrete and redundant roles in maintenance of cation homeostasis in C. neoformans

Deletion of the *ENA1* gene renders *C. neoformans* cells less viable under high pH condition (pH 8.5) and attenuates virulence in mice (Idnurm et al., 2009). To characterize the role of Nha1 and its relationship with Ena1, we constructed the *nha1* and *ena1 nha1* double mutants in the H99 and KN99 strain background as described in Materials and Methods. The mutant set generated in these two strain backgrounds exhibited similar phenotypes and did not show any growth defects at a range of temperatures (25 to 39°C) (data not shown). The *ena1* mutant exhibited reduced survival in the presence of high salt (1 M NaCl or KCl) only under glucose-starved condition, but not under glucose-rich condition (Fig. 3A), as described before (Idnurm et al., 2009; Ko et al., 2009), which is in contrast to the *S. cerevisiae ena1* mutant showing high salt susceptibility under glucose-rich conditions (Prior et al., 1996). In contrast, deletion of the *NHA1* gene alone did not affect susceptibility to salt stress under neutral pH condition (Fig. 3A). However, double deletion of both *ENA1* and *NHA1* genes dramatically enhances K^+ salt stress susceptibility. The *ena1 nha1* double mutant became extremely susceptible to K^+-stress even under glucose rich conditions unlike each single

mutant (Fig. 3A), suggesting that both Ena1 and Nha1 are involved in K^+ homeostasis. In contrast, additional deletion of *NHA1* did not further increase Na^+-stress susceptibility of the *ena1* mutant, indicating that Nha1 is largely dispensable for Na^+ homeostasis.

In *S. cerevisiae,* Ena1 and Nha1 control the intracellular concentration of toxic lithium ion (Li^+) as well as that of Na^+ and K^+ (Haro et al., 1991; Prior et al., 1996). The *C. neoformans ena1* mutant showed increased susceptibility to Li^+-stress compared to wild-type (WT) whereas the *nha1* mutant exhibited WT levels of Li^+ susceptibility (Fig. 3B). Deletion of *NHA1* did not further increase Li^+ susceptibility of the *ena1* mutant, indicating that Ena1, but Nha1, is mainly involved in Li^+ homeostasis. However, *ena1, nha1,* and *ena1 nha1* double mutants showed WT levels of resistance to $CaCl_2$ (Fig. 3B). In conclusion, Ena1 is mainly involved in controlling cellular levels of toxic cations, such as Na^+ and Li^+, whereas both Ena1 and Nha1 are important for influx or efflux of less toxic K^+ ions.

3.4. Both Ena1 and Nha1 play unique roles in survival of C. neoformans under alkaline and acidic pH

Functions of Ena1 and Nha1 are dependent on the electrochemical gradient of protons across the plasma membrane in *S. cerevisiae* (Catty et al., 1997; Sychrova et al., 1999). In yeast, Ena1 is mainly activated in alkaline condition, which has low concentrations of extracellular protons whereas Nha1 is activated in acidic condition, which has higher concentrations of extracellular protons (Sychrova et al., 1999). Moreover, Sod22, an ortholog of Nha1 in *Schizosaccharomyces pombe,* is activated in acidic pH conditions (Papouskova and Sychrova, 2007). In agreement with this finding, Ena1 was also found to be essential in survival of *C. neoformans* under high pH (8.5) conditions (Idnurm et al., 2009).

To further elucidate the role of Ena1 and Nha1 in survival of *C. neoformans* under different pH conditions, we first measured the expression patterns of *ENA1* and *NHA1* in response to high- and low-pH conditions. Supporting the previous finding by Idnurm et al., *ENA1* expression was strongly increased in response to the alkaline condition (pH 8.5) (Fig 4A). Accordingly, the *ena1* mutant, but not the *nha1* mutant, exhibited severe growth defects even in the presence of very low concentration (0.1 M) of Na^+ or K^+ salt under the alkaline condition (Fig 4B). Interestingly, however, the *ena1 nha1* double mutant displayed even higher susceptibility to 0.1 M KCl under high pH condition than the *ena1* mutant. These data show that Ena1 plays a major role in survival at the alkaline pH condition containing low concentration of K^+ or Na^+ whereas Nha1 plays a minor role at the same condition.

In contrast to *ENA1,* Nha1 appears to play a role in survival of *C. neoformans* at the acidic pH condition. *ENA1* expression was transiently induced after 10 min, but rapidly repressed and abolished after 30 min under the acidic condition (pH 4.5). In contrast, *NHA1* expression was weakly induced after any pH changes (Fig. 4A). *C. neoformans* Nha1 was not required for cell survival under low pH (pH 4.5) *per se*. Notably, however, the *nha1* mutant, but not the *ena1* mutant, showed extreme susceptibility to 1.5 M KCl under acidic pH condition compared to WT (Fig. 4C), which was in stark contrast to the finding that the *nha1* mutant was as resistant to 1.5 M KCl as WT under neutral pH (Fig. 3A). This phenomenon was not observed under high Na^+ conditions (Fig. 4C), further indicating that Nha1 mainly controls cellular levels of K^+, not Na^+. Interestingly, the *ena1* mutant did not exhibit increased susceptibility to 1.5 M NaCl under acidic pH condition (Fig. 4C). These data indicate that Nha1 plays a role in survival at acidic pH conditions under high K^+ stress and Ena1 is dispensable for Na^+ or K^+-stress response under acidic conditions. Regardless of the essential role of Nha1, *NHA1* expression was not strongly induced in response to acidic pH and high K^+ stress (Fig. 4D). In conclusion, Ena1 and Nha1 play discrete and overlapping roles in controlling proton homeostasis across the plasma membrane of *C. neoformans* under different pH conditions.

3.5. Ena1 and Nha1 are required for maintenance of plasma membrane potential and stability

The role of Ena1 and Nha1 in intracellular proton homeostasis prompted us to investigate their function in maintenance of plasma membrane potential. For this purpose, we used achemical whose activity is affected by membrane potential. Hygromycin B (HygB), a positively charged aminoglycosidic antibiotic, is known to be incorporated into the cells in proportion to membrane potential and used as a hyper- or hypo-polarization indicator in yeast (Mulet et al., 1999; Perlin et al., 1988). For example, the HygB-resistant *pma1* mutant shows the reduced membrane potential (Perlin et al., 1988) and deletion of Vnx1, a type of vacuolar monovalent cation/H^+ antiporter in *S. cerevisiae,* confers resistance to HygB (Cagnac et al., 2007). To address whether Ena1 and Nha1 control membrane potential in *C. neoformans,* each strain was exposed to HygB in the glucose-rich and -starved conditions. Each *nha1* and *ena1* mutant exhibited slightly increased levels of resistance to HygB in both glucose-rich and -starved conditions. Interestingly, however, the *ena1Δnha1Δ* double mutant was even more resistant to HygB than the *nha1Δ* and *ena1Δ* mutant (Fig. 5A), which is accordance with the previous report showing that overexpression of *NHA1* increases HygB susceptibility of *S. cerevisiae* (Kinclova-Zimmermannova et al., 2006). Therefore Ena1 and Nha1 could modulate membrane potential cooperatively although more direct measurement of membrane potential in the mutants will be needed in future studies.

Related to this finding, inhibition of either Ena1 or Nha1 greatly exacerbated membrane stability. Both *ena1* and *nha1* mutants showed a greatly increased susceptibility to SDS, a membrane destabilizes. The *ena1 nha1* double mutant was even more susceptible to SDS than each single mutant (Fig. 5B). However, the *ena1 nha1* mutant was as resistant to cell wall biosynthesis inhibitors, such as congo red (CR) and calcofluor white (CFW), as WT, indicating that Ena1 and Nha1 affect cell membrane stability, but not cell wall integrity (Fig. 5B). Probably, perturbed membrane potential caused membrane instability in the *ena1* and *nha1* mutants, although other reason(s) might exist.

