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Research Article

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Regulation of *ACT* expression by *VPS41* in response to nitrogen starvation in *Cryptococcus neoformans*

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ABSTRACT

Cryptococcus neoformans is an important opportunistic human fungal pathogen infecting immunocompromised patients and commonly causes life-threatening cryptococcal meningitis. Previous studies have shown that the VPS41 gene plays an essential role in the starvation response, macrophage survival and pathogenicity of C. neoformans. Here we present evidence that expression of an acetyltransferase encoding gene (ACT) under nitrogen starvation conditions was stimulated by VPS41. The transcript level of ACT in the VPS41 deletion mutant vps41Δ was decreased 2.5-fold compared with that in the wild type H99, while reintroduction of the functional VPS41 gene into the vps41Δ cells drastically increased ACT expression. Moreover, constitutive expression of ACT in mutant vps41Δ resulted in greatly improved survival of recombinant strain vps41Δ::ACT under nitrogen deprivation conditions. The above observations strongly suggest that one function of VPS41 in the starvation signaling pathway is to promote ACT expression in response to nitrogen starvation. Better Understanding of the mechanism of the VPS41-mediated starvation response pathway could reveal novel drug targets for designing effective therapy for treating C. neoformans infection.

Key words: *Cryptococcus neoformans*, *VPS41*, *ACT*, qRT-PCR, nitrogen starvation

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INTRODUCTION

Cryptococcus neoformans is a major human fungal pathogen, mainly infecting patients with severe cell immunodeficiency such as AIDS patients and patients receving long-term treatment of large doses of steroid drugs. Cryptococcal yeast is a clinically leading cause of life-threatening fungal meningitis [1-4]. Major established virulence factors of *C. neoformans* include production of laccase [5,6], capsule formation [7], ability to grow at 37℃[8], ability to acquire very low concentrations of iron [9,10] and survival under deprivation of various nutrients such as nitrogen [11,12]. *C. neoformans* is a facultative intracellular pathogen capable of replication inside macrophages. Cells can survive for years as a latent form in the macrophage of immunocompromised patients and eventually proliferate and spread to the neverous systems and cause fatal menagitis if untreated [13,14].

Previous studies have shown that *VPS41* gene plays an essential role in the survival of *C. neoformans* cells in response to nitrogen deprivation since that the *VPS41* knockout mutant showed a severe defect in survival under nitrogen starvation [11], suggesting a crucial role of *VPS41* of cryptococcal cells inveloved in responding appropriately to starvation signals for optimum survival. In an attempt to identify relevant genes in the *VPS41* signaling pathway, digital gene expression (DGE) sequencing was performed to identify differentially expressed genes in the wide type strain H99 and deletion mutant *VPS41Δ* under nitrogen starvation. One of the down-regulated gene revealed is an acetyltransferase encoding gene (*ACT*), which is predicted to encode a member of acetyltransferase (GNAT) superfamily family. The present work investigated: 1) whether *VPS41* promotes *ACT* expression when *C. neoformans* is subject to nitrogen starvation; 2) whether increaing *ACT* expression in mutant *vps41Δ* cells can

suppress defects in cell survival under nitrogen starvation conditions.

EXPERIMENTAL SECTION

Strains, media, and growth conditions

C. neoformans var. *grubii* serotype A H99 was used as a wild-type strain. Mutant *vps41Δ* was used as a starting strain for generating recombinant strain *vps41Δ*::*ACT*. The strains were routinely grown in yeast extract-peptone-dextrose (YPD) medium. Yeast nitrogen base (YNB) with 2% glucose was used for the nitrogen starvation experiments.

