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Snf1/AMPK protein kinase modulates cell wall integrity in the human pathogenic yeast *Cryptococcus neoformans*

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Abstract: [**Objective**] The Snf1/AMPK family of protein kinases is highly conserved among eukaryotes. Our previous study showed that *Cryptococcus neoformans SNF1* played critical roles in the production of virulence factors and virulence itself. In this paper, we report a novel function of *SNF1* in cell wall integrity. [**Methods**] We used Calcofluor white staining epifluorescence microscopy to evaluate the cell wall integrity and cell segregation; tap water with constant flow rate and pressure to wash yeast colonies to evaluate cell-to-agar adhesion capability; growth on Sodium dodecyl sulfate (SDS), Congo red and Fluorescent Brightener 28-containing agar to examine cell wall integrity. [**Results**] The disruption mutant of *SNF1* was sensitive to SDS and Congo red, suggesting impairments in the cell wall. The mutant cells showed abnormal separation, defects in adhesion to agar surface, and growth defects at high temperature which could be suppressed by osmotic stabilization. [**Conclusion**] *C. neoformans SNF1* was essential for cell wall integrity that was likely responsible for normal adhesion of the cells to agar and resistance to heat.

Keywords: Snf1/AMPK, cell wall integrity, Sodium dodecyl sulfate (SDS)
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The basidiomycetous yeast, *Cryptococcus neoformans* is a major pathogenic fungus and infects immunocompromised people via respiratory tract[1-3]. Clinic manifestation of the infection is the life-threatening meningoencephalitis if untreated and lifelong medication is required[4-8]. As an opportunistic pathogen, *C. neoformans* faces challenges from both the environment and the host defense system. Thus, maintenance of cell wall integrity is essential for survival in the variable conditions[9-10]. Besides, the cell wall of *C. neoformans* associates with two important virulence factors. Polysaccharide capsule, recognized as one major virulence factor, attaches to the outer portion of the cell wall and protects the organism from the attack of the host immune system[11]. The wall lacking α (1-3) glucan loses the attachment to the capsule fibers[12]. The high-molecular-weight pigment melanin is another cell wall-associated virulence factor which protects *C. neoformans* from damage by hypochlorite, permanganate and so on[3]. The only enzyme laccase that catalyzes substrate such as L-Dopa to form melanin

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is localized to the cell wall with covalent bonds[13].

Investigation on cell wall biosynthesis has received particular attention, given that it is an ideal target for antifungal drug development. By far, it is known that glucan and chitin are two essential components of *C. neoformans* cell wall[14-16]. *FKS1* encodes a subunit of glucan synthase, essential for the viability of *C. neoformans*[15]. The alpha glucan synthase gene, *AGS1* regulates the cell wall formation and cell division[17]. *KRE* family is involved in β-1, 6-glucan synthesis and *KRE5*, *KRE6*, *SKN1* are the three identified genes of this family. Mutant of *KRE5*, double mutant of *KRE6* and *SKN1* grew slowly with aberrant cell morphology, showed highly sensitivity to environmental stress and were avirulent in mouse model[18]. Chitin synthase *CHS3* and its regulator *CSR2* take part in the maintenance of cell wall and the cellular viability at elevated temperature as well[16]. Moreover, the conserved classic Pkc1-Mpk1 signaling pathway regulates cell wall integrity[19-20].

Previously, we identified a Snf1/AMPK protein kinase counterpart *SNF1* in *C. neoformans* serotype D strain JEC21 and demonstrated its critical roles in virulence factor biosynthesis, carbon source utilization and cation stress tolerance[21]. Here, we report a further finding that Snf1 protein kinase is a key regulator of cell wall integrity in *C. neoformans*. The disruption mutant *snf1Δ* showed a high sensitivity to cell wall disturbing agent SDS and exhibited an aberrant cell separation and a growth defect at elevated temperature which was suppressed by osmotic equilibrium. The mutant *snf1Δ* displayed loss of cell-to-agar adhesion. These results suggest a possible interaction between Snf1 and other signaling pathways in cell wall maintenance in this fungus.

1 MATERIALS AND METHODS

1.1 Strains and Media

C. neoformans JEC21, a serotype D strain, was used as the wild type. *snf1Δ* and *snf1Δ*-C were a *SNF1*-disrupted mutant and its complemented strain, respectively[21]. The yeast was normally cultured on YPD agar (1% yeast extract, 2% peptone, 2% glucose and 2% agar, pH 6.0) at 30℃; while the cells for adhesion assay were grown on 0.1% Asn agar (0.1% Asparagine, 0.3% KH_2PO_4, 0.1% glucose and 2% agar, pH 6.0) at 30℃ as well.