3.6. Inhibition of Ena1 and Nha1 increases antifungal drug susceptibility in C. neoformans

Based on the role of Ena1 and Nha1 in maintenance of membrane potential and stability, we hypothesized that inhibition of Ena1 and Nha1 may affect the capability of antifungal drugs to bind to the cell membrane or to be incorporated into the cells through the cell membrane. Polyene drugs, such as amphotericin B (AMB), bind to ergosterol in the cell membrane and form a transmembrane channel, which results in a leakage of monovalent ion (Na^+, K^+, H^+, and Cl^-). Azole drugs, such as fluconazole (FCZ) and ketoconazole (KCZ), inhibit the synthesis of ergosterol and destabilize the cell membrane stability and fluidity. The *ena1* mutant exhibited a slightly increased susceptibility to AMB than WT. In contrast, the *ena1 nha1* double mutant was highly susceptible to AMB, which is similar to the *hog1* mutant (Fig. 6A). In the presence of mild K^+ stress (0.5 M KCl), the *ena1, nha1*, or *ena1 nha1* mutant exhibited even higher susceptibility to AMB (Fig. 6B). Unlike the *hog1* mutant, however, the *ena1 nha1* double mutant also exhibited increased susceptibility to the azole drugs (Fig. 6C). Hogl is known to repress ergosterol biosynthesis in *C. neoformans* and thereby its inhibition leads to increased expression of genes involved in ergosterol biosynthesis and contents of cellular ergosterol, which subsequently enhances azole resistance but reduces polyene resistance (Ko et al., 2009). Therefore, increased polyene and azole susceptibility observed in the *ena1 nha1* double mutant appears to be independent of ergosterol biosynthesis, and instead results from defective membrane stability and cation imbalance. These data indicate that Ena1 and Nha1 play redundant roles in protecting *C. neoformans* from polyene and azole drugs by regulating membrane stability and cation homeostasis.

3.7. Diverse signaling pathways are involved in ENA1 and NHA1 expressions in alkaline- and cation stress

In the budding yeast, *ENA1* expression is under control of diverse signaling cascades besides the HOG pathway. The calcium/calcineurin signaling pathway controls *ENA1* expression through the Crz1 transcription factor (Mendizabal et al., 2001). Under high pH, the Rim101 pathway controls *ENA1* expression through an Nrg1 repressor protein (Lamb and Mitchell, 2003). In *C. neoformans,* however, *ENA1* expression is not modulated by the calcineurin pathway under high pH condition but is regulated by Rim101 in capsule inducing conditions (Idnurm et al., 2009; O'Meara et al., 2010). To further identify signaling pathways, besides the HOG pathway, that control *ENA1* and *NHA1* expression in response to cation shock and high pH condition, we examined expression patterns of the two cation transporters in *rim101, nrg1, cna1*, and *atf1* mutants. Atf1 is a transcription factor that is positively regulated by the Hog1 MAPK (our unpublished results). Northern blot analysis showed that both basal and induced levels of *ENA1* were highly reduced in the *rim101* mutant in both alkaline (pH 8.5) and salt stress conditions in *C. neoformans* (Fig. 7A). In contrast, basal expression levels of *ENA1* expression were increased in the *nrg1* mutant in both conditions. Under high pH conditions, *ENA1* induction was greater in the *nrg1* mutant than WT (Fig. 7A). These data indicate that the Rim101-signaling pathway may control *ENA1* expression through the Nrg1 repressor in *C. neoformans.* In the *atf1* mutant, basal *ENA1* expression was decreased and its induction appeared to be decreased or delayed at both alkaline and salt stress conditions (Fig. 7A), indicating that the HOG pathway controls *ENA1* partly via the Atf1 transcription factor. The calcineurin pathway appeared to be largely dispensable for regulation of *ENA1* in the presence of 1 M NaCl, although down regulation of *ENA1* seemed to be delayed at pH 8.5. In contrast to *ENA1,* Cna1 and Atf1 appeared to be dispensable for induction of *NHA1* (Fig. 7B). Similar to *ENA1* expression, however, induction of *NHA1* was decreased in the *rim101Δ* mutant and slightly increased in the *nrg1Δ* mutant (Fig. 7B), implying that the Rim101-Nrg1 pathway could be involved in regulation of *NHA1*. Taken together, *ENA1* expression is regulated at least by the two signaling pathways, the Hog1-Atf1 and Rim101-Nrg1 pathways, under cation stress and high pH conditions. Nha1 is mainly regulated by the Hog1 pathway in the Atf1-independent manner and the Rim101-Nrg1 pathway.

3.8 Nha1 cellular localization is similar in C. neoformans and S. cerevisiae

We examined the cellular location of Nha1 protein in *C. neoformans.* It has been reported that Nha1 proteins in other fungi including *S. cerevisiae* and *C. albicans* are preferentially localized in the plasma membrane (Kinclova-Zimmermannova and Sychrova, 2007; Kinclova et al., 2001). In order to find cellular localization of CnNha1, we constructed the *nha1/NHA1-GFP* strain that is generated by transforming the *nha1* mutant with the *NHA1* gene fused with the *GFP* gene at the C-terminal region. Phenotypes of the *nha1/NHA1-GFP* strain were equivalent to those of WT and *nha1/NHA1* complemented strains (data not shown), indicating that the Nha1-GFP protein is functional as native Nha1. We examined cellular localization of CnNha1-GFP in comparison with ScNha1 that is similarly fused to GFP at the C-terminal region. Reflecting low RNA expression levels of *NHA1,* GFP-fluorescence signals were weakly detected throughout the cell in both *S. cerevisiae* and *C. neoformans* (Fig. 8). However, both ScNha1 and CnNha1 appeared to be more enriched in the periphery of cells than other parts of cells, although they were not evenly distributed. Therefore, cellular localization of *C. neoformans* Nha1 was similar to yeast Nha1.

3.9. Roles of two cation transporters in regulation of virulence factor production and sexual differentiation in C. neoformans

The HOG pathway plays a major role in production of virulence factors such as capsule and melanin (Bahn et al., 2005). The perturbation of *HOG1* enhances capsule synthesis and

melanin production (Bahn et al., 2005). The fact that expression levels of *ENA1* and *NHA1* are positively regulated by Hog1 led us to examine the role of the two cation transporters in capsule and melanin production. The *ena1* and *nha1* single mutants produced WT-levels of capsule and melanin. However, the *ena1 nha1* double mutant showed slightly enhanced capsule production than WT based on both qualitative and quantitative observations (Fig. 9A). Similarly, the *ena1 nha1* double mutant also displayed increased melanin production (Fig. 9B). In *C. neoformans,* melanin biosynthesis is catalyzed by laccase, encoded by the *LAC1* gene, whose expression is induced by glucose starvation (Pukkila-Worley et al., 2005). In WT, *LAC1* started to be strongly induced after 30 min and peaked at 120 min upon glucose starvation (Fig. 9C). Compared to WT, *LAC1* induction was found to be much increased in the *ena1 nha1* mutant (Fig. 9C). These data indicate that intracellular cation balance contributes to biosynthesis of capsule and melanin in *C. neoformans.*

The Hog1 MAPK pathway is involved in sexual differentiation as well as virulence factor production. The deletion of *HOG1* enhances mating response via increasing pheromone expression (Bahn et al., 2005). To address the role of Ena1 or Nha1 in sexual differentiation, a mating assay was performed with *MAT* and *MAT***a** cells of *ena1* and *nha1* mutants. Disruption *of ENA1* and *NHA1* did not affect sexual differentiation of *C. neoformans* (data not shown). In summary, the cation transporters Ena1 and Nha1 cooperatively regulate melanin and capsule production, but are dispensable for sexual differentiation.