Construction of recombinant strain vps41Δ::ACT

The actin promoter was PCR-amplified using plasmid pPZP-HYG2 as a template with the primer pair F-pACT: 5'-TCTAGACGACGCTCCTACACTCGACCT-3'(Xba I site is underlined); R-pACT : 5'-GGATCCTCTGTACAACCCGCTCAAATG-3' (BamH I site is underlined). The PCR product was cloned into T vector and then released with XbaI/BamHI digestion and inserted into the same sites of plasmid pBluescript to create the plasmid pBPact-L. To achieve constitutive expression of *ACT* in mutant *vps41Δ*, the entire open reading frame (ORF) of *ACT* was PCR-amplified from the genomic DNA of H99 using the primer pair ACT-F : 5'-GGATCCCGTTGCTGCCTGTCCTTCTTCCTTT-3' (the added BamH I site is underlined) and ACT-R : 5'-CTCGAGTAGTGTTGTGCTGCCCCTGCTTTA -3' (the added Xho I site is underlined). The *ACT* ORF was inserted into the BamHI/XhoI sites of plasmid pBPact-L to generate the expression vector pACT-L1.

Nitrogen starvation response assay

Cells of overnight cultures in YPD liquid medium of H99 wild-type, *vps41Δ* and strain *vps41Δ*::*ACT* were collected and resuspended, respectively, to a concentration of concentration of 10^8 cells/ml in 10 ml of YNB (without amino acids and ammonium sulphate) supplemented with 1.5% glucose in a 50 ml falcon tube and then cultivated at 37℃ with shaking at 200 rpm. Samples were taken at 24 h, 48 h for determining cell viability based on cfu counting on YPD agar using 10-fold serial dilutions.

RNA isolation and real-time quantitative RT-PCR

Total RNAs were isolated using Trizol reagents (Promega, USA) and reverse transcription of the first cDNA strand was performed using 2 ug of total RNA with PrimeScript Reverse Transcriptase (TaKaRa, Dalian, PR China) in a 20 ul reaction volume according to the manufacturer's instructions. A 1-ul aliquot of cDNA was used as the template for real-time quantitative PCR of *ACT* gene on 7900 system (Applied Biosystems Inc, USA) with the primer set:

ACT-F: 5'-CTTTTCAACAAGCACCATCTGC-3' (forward),

ACT-R: 5'-TCATTCTCGGTTTGTAGGTCGTCG-3' (reverse).

The amplifications were conducted in a total volume of 20 ul, containing 1ul of SYBR green (CWBIO, Beijing, PR China), 300 nmol/liter of both primers, and 1 ul of cDNA. The amplification was conducted as follows: 10 min at 95°C, followed by 35 cycles consisting of 15 s at 95°C, 30 s at 60°C. The transcript level of actin gene was used as an internal control using the following primer pair: Actin-F: 5'-CTCTTACGTCGGTGACGAAG-3';

Actin-R: 5'-GTCATCTTCTCTCGGTTCTGC-3'.

All the qRT-PCR texperiemnts were conducted in triplicate.

RESULTS AND DISCUSSION

Decreased ACT expression in mutant vps41Δ

The *VPS41* gene knockout mutant *vps41Δ* showed a severe defect in the survival of *C. neoformans* under nitrogen starvation [11]. Initialy the down-regulation of *ACT* gene expression at the transcription level was revealed by DGE sequencing during the investigation of genes that are differentially expressed in the mutant *vps41Δ* compared with those in the wild type H99. *ACT* is predicted to encode a member of acetyltransferase (GNAT) superfamily family. To verify the effect of *VPS41* deletion on the level of *ACT* expression, quantitative RT-PCR (qRT-PCR) was perfomed. It was found that *ACT* expression levels in mutant *vps41Δ* cells was decreased by 2.5-fold compared with that in the H99 under nitrogen starvation for 6 h (Fig. 1), suggesting a potential role of eenhanced *ACT* expression in *VPS41* mediated starvation signaling.