1.2 Detection of cell wall defects in *snf1Δ* with inhibitors

Cells were cultured on YPD agar at 30℃ for 2 days, then washed by sterile water twice and resuspended in double distilled water (ddH_2O) again. Cell concentration was determined by hemocytometer. Cells were spotted onto YPD agar in serial dilution as indicated in the figure legends. Following the protocols by Baker[12], YPD agar was supplemented with inhibitors, Sodium dodecyl sulfate (SDS) (BeiJing DingGuo Biotech. Co. Ltd), Congo red (BeiJing DingGuo Biotech. Co. Ltd) and Fluorescent Brightener 28 (FB28) (Sigma, St. Louis, USA) separately at the indicated concentration, at 30℃ for 1 – 4 days according to specific condition. To examine chitin biosynthesis, aliquots of the cells were stained with Calcofluor white (CFW) (Sigma, St. Louis, USA) as described by the protocol of Pringle[22]. Cells were examined with a Nikon Eclipse 80i fluorescence microscope using a 4, 6-diamidino-2-phenyl indole filter.

1.3 Growth defect of *snf1Δ* at elevated temperature and restoration by osmotic stabilizer

To observe growth at elevated temperature, fresh cells were counted and dropped to YPD agar in serial dilution at 39℃, for 2 days. To check whether an osmotic stabilizer could restore the growth, YPD agar was supplemented with 1 mol/L sorbitol; cells were incubated at 30℃ as control. For further confirmation of the growth restoration by osmotic stabilization, ratio of survival was determined. Briefly, 10^7 yeast cells were incubated in 500 μl ddH_2O which stands for low osmotic condition or ddH_2O with 1 mol/L sorbitol at 39℃, for 2 days, meanwhile took the cultivation at 30℃ under the same conditions as control. Then 10^2 cells were spread onto YPD agar to incubate for 2 more days at 30℃. The number of colonies was counted for

each plate. The survival ratio of JEC21, the deletion strain and the complemented strain were calculated. Survival was expressed as the ratio of colony numbers of the indicated strain from the original cultivation at 39℃ to counted colony numbers of corresponding strain from the control group. The assay was carried out in triplicate and error was expressed as the standard deviation.

1.4 Adhesion assay for *snf1Δ*

The adhesion of yeast cells to agar was assayed by following the method of Gulda[23]. Briefly, JEC21, *snf1Δ* and the complemented strain *snf1Δ*-C were cultured on YPD agar at 30℃ for 2 days. Cells were scraped from the agars, washed by sterile water twice and resuspended in TE buffer described by Vyas[24] (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5). 4×10^6 cells were collected and dropped onto 0.1% Asn plate and cultured at 30℃. The plates were photographed on day 4 before washing. Then the cells were washed with constant water from the faucet water as described by Guldal for about 20 seconds or longer without damaging the agar. The plates were dried in the hood with sterile air and pictured.

2 RESULTS

2.1 Sensitivity of *snf1Δ* to cell wall inhibitors and aberrant cell morphology of the mutant

To find out the consequence of the disruption of *SNF1* in the cell wall integrity, we adopted the conventional assay to test the sensitivity of the mutant to three cell wall inhibitors, *i.e.* SDS, Congo red and Fluorescent Brightener 28. SDS has been used extensively to detect any cell wall impairments in the wall. Congo red is regarded to inhibit microfibril assembly of the cell wall and CFW could disrupt the assembly of chitin[12]. *snf1Δ* displayed extreme sensitivity to SDS. The mutant *snf1Δ* could not grow on 0.03% SDS plate, whereas the wild type grew normally at this concentration of SDS. The complemented strain restored the growth of the mutant, verifying that the gene *SNF1* is essential for cell wall formation (Fig. 1A). In the meanwhile, 0.5% Congo red showed significant toxicity to *snf1Δ* whereas Fluorescent Brightener 28 exhibited little effect on the growth of the mutant (Fig. 1A). Sensitivity of *snf1Δ* to SDS and Congo red suggests a disruption of cell wall integrity.