3.10. Ena1 and Nha1 play different roles in the virulence of C. neoformans

As Ena1 plays an essential role in virulence of *C. neoformans* (Idnurm et al., 2009; Lee et al., 2010; Liu et al., 2008), we examined the role of Nha1 in virulence by using a murine model of systemic cryptococcosis. In this virulence assay, we used WT strain and *ena1, nha1, ena1 nha1*, and *nha1/NHA1* strains that were constructed in the H99 strain background. In agreement with the previous study (Idnurm et al., 2009), the *ena1* mutant constructed in the H99 strain background was a virulent in a mouse model as expected (Fig. 10). In contrast, the *nha1* mutant was as virulent as WT, indicating that Nha1 is not involved in virulence of *C. neoformans* (Fig. 10). The *ena1 nha1* mutant was as a virulent as the *ena1* mutant (Fig. 10). The virulence attenuation observed in both *ena1* and *ena1 nha1* mutant was not due to clearance of the infection, as it was still possible to detect low levels of CFUs in the lungs of *ena1Δ-* or *ena1Δnha1Δ-* infected animals at day 43 post infection (data not shown). In addition, there was little or no dissemination of these mutants from the lungs, as few (only 1) or no CFUs were detected in the brains of infected animals (data not shown). We therefore concluded that Ena1 is essential and Nha1 is dispensable for virulence of *C. neoformans.*

4. Discussion

In this study we aimed to elucidate the role of two cation transporters, Ena1 and Nha1, in the human pathogenic fungus *C. neoformans,* as key downstream effectors of the HOG signaling pathway. Our previous transcriptome analysis revealed that expression of *ENA1* and *NHA1* is differentially regulated in response to osmotic and oxidative stresses in a Hog1-dependent manner (Ko et al., 2009). The function of Ena1, a P-type ATPase, has been recently investigated in *C. neoformans* (Idnurm et al., 2009; Ko et al., 2009). Unlike other fungal Ena1 orthologs, however, cryptococcal Ena1 appears to play a limited role in counter acting salt stress, important only under glucose-starved conditions (ldnurm et al., 2009; Ko et al., 2009), indicating that another cation transporter may compensate for the loss of Ena1 for maintenance of cation homeostasis and salt stress response.

Here we provide several lines of evidence showing that Nha1, Na^+/H^+ antiporter, plays redundant and discrete roles with Ena1 in controlling cation homeostasis via the HOG

pathway in *C. neoformans.* First, both *ENA1* and *NHA1* expression were induced by either Na$^+$ or K$^+$ salt stress in WT, but not in the *hog1* mutant, although *ENA1* was much more strongly induced than *NHA1.* Second, regardless of the expression patterns, Ena1, but not Nha1, appears to be a key player for counter acting salt stress conferred by toxic cations, such as Na$^+$ or Li$^+$. The *nha1* mutant did not show any increased susceptibility to Na$^+$ stress compared to WT under either glucose-rich or –starved conditions. Moreover, deletion of *NHA1* did not further increase Na$^+$ or Li$^+$ susceptibility of the *ena1* mutant. Third, both Ena1 and Nha1 play redundant roles in responding and adapting to the less toxic K$^+$ cation. Disruption of *NHA1* further increased K$^+$ susceptibility of the *ena1* mutant, particularly under glucose-rich condition. Fourth, Ena1 and Nha1 work oppositely in response to changes of extracellular pH. Under alkaline condition, Ena1 was highly induced in transcript levels and was required for survival even in the presence of low levels of Na$^+$ or K$^+$ salt. For survival under the high pH and low K$^+$ salt, Nha1 is also required. In contrast, Nha1, but not Ena1, was essential for survival at acidic condition (pH 4.5) under high K$^+$ stress. Indeed, this (pH 4.5, 1.5 M KCl) was the only condition where the *nha1* mutant showed obvious growth defects. To sum up, Ena1 and Nha1 are two major cation transporters for controlling cation homeostasis in *C. neoformans* in both cooperative and opposite manners.

The Nha1 cation transporter is structurally conserved but functionally divergent in *C. neoformans.* Most yeast alkali metal cation/H$^+$ antiporters, except SpSod2, consist of N-terminal hydrophobic region and C-terminal hydrophilic region. About 10 to 12 transmembrane regions appear to be present in the N-terminus of the Nha1 orthologs. CnNha1 also has 11 or 12 hydrophobic segments at its N-terminus based on the hydrophilicity plot. Although fungal Nha1 orthologs show high sequence homology to each other, they are divided into two groups depending on substrate specificity. One group, such as Na$^+$/H$^+$ antiporter in *S. pombe,* has a substrate specificity for Na$^+$ and Li$^+$ (Jia et al., 1992). The other group has a broad substrate specificity to regulate cation homeostasis in cells (Pribylova et al., 2006). Interestingly, unlike other Nha1, CnNha1 is involved in potassium homeostasis, but not sodium and lithium homeostasis. Even though the *nha1Δ* mutant displayed WT levels of resistance to NaCl or KCl, the *ena1 nha1* double mutant was significantly more susceptible to KCl, but not to NaCl, than the *ena1* mutant in the glucose-rich condition (Fig 3A). Furthermore, the *ena1 nha1* double mutant was as resistant to K$^+$ stress, but not to Na$^+$ stress, as the *nha1Δ* mutant under low pH condition (pH 4.5), indicating that CnNha1 has substrate preference to potassium, but not to sodium.

One notable finding made in this study is that disruption of *ENA1* and *NHA1* enhances susceptibility to antifungal drugs, particularly azoles, in *C. neoformans.* The primary mode of action of azole drugs is to reduce sterol biosynthesis by inhibiting P450 cytochrome activity. However, a recent study showed that these azole drugs, including ketoconazole and miconazole, alter potassium homestasis in *S. cerevisiae* (Calahorra et al., 2011). The present study also showed that cation transporters play critical roles in conferring resistance against polyene (amphotericin B) as well as azoles (fluconazole and ketoconazole), indicating that cation transporters regulate antifungal drug susceptibility through cation balance between outside and inside membrane regardless of ergosterol biosynthesis. The finding could apply to therapeutic target of cryptococcosis with azole or polyene drugs with inhibitors of cation transporters.

In *S. cerevisiae,* the HOG pathway is also known to control *ENA1* induction through the Sko1-Ssn6-Tup6 corepressor complex, which is recruited as a repressor to the *ENA1* promoter (Marquez et al., 1998; Proft and Serrano, 1999). Upon incoming cation stress, activated Hog1 phosphorylates Sko1, which is subsequently dissociated from the Ssn6-Tup6 complex and activates *ENA1* expression. Although none of the Sko1-like bZIP transcription factors have been discovered in the *C. neoformans* genome database, the Atf1 transcription

factor, which is a distant ortholog of Sko1 in *C. neoformans,* is likely to be a good candidate because *ENA1* expression is partly defective in the *atf1* mutant. Connection between Hog1 and Atf1 for regulation of *ENA1* expression should be further investigated in future.

Besides the HOG pathway, diverse signaling pathways are known to be involved in *ENA1* expression in the budding yeast. The calcineurin pathway regulates *ENA1* expression by Crz1 transcription factor under high pH and salt stress (Mendizabal et al., 2001). In response to high pH, the Rim101-Nrg1 and Snf1-Mig1/2 signaling pathways control *ENA1* expression. Compared to expression patterns of *ENA1* in *S. cerevisiae, ENA1* expression in *C. neoformans* exhibited similar and different patterns. First, this study revealed that the calcineurin pathway did not affect the expression level of *ENA1* salt stress condition. Previously, Idnurm and co-workers also reported similar results (ldnurm et al., 2009). Compared to *ENA1* in *S. cerevisiae,* Crzl binding sites (5′-GAATGGCTG-3′ and 5′-GGGTGGCTG-3′) are not found in the promoter region of *ENA1* in *C. neoformans.* Second, the *nrg1* mutant in *C. neoformans* shows increased susceptibility to NaCl whereas the *nrg1* mutant in *S. cerevisiae* confers tolerance to NaCl compared to WT (Cramer et al., 2006; Lamb and Mitchell, 2003). Nevertheless, the expression levels of *ENA1* in the *nrg1* mutant are enhanced in both *S. cerevisiae* and *C. neoformans* and the promoter region of cryptococcal *ENA1* also has conserved Nrg1 binding sequences (5′-CCCCT-3′). Third, we found that *ENA1* expression is blocked in the *rim101* mutant in alkaline and cation stress (1 M NaCl) conditions. According to the study by O'Meara, the promoter of *ENA1* has conserved predicted Rim101 binding sites (5′-GCCAAG-3′) (O'Meara et al., 2010). The one notable finding is that *ENA1* expression was abolished in the *hog1* mutant whereas *ENA1* was slightly induced in *rim101* mutant in response to 1 M NaCl shock, implying that the Rim101 signaling pathway could be modulated by the HOG pathway (Fig. 11). According to our previous transcriptome analysis, expression of *RIM101* appears to be delayed in the *hog1* mutant under 1 M NaCl shock (Ko et al., 2009). It indicates that the HOG1 pathway could cross talk with the Rim101 pathway. The connection between the Hog1 and Rim101 pathways is a topic of future research.

