A Nudix Hydrolase Protein, Ysa1, Regulates Oxidative Stress Response and Antifungal Drug Susceptibility in Cryptococcus neoformans

Kyung-Taemy Lee, Hyojeong Kwon, Dohyun Lee and Yong-Sun Bahn

1Department of Biotechnology, Center for Fungal Pathogenesis, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Korea
2Nutrex Technology Co., Ltd., Seongnam 463-400, Korea

Abstract A nucleoside diphosphate-linked moiety X (Nudix) hydrolase-like gene, YSA1, has been identified as one of the gromwell plant extract-responsive genes in Cryptococcus neoformans. Ysa1 is known to control intracellular concentrations of ADP-ribose or O-acetyl-ADP-ribose, and has diverse biological functions, including the response to oxidative stress in the ascomycete yeast, Saccharomyces cerevisiae. In this study, we characterized the role of YSA1 in the stress response and adaptation of the basidiomycete yeast, C. neoformans. We constructed three independent deletion mutants for YSA1, and analyzed their mutant phenotypes. We found that ysa1 mutants did not show increased sensitivity to reactive oxygen species-producing oxidative damage agents, such as hydrogen peroxide and menadione, but exhibited increased sensitivity to diamide, which is a thiol-specific oxidant. Ysa1 was dispensable for the response to most environmental stresses, such as genotoxic, osmotic, and endoplasmic reticulum stress. In conclusion, modulation of YSA1 may regulate the cellular response and adaptation of C. neoformans to certain oxidative stresses and contribute to the evolution of antifungal drug resistance.

Keywords Cryptococcus neoformans, Nudix hydrolase, Oxidative stress, Ysa1

The nucleoside diphosphate-linked moiety X (Nudix, NDP-X) hydrolase superfamily is ubiquitous in all organisms ranging from bacteria to mammals, and catalyzes degradation of NDP-X to NMP plus P-X [1]. In bacteria and fungi, but not in mammals, the number of Nudix hydrolases is generally proportional to genome size [1]. In the budding yeast model organism, Saccharomyces cerevisiae, six Nudix hydrolases have been discovered and partially characterized [1]: Npy1p is an NAD(P)H/NAD(P)⁺ hydrolase localized in peroxisomes [2, 3]; Pcd1p is a CoA hydrolase that is also localized in peroxisomes [4]; Dcp1p is a diadenosine hexaphosphate hydrolase, and belongs to the diphosphoinositol polyphosphate phosphohydrolase family [5, 6]; Dcp2p is a Nudix hydrolase, which is the catalytic subunit of the Dcp1-Dcp2 mRNA decapping enzyme complex [7]; Ysa1p is an NDP-sugar hydrolase [8]; and YJR142W is an uncharacterized Nudix hydrolase.

Among the S. cerevisiae Nudix hydrolases, Ysa1p catalyzes the enzymatic degradation of ADP-ribose (ADPr) or O-acetyl-ADP-ribose (OAADPr) to AMP and acetylated phosphoribose, and belongs to the diphosphoinositol polyphosphate phosphohydrolase family [5, 6].
result in enhanced cellular resistance to oxidative stress (e.g., H₂O₂) and ROS-generating copper ions [9].

In a previous study, we performed a global transcriptome analysis of eukaryotic genes affected by gromwell extract by using *Cryptococcus neoformans* as a eukaryotic model system [12]. Gromwell is a perennial herb, which belongs to the family Boraginaceae, and is known to have a plethora of pharmacological, cosmetic, and nutritional properties [12]. Interestingly, a gene (CNAG_02986) that is highly orthologous to the *S. cerevisiae* YSA1 gene was found to be downregulated by gromwell extract treatment [12]. Based on this finding, we hypothesized that gromwell extract could potentially decrease expression levels of YSA1, which may contribute to increased intracellular levels of ADPr and OAADPr, and subsequently help cells to resist oxidative stress and efficiently counteract ROS. However, it remains unclear whether Ysa1 plays similar biological roles in other eukaryotes such as *C. neoformans*.

In this study, we characterized the functions of Ysa1 through construction of targeted gene deletion mutants by homologous recombination in *C. neoformans* and analyzed their phenotypes, providing further insight into the Nudix hydrolase-dependent stress defense mechanism in eukaryotic cells.