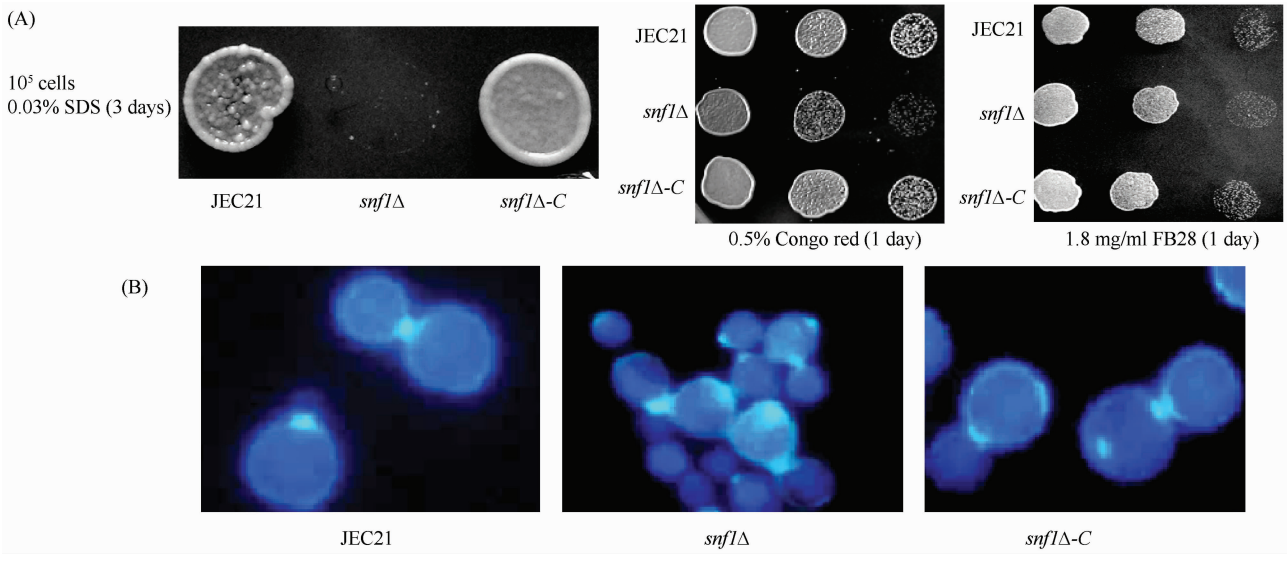

Fig. 1 *snf1Δ* showed sensitivity to cell wall inhibitors. A: Equal concentration of fresh cells were spotted onto YPD agars respectively supplemented with 0.03% SDS, 0.5% Congo red and 1.8 mg/ml Fluorescent Brightener 28 (FB28), cultured at 30℃ for 1 – 4 days. B: Calcofluor white-staining of indicated *C. neoformans* strains. The mutant showed an aggregation phenotype and an abnormal chitin distribution pattern. All photos were at the same magnification (×1,000).

CFW staining fluorescence microscopy revealed that *snf1Δ* cells from SDS agar exhibited aberrant cell morphology and tended to aggregate together (Fig. 1B). Under the microscope, the mutant cells remained linked at the budding sites, indicating an incomplete separation of the mother/daughter cells. The distribution pattern of chitin stained by CFW revealed an alteration in the mutant. Taking a care look, we noticed that chitin in the wild type and the complemented cells were located largely on the cell wall forming a brighter ring around the cells, and also at the budding sites. In contrast, most of chitin fluorescence seemed to be localized on the budding scars and the mother/daughter cell conjunctions in the mutant. The fluorescence microscopy supports that cell wall has suffered impairments in *snf1Δ*.

2.2 Osmotically remediable growth defect of *snf1Δ* at high temperature

Previous studies have demonstrated that cell wall defects result in sensitivity to heat shock and high temperature in fungi[19-20, 25], which may be rescued by the addition of osmotic stabilizer into the media[25]. We indeed observed that growth defect of *snf1Δ* at 39℃ is restorable by the addition of 1 mol/L sorbitol (Fig. 2). *snf1Δ* displayed a similar growth rate to that of the wild type and the complement in sorbitol at 39℃. The cells grown at 30℃ showed no difference for both the mutant and the wild type. Further, viability of *snf1Δ* was examined from the same conditions to reassure that osmotic stabilization rescued the growth defect of *snf1Δ*. The cells were incubated in ddH_2O or sorbitol-containing ddH_2O at 39℃ for 2 days. The viability was described in section 1.3. Cells were grown at 30℃ to allow colonies to form. The survival ratio of each strain was shown in Fig. 2, right panel. In a precise concomitance, *snf1Δ* displayed a severe sensitivity to heat incubation and 1 mol/L sorbitol protected the cells from heat killing. Putting together, *SNF1* modulates the cell wall integrity in our yeast.