In contrast to *ENA1,* the regulatory mechanism of *NHA1* remains elusive. In *Candida* species, expression levels of *NHA1* do not appear to change significantly in response to salt stresses, similar to *S. cerevisiae* (Krauke and Sychrova, 2011; Soong et al., 2000). Upon osmotic shock in *S. cerevisiae,* however, the Hog1 kinase rapidly phosphorylates Nha1 at its C-terminal region through an unknown mechanism (Proft and Struhl, 2004). Phosphorylation of Nha1 via Hog1 results in reassociation of the general transcriptional machinery. In contrast, our study revealed that expression levels of *NHA1* were induced under salt stress in a Hog1-dependent manner. Furthermore, *NHA1* mRNA abundance was also regulated by the Rim101-Nrg1 pathway. The promoter region of Nha1 also has both Nrg1 binding site (5′-CCCCT-3′) and Rim101 binding site (5′-GCCAAG-3′) similar to that of Ena1. However, it is unclear whether Rim101 controls expression levels of *NHA1* via Nrg1 repressor under K^+ shock. Taken together, the HOG pathway and Rim101-Nrg1 pathway are key signaling cascades to control expression and activity of Nha1, although its regulatory mechanism is diverse among fungi.

An additional interesting finding of this study is that Ena1 and Nha1 play redundant roles in negatively regulating production of capsule and melanin in *C. neoformans.* Antioxidant melanin and antiphagocytic capsule are well established virulence factors in *C. neoformans.* Lac1 (laccase 1) mainly governs melanin production, whereas Lac2 is partially contributes to melanin biosynthesis (Pukkila-Worley et al., 2005; Williamson, 1994). Furthermore, cations such as Fe^{3+}, Cu^{2+}, and K^+, are involved in phenoloxidase (laccase) activity in *C. neoformans* (Palumbo et al., 1985; Vidotto et al., 2002). This study showed that deletions of both *ENA1* and *NHA1* induce melanin biosynthesis through enhanced expression levels of

LAC1. This result may result from imbalance of intracellular cations, which induces to increase expression of laccase. At this point, it is not clear whether perturbed cation homeostasis also affects activity of laccases. Metal ions, such as iron, are also known to be required for capsule production (Lian et al., 2005). In conclusion, cation homeostasis mediated by Ena1 and Nha1 is required for tight regulation of melanin and capsule biosynthesis.

Intracellular cation homeostasis is essential for survival and virulence in fungi. The perturbation of *ENA1* or *VPH1,* encoding a vesicular (H$^+$)-ATPase proton pump, results in severe virulence defects in *C. neoformans* (*Erickson* et al., 2001; Idnurm et al., 2009). Furthermore, *CN/H1*, a Na$^+$/H$^+$ antiporter in *C. albicans,* is involved in morphological change and shows attenuated virulence in deletion mutants (Soong et al., 2000). However, our animal study exhibited that Nha1 is dispensable for virulence in *C. neoformans*. In fact this finding is not surprising because Nha1 is only involved in less toxic K$^+$ stress, but not in toxic Na$^+$ stress like Ena1. The only growth condition (acidic pH and high K$^+$) where the *nha1* mutant exhibited severe growth defects may not be experienced by *C. neoformans* during host infection and disease progressions contrast, the role of Ena1 in counteracting high concentration of toxic ions, such as sodium ion, and growth at alkaline pH appears to be required for the pathogen to survive in the host. Although the exact reason is not clear at this point, Idnurm and colleagues proposed that Ena1 might be essential for survival in the localized alkaline microenvironments generated by the virulence factor urease in *C. neoformans* within the host (Erickson et al., 2001; Idnurm et al., 2009). However, other explanation may exist for such a dramatic role of Ena1 in virulence of *C. neoformans.*

In summary, we demonstrated that cation transporters, Ena1 and Nha1, have redundant and discrete roles in cation homeostasis, pH regulation, membrane potential, and virulence in *C. neoformans* as summarized in Figure 11. Based on our data, we suggest that cation transporters could be good targets for combination antifungal drug therapy for treatment of cryptococcosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Cation transporters Ena1 and Nha1 play redundant and discrete roles in cation homeostasis of *Cryptococcus.*

- Ena1 and Nha1 affect antifungal drug susceptibility in *Cryptococcus.*

- Both Ena1 and Nha1 regulate production of capsule and melanin in a cooperative manner.

- The HOG and Rim101 pathways control expression of *ENA1* and *NHA1* in *Cryptococcus*
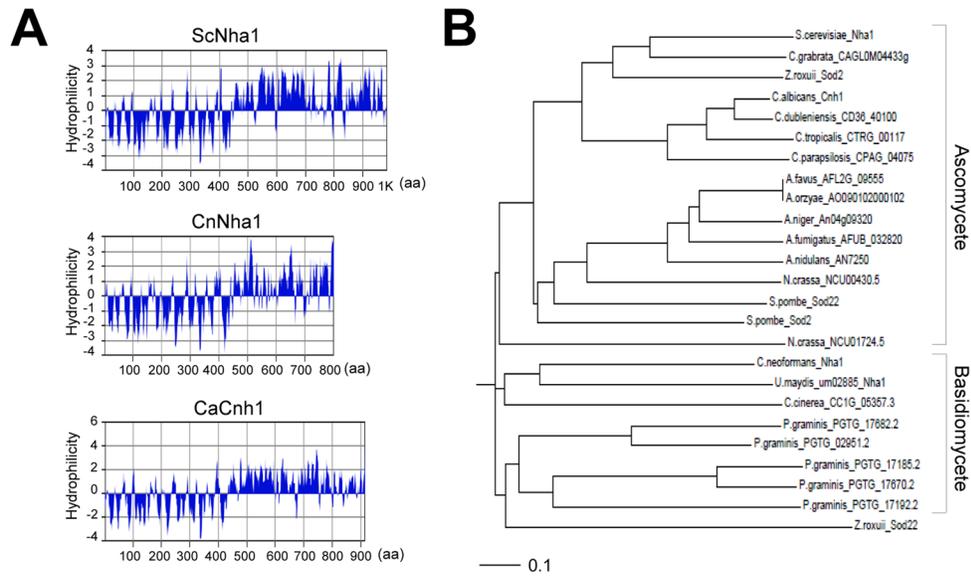
Fig. 1.