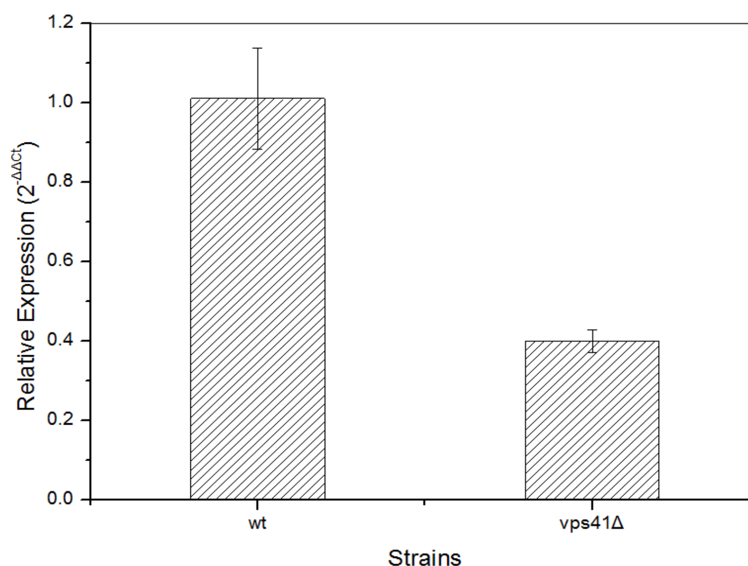

Fig. 1. Effects of *VPS41* function on the transcript level of *ACT*

The cultures of wild type H99 and mutant *vps41Δ* were subject to 6 h nitrogen starvation in liquid medium of YNB (without amino acids and ammonium sulphate) supplemented with 1.5% glucose at at 37℃ with shaking (200 rpm). The transcript levels of *ACT* in both strains were measured by qRT-PCR using the actin gene transcript as an internal control. Strains wt: H99, *vps41Δ*: *VPS41* knowckout mutant.

Dramatically increased ACT expression in complementated strain vps41Δ::VPS41

To further probe the promoting effects of functional V*PS41* gene on *ACT* expression in *C. neoformans* under nitrogen starvation, we tested whether reintroduction of a functional *VPS41* can enhance the *ACT* expreesion under nitrogenstarvation. qRT-PCR was employed to measure the levels of *ACT* expression after 6 h nitrogen starvation in a complementated strain *vps41Δ*::*VPS41*. As shown in Fig. 2, The levels of *ACT* expression in the complemented strain *vps41Δ*::*VPS41* exhibited about 58% increase compared to that in mutant *vps41Δ*. This observation further reenforced the idea that the function of *VPS41* enhances the transcription of *ACT* under nitrogen starvation.

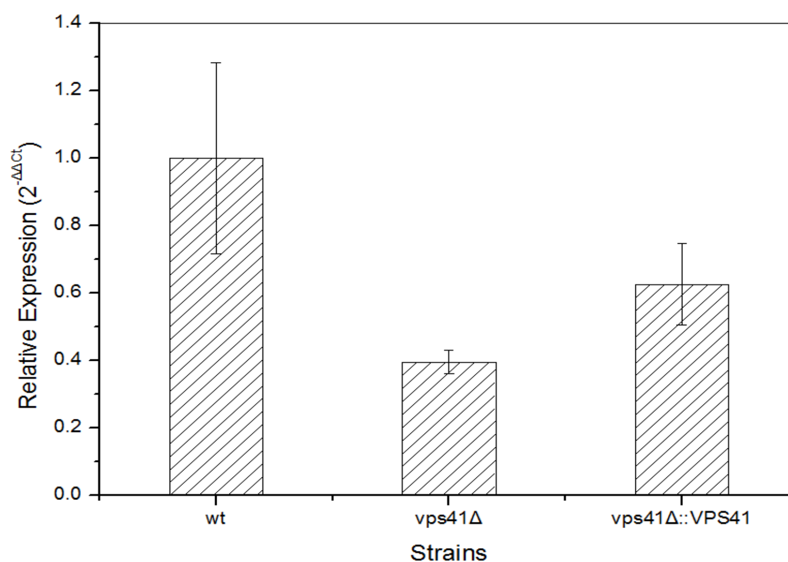

Fig. 2. Effects of reintroduction of *VPS41* on the expression of *ACT*

The cultures of H99, mutant *vps41Δ* and *ACT*: *VPS41* strains were subject to 6 h nitrogen starvation in liquid medium of YNB supplemented with 1.5% glucose at at 37℃ with shaking (200 rpm). The transcript levels of *ACT* in each strain were measured by qRT-PCR using the actin gene transcript as an internal control. Strains wt: H99, *vps41Δ*: *VPS41* knowckout mutant, *vps41Δ::ACT*: *VPS41* complement