2.3 Loss of cell-to-agar adhesion in *snf1Δ*

Change of cell wall structure or components may affect the ability of yeast adhesion to substrate[26]. Thus, we examined the capability of *snf1Δ* to attach to agar surface. Fresh cells were spotted on Asn agar with 0.1% glucose as the sole carbon source which favors

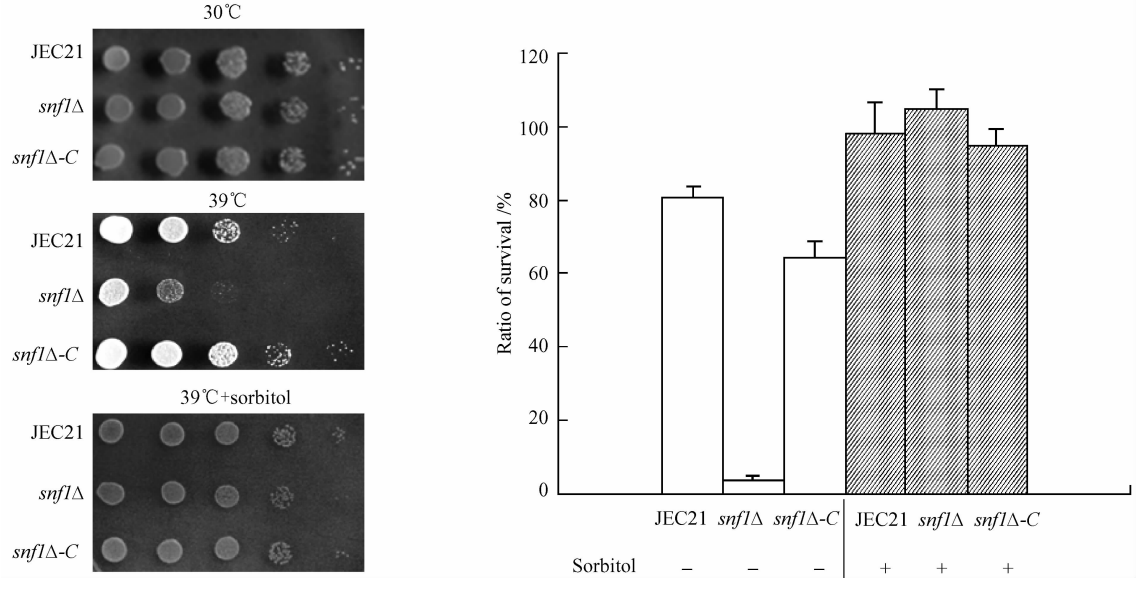

Fig. 2 *snf1Δ* displayed an osmotically remediable growth defect at 39℃. Left panel: Cells were grown on YPD agar alone or supplemented with 1 mol/L sorbitol at 39℃ for 2 days. The cells grown at 30℃ were set for control. Right panel: 10^7 cells of indicated strains were incubated in 500 μl ddH_2O and ddH_2O with 1 mol/L sorbitol at 39℃ for 2 days. 10^2 cells were spread onto YPD media and cultured as described. The survival ratio was determined for each strain as the description in the Materials and Methods.

cell-to-surface adhesion[27]. After incubation at 30℃ for 4 days, the plates were subject to a wash of tap water with consistent flow for 20 s or longer. As shown in Fig. 3, after wash, cells (drops in triplicate) of *snf1Δ* stripped off the agar surface, reflecting an attenuated adhesion (the second row on right panel), by comparison to the wild type JEC21. And the complemented strain restored a similar adhesion level to the wild type. We obtained a consistent result by extending the washing time to 40 seconds (data not shown). This result confirms a crucial role of *SNF1* in adhesion of the yeast cells to substrate, which may be a result of the defects in the cell wall.