Identification of the *NHA1* gene in *C. neoformans.* (A) The hydrophilicity plot of *C. neoformans* Nha1 (CnNha1), *S. cerevisiae* Nha1 (ScNha1), and *C. albicans* Cnh1 (CaCnh1). The hydrophilicity plots were depicted by protein analysis tool from MacVector software (version 7.2.3, Accelrys). (B) Phylogenetic tree of Nha1 proteins in *C. neoformans* and other fungi. The phylogenetic tree was generated by philodendron phylogenetic tree printer (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html). The scale bar line represents an evolutionary distance of 0.1.
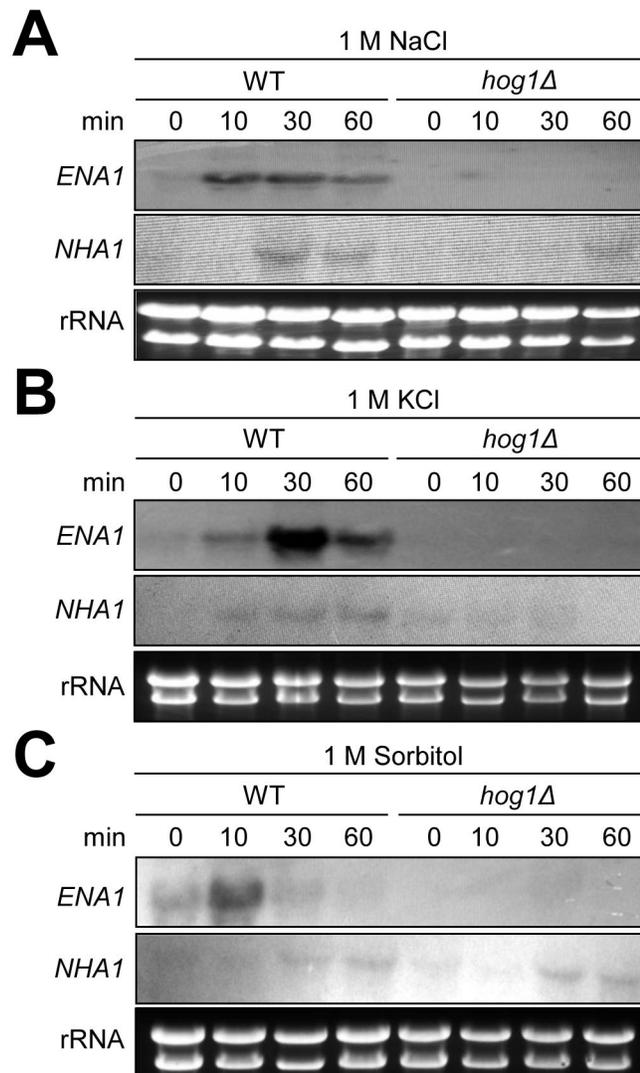
Fig. 2.
Expression analysis of *ENA1* and *NHA1* under cation and osmotic stress conditions. Northern blot analysis was performed with total RNA isolated from WTH99 strain and *hog1* mutants grown in YPD medium containing 1 M NaCl (A), 1 M KCl (B), or 1 M sorbitol (C) at different time points (0, 10, 30 and 60 min). Each membrane was hybridized with the radioactively labeled *ENA1* specific probe, washed, and developed. Subsequently the same membrane was deprobed, re-hybridized with the *NHA1* specific probe, washed, and developed.
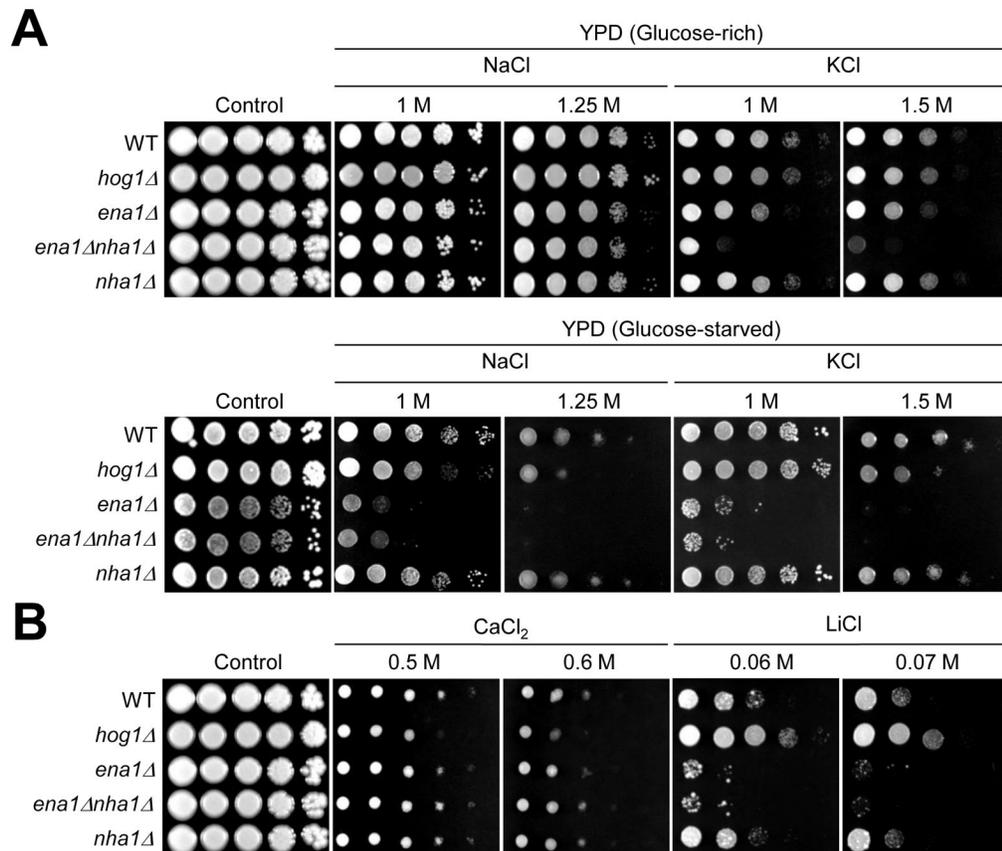
Fig. 3.
The role of Ena1 and Nha1 in cation homeostasis. (A) WT H99 strain and *hog1, ena1, ena1 nha1*, and *nha1* mutants were grown for 16hr at 30°C in liquid YPD medium, 10-fold serially diluted and spotted on YPD (glucose-rich) or YP (glucose-starved) media containing indicated concentrations of KCl or NaCl. (B) Cells were incubated for 16hr at 30°C in liquid YPD medium, 10-fold serially diluted and spotted on YPD medium containing indicated concentrations of CaCl$_2$ or LiCl.
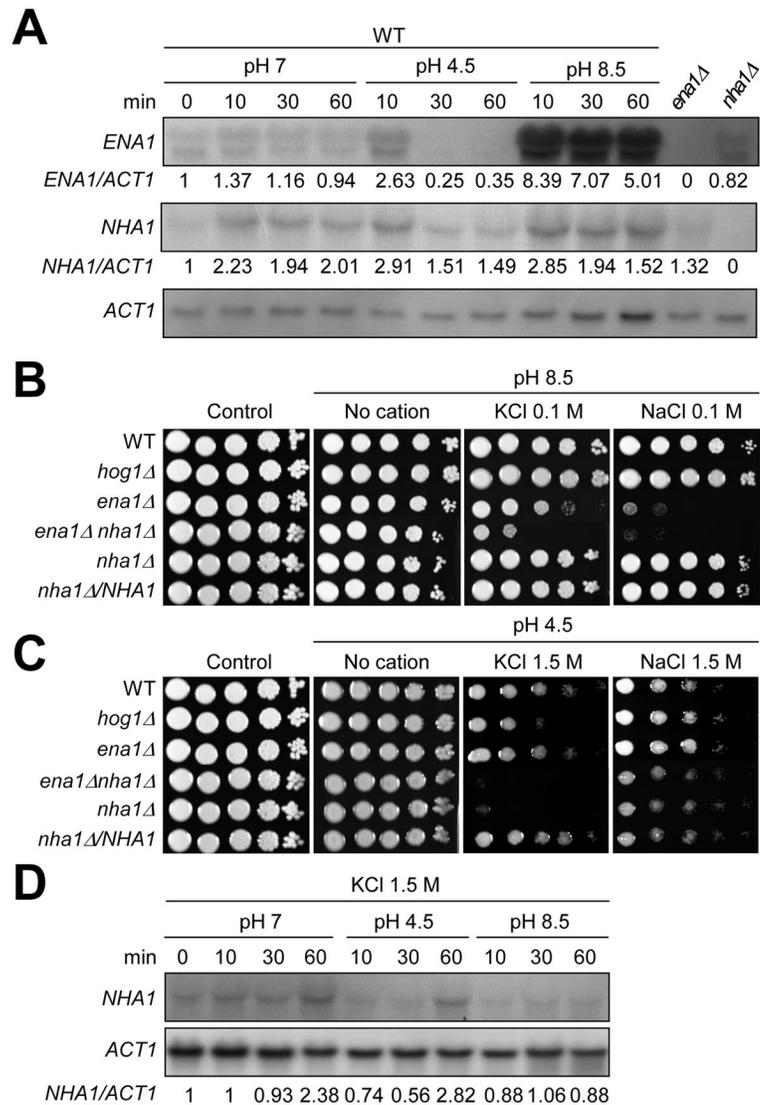
Fig. 4.