**MATERIALS AND METHODS**

**Strains and media.** *C. neoformans* strains listed in Table 1 [13] were cultured and maintained in yeast extract-peptone-dextrose (YPD) medium or yeast extract-peptone

| Name   | Genotype   | Parent   | Reference |
|--------|------------|----------|-----------|
| H99    | MATα       | H99      | [32]      |
| YSB2544| MATα ysa1∆::NAT-STM#177 | H99 | This study |
| YSB2545| MATα ysa1∆::NAT-STM#177 | H99 | This study |
| YSB2546| MATα ysa1∆::NAT-STM#177 | H99 | This study |

**Table 1. Strains used in this study**
was also amplified from a plasmid that contains the B5570, respectively (Fig. 1C). A dominant selection marker were amplified with primer pairs B5567/B5568, and B5569/ B5570, respectively (Fig. 1C). A dominant selection marker was also amplified from a plasmid that contains the NAT gene with the primer pair B1026/B1027. In the second round of PCR, the 3' or 5'-flanking regions of YSA1 were amplified with DLP-PCR with primer pairs B5567/B1455 and B1454/B5570, respectively, by combining the first-round PCR products (Fig. 1C). These split cassettes were then introduced into the wild-type H99 strain by biolistic transformation. Stable transformants selected on YPD medium containing 100 µg/mL nourseothricin were screened using diagnostic PCR with the primer pair B5566/B79. Finally, the correct genotypes of positive YSA1::NAT disruption cassettes with the 5' or 3' NAT-split markers were amplified by DJ-PCR with primer pairs B5567/B1455 and B1454/B5570, respectively, of stress-inducers and antifungal drugs. Stress-inducers included oxidizing agents (hydrogen peroxide, tert-butyl hydroperoxide, menadione, and diamine), a reducing reagent (dithiothreitol [DTT]), an endoplasmic reticulum (ER) stress-inducing agent (tunicamycin [TM]), a cell membrane destabilizer (sodium dodecyl sulfate), cell wall stress agents (calcofluor white and Congo red), genotoxic agents (hydroxyurea [HU], methyl methanesulfonate [MMS]), osmotic shock agents (NaCl and KCl), and heavy metal stress agents (CdSO₄ and CuSO₄). The antifungal drugs tested included a polyene drug (amphotericin B) andazole drugs (fluconazole, ketoconazole, and itraconazole). To measure cellular sensitivity to ultraviolet (UV) radiation, cells were spotted onto YPD medium and exposed to UV (300 j/m²) using a UV cross-linker (model CK-2000; UVP, Upland, CA, USA). The plates were then further incubated for 2-4 days at 30°C and photographed.

RESULTS

Discovery and disruption of a Nudix hydrolase gene, YSA1, in C. neoformans. The structural features of CNAG_02986, which was previously found to be a gromwell extract-responsive gene [12], were analyzed to confirm that CNAG_02986 was indeed a Ysa1 ortholog in C. neoformans. The annotated C. neoformans var. grubii H99 strain genome appeared to have 11 Nudix domain-containing proteins (CNAG_01864, 02986, 04731, 04732, 04852, 01900, 06903, 03396, 00265, and 00076). Six of these were homologous to Nudix hydrolase family proteins in S. cerevisiae (Fig. 1A). However, a BLAST search of an S. cerevisiae Ysa1 ortholog in the C. neoformans genome database (http://www.yeastgenome.org) generated a single hit: CNAG_02986 (score of 130.183 and e-value of 3.36E-31). The expected protein size of CNAG-02986 (215 amino acids [aa]) was comparable to that of S. cerevisiae Ysa1 (231 aa). A reverse BLAST search of the CNAG_02986 ortholog in the S. cerevisiae genome database (http://www.yeastgenome.org) also revealed Ysa1 as its closest hit. Furthermore, based on protein domain analysis conducted using Pfam (http://www.pfam.janelia.org), CNAG_02986 contains a typical Nudix domain (PF00293), which is widely found in a protein family of phosphohydrolases, with the Nudix motif (GxxxxxExxxxxxREUXEExGU, where x is any amino acid) (Fig. 1B). Therefore we named CNAG_02986 YSA1.