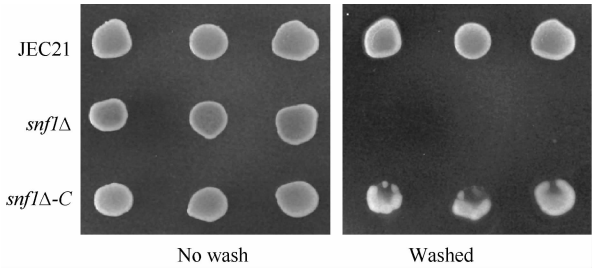

Fig. 3 *SNF1* is required for cell-to-agar adhesion in *C. neoformans*. Equal numbers of cells in triplicate were spotted onto Asn (pH 6.0) agar cultured at 30℃ for 4 days. Adhesion assay was carried out as the description in the Materials and Methods. *snf1Δ* showed a defect in cell-to-agar adhesion on Asn agar compared to JEC21 and the complemented strain (second row from top on right panel).

3 DISCUSSION

Here we described a novel function of *SNF1*, *i.e.*, its modulating the cell wall integrity in *C. neoformans*. This is revealed by the observation that the disruption mutant *snf1Δ* was sensitive to SDS, Congo red. The phenotype of cell wall defects was also evidenced by the fact that *snf1Δ* was unable to grow at high temperature and sensitive to heat shock (Fig. 2). This growth delay was restored by the addition of 1 mol/L sorbitol to the media, further suggested that cell wall defects were caused by the disruption of *SNF1*. Fungal cells frequently faces extreme conditions in the environment and cell wall is the essential barrier in protecting cells from harmful effect caused by the extremes[9-10, 14]. In *C. neoformans*, several conventional pathways have been identified for the control of cell wall integrity, for example, the Pkc1, Mpk MAP kinase and Ras signal transduction pathways[19-20, 28]. More than ten genes involved in these pathways have already been analyzed. Our work suggests that cross talks between Snf1 protein kinase pathway and the others may coordinate in modulating cell wall biogenesis and integrity maintenance.

We also found that the *SNF1* mutant displayed impaired cell-to-agar adhesion ability compared to the control strains. Adhesion plays an important part in mediating colonial interactions as well as host-pathogen interactions[29]. In many cases it is required for biofilm formation in certain fungi. The reduced biofilm formation in *C. neoformans* results in the failures to survive the hostile environment and the resistance to other microbes[27]. Former studies have shown that cell wall glycoproteins were involved in the process of *Sporothrix schenckii* adhesion to epithelial cells, and previous study also demonstrated that *SNF1* was required for the expression of cell wall relating enzymes in two Maize pathogens *Cochliobolus carbonum* and *Ustilago maydis*[30-31]. Putting together with what we have observed, the cell wall impairment in *snf1Δ* may cause the change of cellular surface components such as glycoprotein that results in the loss of adhesion capability. Unfortunately, the mechanism of the regulation on adhesion ability was still unknown in *C. neoformans*. Thus, the finding of *SNF1* in cellular adhesion may help to open a new avenue to this issue.

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人类致病菌新型隐球酵母 Snf1/AMPK 蛋白激酶调节细胞壁的完整性

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摘要:【目的】Snf1/AMPK 在真核生物中是重要的且高度保守的一类蛋白激酶。在新型隐球酵母中,*SNF1* 基因在调节致病因子的生物合成和细胞毒力方面具有重要作用。本文进一步报道了该基因在维持细胞壁完整方面的新功能,这一功能在其他微生物中未见报道。【方法】利用荧光增白剂染料(Calcofluor white dye)染色,荧光显微观察细胞分离、胞壁完整性;利用恒定流速和压力水流冲击菌落,测定细胞黏附琼脂表面能力;在含有十二烷基硫酸钠(Sodium dodecyl sulfate, SDS),刚果红(Congo red)染料和增白剂(Fluorescent Brightener 28)的培养基上观察突变株的生长情况,以验证细胞壁完整性。【结果】*SNF1* 基因突变菌株对细胞壁抑制剂 SDS 等敏感,表明细胞壁完整性的损坏;在葡萄糖固体培养基上表现为细胞与琼脂间的黏附力丧失;在热击压力下,该菌株不能正常生长,而这种生长缺陷能够被渗透平衡抑制。【结论】新型隐球酵母 *SNF1* 基因对于维持细胞壁完整性是非常重要的,并且影响细胞与琼脂间黏附作用以及细胞对抗热的能力。

关键词: snf1/AMPK, 细胞壁完整性, 十二烷基硫酸钠(Sodium dodecyl sulfate, SDS)

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