The role of Ena1 and Nha1 in growth under diverse pH conditions. (A) Northern blot analysis for monitoring *ENA1* or *NHA1* expression in response to different pH conditions. Total RNA was isolated from WT strain H99 grown in different pH conditions as described in Materials and Methods. The relative expression level of *ENA1* or *NHA1* was quantitatively measured with phosphoimager analysis by normalization with *ACT1* expression levels. (B and C) Cells (WT, *hog1, ena1, ena1 nha1, nha1* mutants and *nha1/ NHA1* complemented strain) were spotted on YPD agar medium that was adjusted to acidic (pH 4.5) or alkaline pH (pH 8.5) YPD and contained indicated concentration of KCl or NaCl. (D) Expression levels of *NHA1* were monitored under different pH condition (pH 7, pH 4.5, and pH 8.5) in the presence of 1.5 M KCl. The relative expression level of *NHA1* was quantitatively measured as described above in (A).
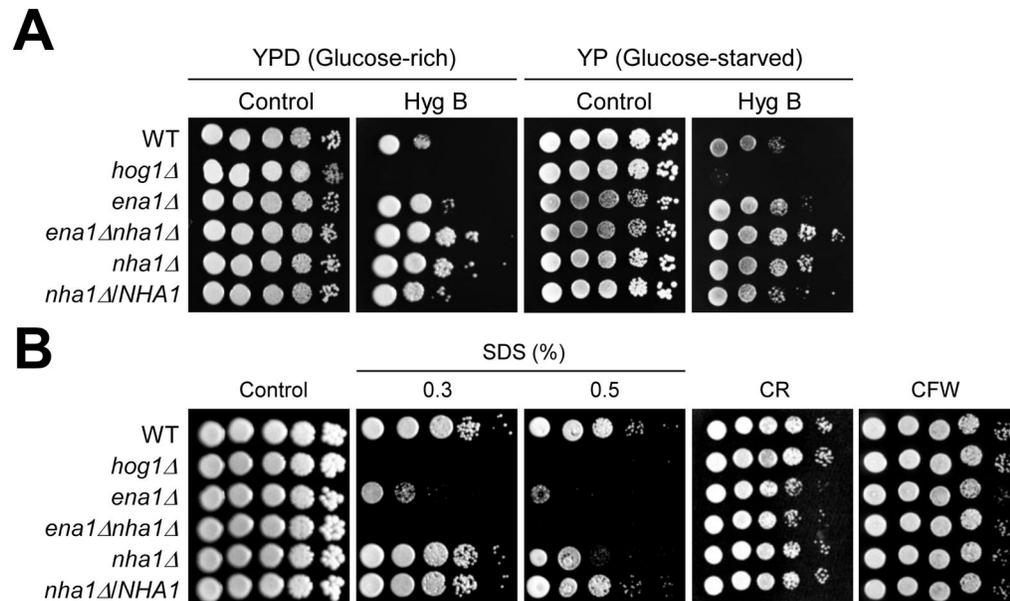
Fig. 5.
The role of Ena1 and Nha1 in maintenance of membrane potential and stability. (A) Cells were spotted onto the YPD or YP medium containing the indicated concentration of Hyg B (Hygromycin B 60 μg/ml and 40 μg/ml in glucose-rich and-starved conditions, respectively). Strains were incubated at 30°C for 3 to 4 days and were photographed. (B) Cells were spotted onto the YPD medium including the indicated concentration of SDS, CR (Congored, 1%) or CFW (Calcofluor white, 2 mg/ml).
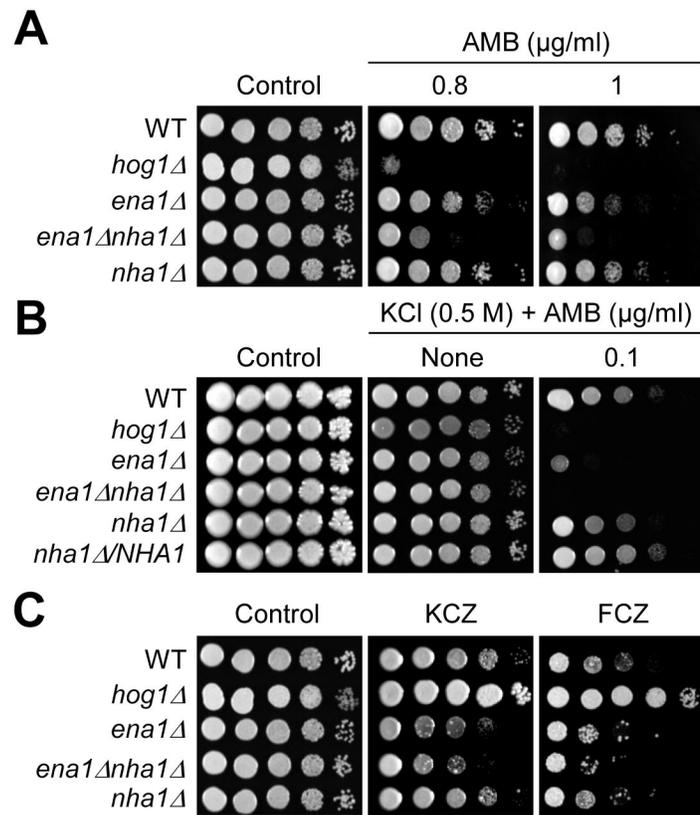
Fig. 6.
The role of Ena1 and Nha1 in antifungal drug resistance. (A and C) Strains (WT H99 strain and *hog1, ena1, ena1 nha1*, and *nha1* mutants) were spotted onto YPD medium including the indicated concentration of polyene [AMB (amphotericin B)] or azole [FCZ (fluconazole, 14 μg/ml), KCZ (ketoconazole, 0.3 μg/ml)] drugs. (B) Each *C. neoformans* strain was grown for 16hr at 30°C in YPD liquid medium, 10-fold serially diluted and spotted YPD (0.5 M KCl) agar containing indicated concentrations of AMB. Cells were incubated at 30°C for 72hr and photographed.