To characterize the functions of YSA1, we performed a gene knockout study. To this end, we constructed ysa1Δ mutants in the C. neoformans H99 strain genetic background by employing DJ-PCR with NAT-split markers and biolistic transformation (Fig. 1C) as previously reported [14, 15]. Positive ysa1Δ mutants were initially screened using diagnostic

| Name | Sequence | Description |
|------|----------|-------------|
| B79  | TGTGGATGCTGCGGAGGATA | Screening primer |
| B1026| GTAAAAACGAGGCCAGTGGAG  | M13 forward primer |
| B1027| CAGGAAAACGACATCAGCACATG | M13 reverse primer |
| B1454| AAGGGTGTCCCAGACGAGAACACG | NAT' split marker primer 1 |
| B1455| AACCTCGTGCCAGGCCCCCATCACA | NAT split marker primer 2 |
| B5567| TGTTGCTTCTTCGGTCGTCG | Primer for the 5' flanking region of YSA1 |
| B5568| TCACTGGGCGGCTGCTTTACAGGTTCCTCGGG | Primer for the 5' flanking region of YSA1 |
| B5569| CATGGTCAAGCTGCTTTTCCTGACTAGTGCCAGGAGGTCTTTTG | Primer for the 3' flanking region of YSA1 |
| B5570| TGAACGAGTGTATTGTCGTCG | Primer for the 3' flanking region of YSA1 |
| B5566| TGGCTTGTTCATAGGTTG | Screening primer of YSA1 |
| B5555| ATGACGCGTGAAGCCAGCAG | Primer for the YSA1 probe (with B5567) |
The Functions of Ysa1 in \textit{C. neoformans}

PCR (data not shown), and the correct genotype was confirmed by Southern hybridization (Fig. 1D). To confirm Ysa1-dependent phenotypic traits, and exclude phenotypes caused by unwanted mutation or genome alteration during the transformation and gene disruption process, we generated three independent \( ysa1 \Delta \) mutants (YSB2544, YSB2545, and YSB546). All of these mutants grew as well as the wild-type strain at different temperature ranges (from 25°C to 39°C), suggesting that Ysa1 is dispensable for the growth of \textit{C. neoformans} (data not shown).

\textbf{Ysa1 was dispensable in \textit{C. neoformans} for stress response to ROS-generating oxidizing agents, but promoted cellular resistance to diamide, a thiol-specific oxidant.} The well-known function of Ysa1 in \textit{S. cerevisiae} is its ability to control intracellular ROS levels. Accordingly, \textit{S. cerevisiae} \( ysa1 \Delta \) mutants show increased resistance to \( \text{H}_2\text{O}_2 \) [9]. Therefore, we first measured the \( \text{H}_2\text{O}_2 \) sensitivity of \textit{C. neoformans} \( ysa1 \Delta \) mutants. Unexpectedly, \( ysa1 \Delta \) mutants were as resistant to \( \text{H}_2\text{O}_2 \) as the wild-type strain (Fig. 2A). In response to organic peroxides, such as tert-butyl hydroperoxide, the \( ysa1 \Delta \) mutants also showed wild-type levels of resistance (data not shown). We tested another ROS-generating agent, menadione, which is a superoxide generator. Similarly, the \( ysa1 \Delta \) mutants exhibited wild-type levels of resistance to menadione (Fig. 2A). In addition to peroxides and menadione, copper ions are also able to generate endogenous ROS as a transition metal [16, 17]. In fact, \textit{S. cerevisiae} \( ysa1 \Delta \) mutants also exhibit enhanced resistance to CuSO\(_4\) [9]. Therefore, we also measured copper ion sensitivity in the \textit{C. neoformans} \( ysa1 \Delta \) mutants. Similarly, the \( ysa1 \Delta \) mutants showed wild-type levels of resistance to CuSO\(_4\) (Fig. 2B).