Suppression of nitrogen starvation defects by constitutive expression of ACT in vps41Δ
Given that the essential role of the *VPS41* gene in the starvation response and virulence of C. neoformans [11] and the fact that *VPS41* was shown to have a positive effects on *ACT* transcription under nitrogen starvation. The question to ask is whether up-regulation of *ACT* transcription in response to nitrogen starvation has a functional role in the survival of *C. neoformans* cells under nutrient limitations. To examine this possibility, we constructed an *ACT* expression vector pACT-L1 in an attempt to restore *ACT* expression in the *vps41Δ* genetic background to levels comparable to that in H99. The *ACT* expression is under the control of the constitutive actin promoter (pACT) derived from *C. neoformans*. Hygromycin resistance was used as a selection marker. The pACT-L1 was introduced into mutant *vps41Δ* by electroporation to generate the recombinant strain *vps41Δ::ACT*. As shown in Fig. 3, The restoration of *ACT* expression in *vps41Δ* cells resulted in dramatic suppression of starvation defects in strain *vps41Δ*.

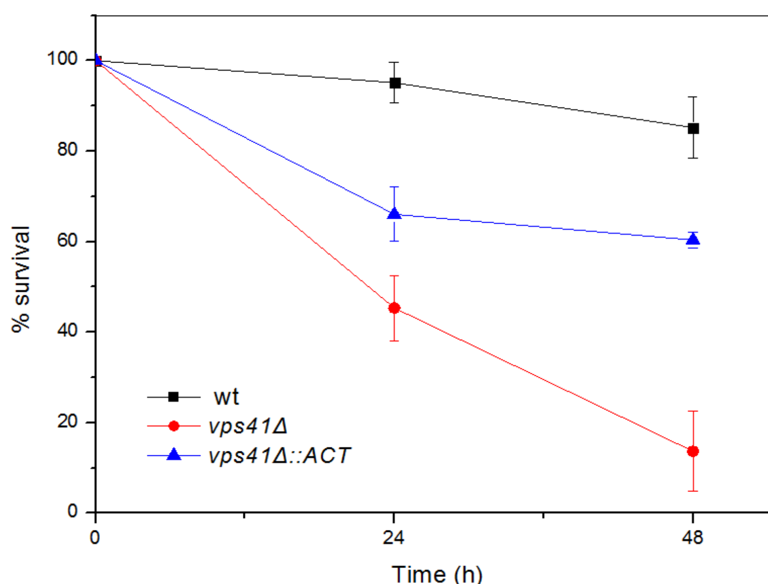

Fig. 3. Effects of constitutive *ACT* expression on survial of mutant *vps41Δ* cells

The cultures of H99, mutant *vps41Δ* and *vps41Δ::ACT* strains were subject to 24 h and 48 h nitrogen starvation in liquid medium of YNB supplemented with 1.5% glucose at at 37℃ with shaking (200 rpm). Strains wt: H99, *vps41Δ*: *VPS41* knockout, *vps41Δ::ACT*: constitutive expression *ACT* in mutant *vps41Δ*. Samples were taken at 24 h, 48 h for determining cell viability based on cfu counting on YPD agar using 10-fold serial dilutions.

CONCLUSION

The regulatory role of *VPS41* on *ACT gene* expression under nitrogen stravation in *C. neoformans* was demonstrated. Using the qRT-PCR technique, we showed that *VPS41* function is required for the enhanced transcription of *ACT* of *C. neoformans* in response to nitrogen starvation, which is essential for the optimum survival of this important human pathogen in the nutrient deprivation conditions given that engineered increases of *ACT* expression in mutant *vps41Δ* lead to their dramatically improved survival, suggesting that enhanced expression of *ACT* is an important component of the underlying mechanism of *VPS41* in response to nitrogen starvation in the environment.

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