A

B
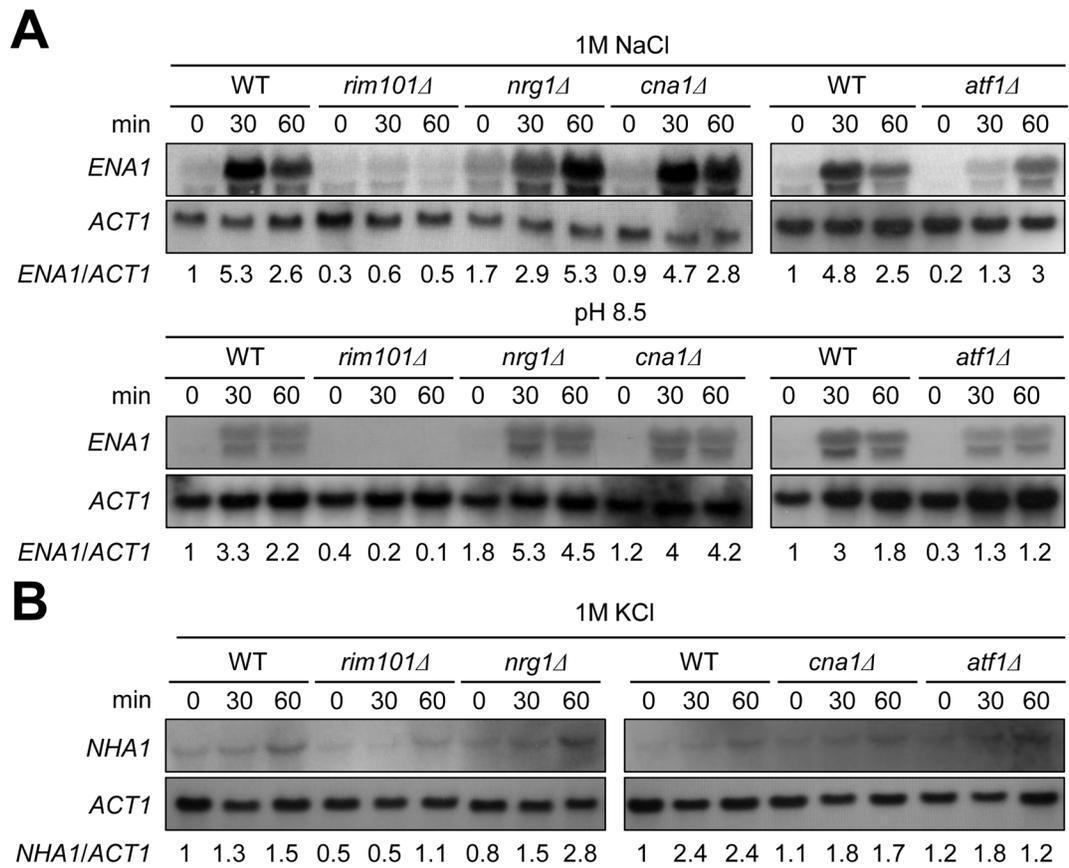
Fig. 7.
Expression patterns of *ENA1* and *NHA1* in diverse signaling pathways. Total RNA was isolated from WT H99 strain and *rim101, nrg1, cna1* and *atf1* mutants grown in YPD liquid medium containing 1 M NaCl (A, upper panel) or 1 M KCl (B), or YPD medium adjusted to high pH (8.5) (A, lower panel) at different time points (0, 30, 60 minutes). Each membrane was hybridized with the *ENA1* (A) or *NHA1* (B) specific probes, washed, and developed. Subsequently the same membrane was deprobed, re-hybridized with the *ACT1* specific probe, washed, and developed. Expression levels of *ENA1* or *NHA1* relative to *ACT1* were measured by phosphoimager analysis.
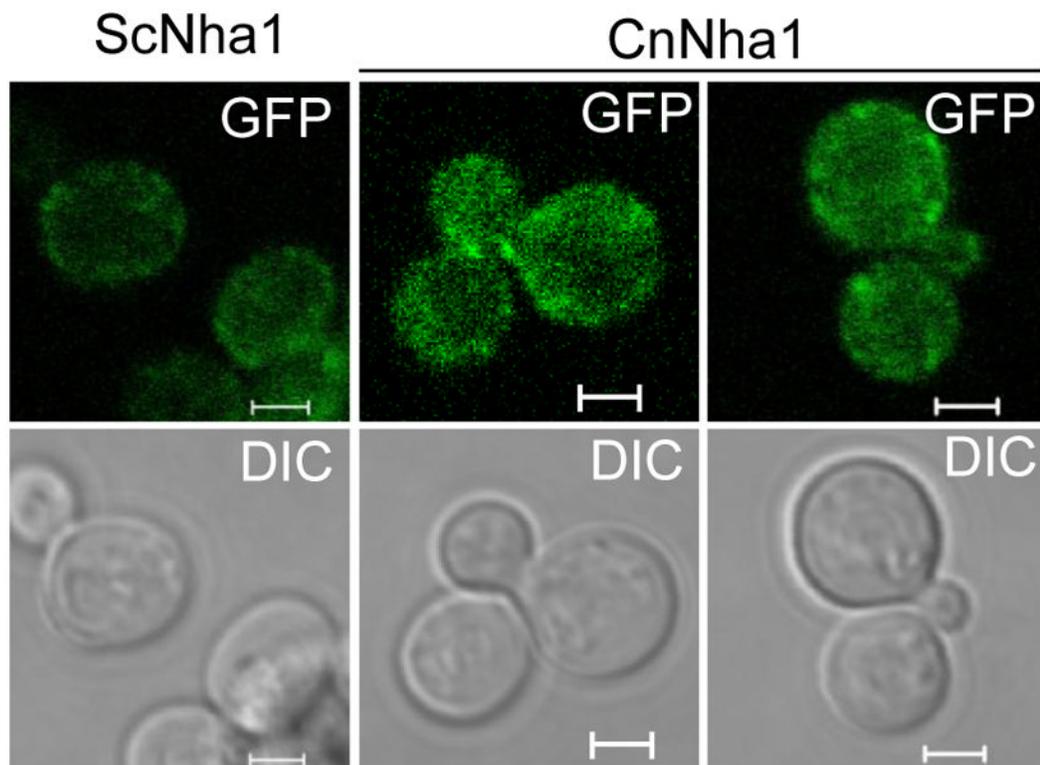
Fig. 8.
Localization of Nha1 in *C. neoformans. C. neoformans* and *S. cerevisiae* cells expressing
Nha1-Gfpfusion proteins (CnNha1 and ScNha1, respectively) were cultured in SC liquid
media at 30°C for 16 hr and were subcultured in SC liquid containing 1 M KCl for 1 h.
Cellular localization of Nha1-Gfp proteins were visualized by confocal microscope (Carl
Zeiss). The scale bar represents 2 μm.
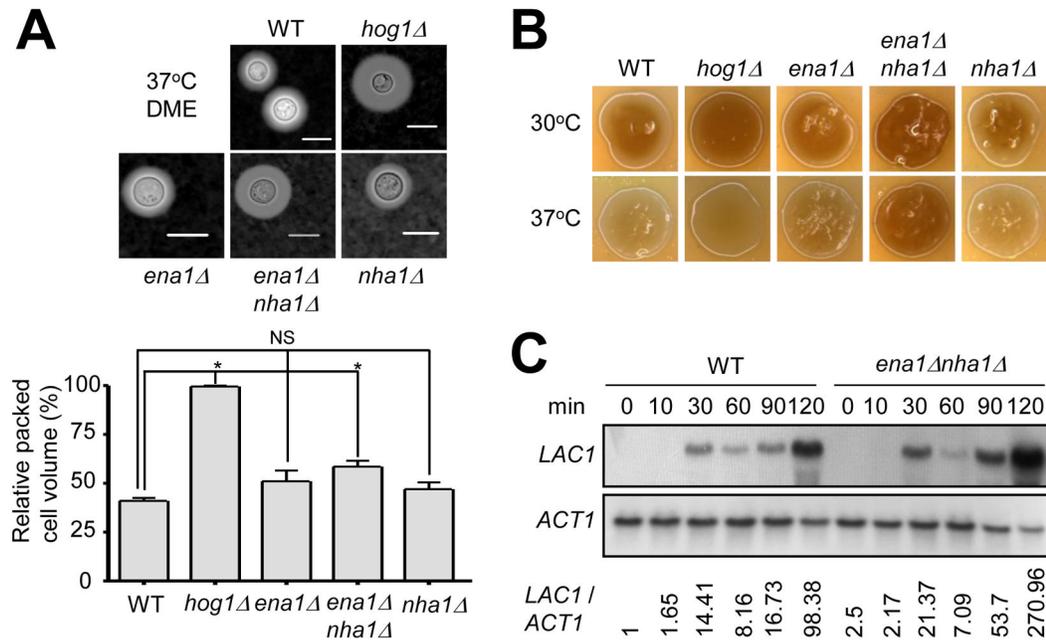
Fig. 9.