Next, we tested the cellular susceptibility of the \( ysa1 \Delta \) mutants to diamide, a different type of oxidizing agent. Diamide is a thiol (SH) group-specific oxidant, but does not generate endogenous ROS when exogenously introduced. Surprisingly, all three \( ysa1 \Delta \) mutants exhibited enhanced sensitivity to diamide (Fig. 2C), suggesting that Ysa1 promotes cellular resistance to diamide in \textit{C. neoformans}. Diamide is able to induce abnormal disulfide bond formation and perturb normal protein structure. Therefore, we examined whether the \( ysa1 \Delta \) mutants also exhibit enhanced sensitivity to DTT, which is a reducing agent that also perturbs protein structure by breaking disulfide bonds. Unlike diamide, the \( ysa1 \Delta \) mutants showed wild-type levels of sensitivity to DTT (Fig. 2D). In conclusion, Ysa1 appears to play a specific role in diamide resistance in \textit{C. neoformans}.

\textbf{Ysa1 was dispensable for genotoxic stress and other environmental stress responses in \textit{C. neoformans}.} Based on a large-scale survey of \textit{S. cerevisiae} phenotypes, \textit{S. cerevisiae} \( ysa1 \Delta \) mutants also exhibit increased sensitivity to MMS, indicating that Ysa1 could also be involved in genotoxic stress responses. Furthermore, under massive genotoxic stress, NAD\(^+\)-dependent PARPs, which are involved in ADPr production along with poly(ADP-ribose) glycohydrolases (PARGs), are over-activated and deplete cellular NAD\(^+\), resulting in ATP loss and cell death [18, 19]. In \textit{C. neoformans}, however, \( ysa1 \) mutants exhibited wild-type levels of resistance to UV radiation and genotoxic...
agents such as HU and MMS (Fig. 3A), suggesting that Ysa1 is mostly dispensable for genotoxic stress responses in *C. neoformans*. In addition, we evaluated whether Ysa1 is involved in cellular responses and adaptations to other types of stresses. In response to osmotic stress conferred by high salt concentrations (1–1.5 M NaCl or KCl), *ysa1Δ* mutants were as resistant as the wild-type strain (Fig. 3B). Ysa1 also appeared to be dispensable for resistance to cell membrane and wall stress (Fig. 3C). Furthermore, in response to an ER stress agent (TM) and a heavy metal (CdSO₄), *ysa1Δ* mutants showed wild-type levels of resistance (Fig. 3D). In conclusion, Ysa1 appears to be generally dispensable for the response and adaptation to environmental stresses.

**Deletion of YSA1 promoted cellular resistance to anazole drug, itraconazole, in *C. neoformans***. Finally, we examined the role of Ysa1 in antifungal drug resistance. Notably, the activity of some antifungal drugs has been related to ROS production. Amphotericin B, which binds to ergosterol and forms a lethal pore through the plasma membrane, has been shown to produce ROS through autooxidation, resulting in lipid peroxidation in fungi [20, 21]. Itraconazole, which inhibits sterol 14-α-demethylase and sterol biosynthesis, is also known to produce ROS, and causes lipid peroxidation in *Cryptococcus gattii* [20]. Therefore, we also measured the cellular susceptibility of *ysa1Δ* mutants to polyene (amphotericin B) andazole drugs (fluconazole, itraconazole, and ketoconazole). Interestingly, the *ysa1Δ* mutants exhibited weakly enhanced resistance to itraconazole, but not to amphotericin B and other azole drugs (fluconazoles and ketoconazoles) (Fig. 4). In summary, inhibition of *YSA1* may promote resistance to itraconazole in *C. neoformans*. 

**Fig. 3.** Ysa1 was dispensable for the genotoxic stress response and other general environmental stress responses. *Cryptococcus neoformans* strains (wild-type [H99] and *ysa1Δ* [YSB2544-2546]) were grown for 16 hr at 30°C in liquid yeast extract-peptone-dextrose (YPD) medium, 10-fold serially diluted (1–10⁴ dilutions), and spotted (3 µL of dilution) onto YPD agar containing the indicated levels of ultraviolet (UV) radiation (250 J/m²), hydroxyurea (HU; 110 mM), methyl methanesulfonate (MMS; 0.04%) (A), sodium dodecyl sulfate (SDS; 0.04%), Congo red (CR; 1%), Calcofluor white (CFW; 5 mg/mL) (C), tunicamycin (TM; 0.3 µg/mL), or CdSO₄ (32.5 µM) (D). For measuring osmosensitivity, cells were spotted on YP agar containing 1 M or 1.5 M NaCl or KCl (B). Cells were incubated at 30°C for 3 days and photographed.