The role of Ena1 and Nha1 in capsule and melanin production. (A) For capsule production measurement, each strain (WT H99 strain and *hog1, ena1, ena1 nha1*, and *nha1* mutants) was spotted and cultured on DME agar medium at 37°C for 2 days. Capsules were visualized by India ink staining (upper panel) and the relative capsule volume was measured by calculating the ratio of the length of packed cell volume phase per length of total volume phase (lower panel). Three independent experiments with technical triplicates were performed. Statistical analysis was performed by using Bonferroni multiple comparison test. *, $P < 0.01$ and NS, not significant ($P > 0.05$). (B) Each *C. neoformans* strain was spotted, cultured on Niger seed medium containing concentration of glucose 0.1% at 30°C or 37°C for 1 day, and photographed. (C) The role of Ena1 and Nha1 in controlling *LAC1* expression. WT H99 strain and *ena1 nha1* mutants grown to a logarithmic phase ($OD_{600nm} = 1.0$) in YPD liquid medium (zero time control) were shifted to YNB liquid medium (without glucose), and further incubated at 30°C. Northern blot analysis was performed with total RNA isolated from each cell grown at the indicated time points. Each membrane was hybridized with the *LAC1* specific probe, washed, and developed. Subsequently the same membrane was deprobed, re-hybridized with the *ACT1* specific probe, washed, and developed. The expression levels of *LAC1* relative to *ACT1* (*LAC1/ACT1*) were measured via phosphoimager analysis.
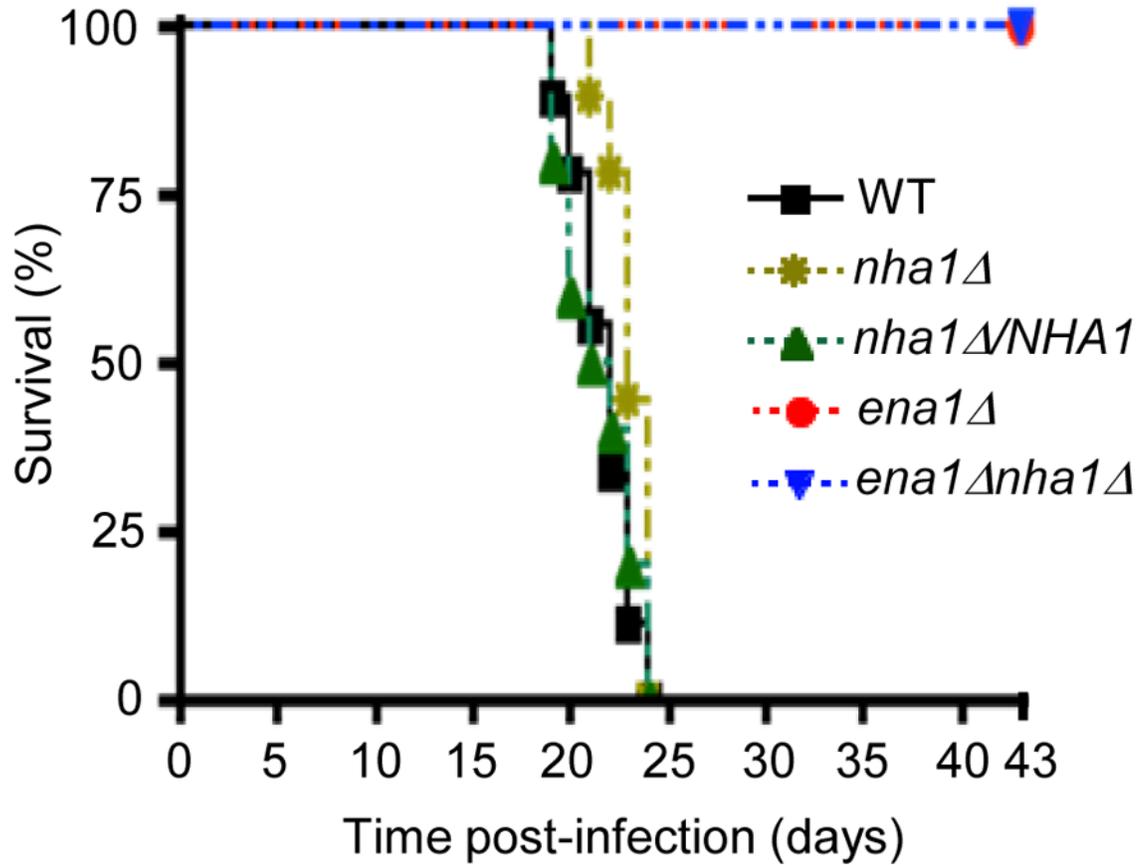
Fig. 10.
Nha1 is not required for virulence in *C. neoformans.* For virulence assays, seven week old A/J female mice were infected with 5×10^4 cells of WT, *ena1, ena1 nha1, nha1*, and *nha1/ NHA1* strains by intranasal inhalation. Survival (%) was monitored daily for 43 days after infection.
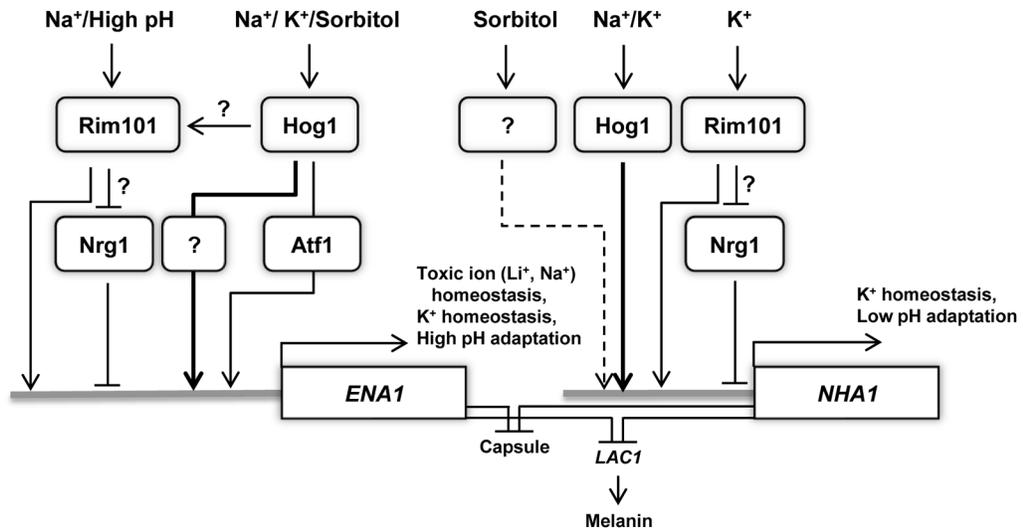
Fig. 11.
The proposed model for regulation and function of *ENA1* and *NHA1* in *C. neoformans.* Under cation shock and high pH, *ENA1* expression is controlled by diverse signaling proteins. *ENA1* is positively and negatively regulated by Rim101 and Nrg1 proteins, respectively. However, it is unclear whether Rim101 controls expression of *ENA1* through the Nrg1 repressor protein. Hog1 appears to positively regulate *ENA1* expression, partly through the Atf1 transcription factor. It is possible that Hog1 controls the Rim101 pathway directly or indirectly to regulate *ENA1* expression. Upon Na^+/K^+ shock, *NHA1* expression is induced in a Hog1-dependent manner. Under osmotic shock, such as high sorbitol addition, *NHA1* is induced in a Hog1-independent manner. Under K^+ shock, Rim101 positively regulated mRNA levels of *NHA1*. However, it remains unclear whether the Ena1-dependent regulation of *NHA1* is mediated via the Nrg1 repressor. In *C. neoformans,* Ena1 plays a major role in toxic ion (Li^+ and Na^+) and K^+ homeostasis as well as high pH adaptation whereas CnNha1 is involved in K^+ homeostasis and low pH adaptation. Furthermore, Ena1 and Nha1 play redundant roles in negatively regulating production of melanin and capsule.

Table 1

Strains used in this study

Strain	Genotype	Parent	Reference
C. neoformans			
H99	*MAT*		(Perfect et al., 1993)
KN99**a**	*MAT***a**		(Nielsen et al., 2003)
YSB64	*MA hog1 ::NAT-STM#177*	H99	(Bahn et al., 2005)
YSB586	*MA nha1 ::NEO*	H99	This study
YSB1161	*MA ena1 ::NAT-STM#58*	H99	This study
YSB1169	*MAT ena1 ::NAT-STM#58 nha1 ::NEO*	YSB586	This study
YSB1201	*MAT nha1 ::NEO NHA1-NAT*	YSB586	This study
YSB1171	*MAT***a***nha1 ::NAT*	KN99	This study
YSB1371	*MAT***a***ena1 ::NEO*	KN99	This study
YSB1374	*MAT***a***ena1 ::NEO nha1 ::NAT*	YSB1171	This study
YSB1548	*MAT nha1 ::NEO NHA1-NAT-GFP*	YSB586	This study
KN99	*MAT*		(Nielsen et al., 2003)
AI167	*MAT ena1 ::NAT*	FJW18 and KN99**a**	(Idnurm et al., 2009)
YSB590	*MAT ena1 ::NAT nha1 ::NEO*	AI167	This study
YSB1111	*MA nha1 ::NEO*	KN99	This study
YSB1283	*MA nha1 ::NEO NHA1-NAT*	YSB1111	This study
S. cerevisiae			
	*MAT***a***his3 1 leu2 0 met15 0 ura 0 NHA1-GFP::His3MX6*	BY4741	(Huh et al., 2003)

Each *NAT-STM#* indicates the Nat[r] marker with a unique signature tag.