**Fig. 4.** Ysa1 was involved in itraconazole resistance of *Cryptococcus neoformans*. *C. neoformans* strains (wild-type [H99] and *ysa1Δ* [YSB2544-254]) were grown for 16 hr at 30°C in liquid yeast extract-peptone-dextrose (YPD) medium, 10-fold serially diluted (1–10⁴ dilutions), and spotted (3 µL of dilution) onto YPD agar containing the indicated concentration of amphotericin B, fluconazole, itraconazole, or ketoconazole (KCZ). Cells were incubated at 30°C for 3–4 days and photographed.
DISCUSSION

In this study, we characterized the function of a Nudix hydrolase gene, YSA1, for the first time in C. neoformans, which appeared to contain 11 putative Nudix hydrolase genes in its genome. The functions of C. neoformans Ysa1 were shown to differ from those of S. cerevisiae Ysa1p. First, C. neoformans ysa1Δ mutants did not show any growth defects, whereas S. cerevisiae ysa1Δ mutants do have growth defects [22]. Second, C. neoformans ysa1Δ mutants were as resistant to ROS-generating oxidizing agents (H₂O₂ and CuSO₄) as the wild-type strain, whereas S. cerevisiae ysa1Δ mutants exhibited enhanced resistance to these agents [9]. By contrast, Ysa1 appeared to be involved in resistance to diamide in C. neoformans, which is a thiol-specific oxidant but does not generate ROS. Therefore, it remains unclear whether Ysa1 directly controls intracellular ROS levels in C. neoformans. However, it is possible that other ROS defense systems sufficiently compensate for a loss of Ysa1 in C. neoformans. In fact, our study showed that the C. neoformans ysa1Δ mutants exhibited weakly increased resistance to itraconazole, but not to fluconazole. Interestingly, itraconazole, but not fluconazole, has the ability to produce intracellular ROS in addition to its Erg11 inhibitory activity [20]. Therefore, we cannot exclude the possibility that Ysa1 may modulate intracellular ROS levels under certain circumstances. Third, C. neoformans ysa1Δ mutants did not show any altered sensitivity to MMS, whereas S. cerevisiae ysa1Δ mutants show decreased resistance to MMS [23]. Taken together, our data demonstrate that the functions of Ysa1 are likely to be divergent among eukaryotic species.

The involvement of Ysa1 catalytic activity in the degradation of ADPr/OAADPr has not been biochemically demonstrated in C. neoformans. Therefore, whether or not Ysa1 is the key regulator for modulating intracellular levels of ADPr/OAADPr in C. neoformans remains elusive. Twelve Nudix hydrolases were discovered in the Aspergillus nidulans genome. Among these, NdxA and NdxC are able to hydrolyze NAD⁺, NADH, NADPH, and ADPr, like S. cerevisiae Nyp1, whereas NdxB and NdxD can hydrolyze ADPr, but not NAD(H) or NADPH, like S. cerevisiae Ysa1 [24]. However, the cellular functions of NdxB, which is the closest Ysa1 ortholog, have not been characterized. The role of ADPr/OAADPr as second messenger signaling molecules in C. neoformans needs to be addressed in future studies.

Regardless of the limited role of Ysa1 in C. neoformans, complete elucidation of the biological roles of ADPr derivatives, which could be major Ysa1 substrates, in the pathogen requires further study. Mono- or poly(ADP-ribo)sylation of proteins is an important post-translational modification for bacterial toxin activity as well as mammalian cell signaling and cell cycle regulation [19, 25]. Some PARPs, such as PARP-1 and PARP-2 in mammals, serve as genotoxic sensors by targeting and modifying a number of proteins involved in chromatin structure and DNA repair, with NAD⁺ as a substrate [19]. The breakdown of poly(ADP-ribose) (PAR) is mediated by PARPs, generating a free ADPr. Mice deleted of all PARP isoforms have been reported to die at the embryonic stage due to accumulated PAR and subsequent apoptosis [26]. Therefore, coordinated action of PARP and PARG is critical for cell survival as well as ADPr metabolism. Furthermore, OAADPr, which is generated during the deacetylation of lysine residues by the SIRT family of NAD⁺-dependent histone deacetylases, also acts as a cellular signaling molecule, modulating gene silencing and DNA repair [27]. Interestingly, it has been reported that poly(ADP-ribo)sylation does not occur in S. cerevisiae, but this organism does contain a sirtuin gene, SIR2 [28], and nutrient restriction is known to extend the lifespan of the budding yeast through activation of Sir2 [29, 30]. In filamentous fungi (e.g., Aspergillus fumigatus and Neurospora crassa) and mushrooms, however, PARPs were identified [25, 31, 32]. C. neoformans appears to have a single gene (CNAG_02941) containing a PARP-like domain, suggesting that poly(ADP-ribo)sylation may also occur in C. neoformans. There is no obvious yeast ortholog to CNAG_02941. Furthermore, C. neoformans has two Sir2-like NAD⁺-dependent histone deacetylases (CNAG_04866.7 and _07712.7) in its genome. Therefore, the role of PARP/Sir2-dependent NAD⁺ metabolism and ADPr/OAADPr in cell signaling should be further addressed in other diverse fungi, as well as in C. neoformans.

ACKNOWLEDGEMENTS

This work was supported by a grant of the 3G-Bio based eco-Biometric R&D Project, Ministry of knowledge economy, Republic of Korea (No. R0000480) (D.H.L.) and in part by the National Research Foundation of Korea (NRF) grants funded by the Korea government (MEST) (No. 2010-0029117 and No. 2008-0061963) (Y.S.B.).

REFERENCES

1. McLennan AG. The Nudix hydrolase superfamily. Cell Mol Life Sci 2006;63:123-43.
2. Xu W, Dunn CA, Bessman MJ. Cloning and characterization of the NADH pyrophosphatases from Caenorhabditis elegans and Saccharomyces cerevisiae, members of a Nudix hydrolase subfamily. Biochem Biophys Res Commun 2000;273:753-8.
3. AbdelRaheim SR, Cartwright JL, Gasi M, McLennan AG. The NADH diphosphatase encoded by the Saccharomyces cerevisiae NPY1 Nudix hydrolase gene is located in peroxisomes. Arch Biochem Biophys 2001;388:18-24.
4. Cartwright JL, Gasi M, Spiller DG, McLennan AG. The Saccharomyces cerevisiae PCDI gene encodes a peroxisomal Nudix hydrolase active toward coenzyme A and its derivatives. J Biol Chem 2000;275:32925-30.
5. Safarany ST, Ingram SW, Cartwright JL, Falck JR, McLennan AG, Barnes LD, Shears SB. The diadenosine hexaphosphate hydrolases from Schizosaccharomyces pombe and Saccharomyces cerevisiae are homologues of the human diphosphoinositol...
polyphosphate phosphohydrolase. Overlapping substrate specificities in a MutT-type protein. J Biol Chem 1999;274:21735-40.

6. Cartwright JL, McLennan AG. The *Saccharomyces cerevisiae* YOR163w gene encodes a diadenosine 5',5''-P1,P6-hexaphosphate (Ap6A) hydrolase member of the MutT motif (Nudix hydrolase) family. J Biol Chem 1999;274:8604-10.

7. Dunckley T, Parker R. The DCP2 protein is required for mRNA decapping in *Saccharomyces cerevisiae* and contains a functional MutT motif. EMBO J 1999;18:5411-22.

8. Dunn CA, O'Handley SF, Frick DN, Bessman MJ. Studies on the ADP-ribose pyrophosphatase subfamily of the Nudix hydrolases and tentative identification of trgB, a gene associated with tellurite resistance. J Biol Chem 1999;274:32318-24.

9. Tong L, Lee S, Denu JM. Hydrolase regulates NAD+ metabolites and modulates cellular redox. J Biol Chem 2009;284:11256-66.

10. Belenky P, Bogan KL, Brenner C. NAD+ metabolism in health and disease. Trends Biochem Sci 2007;32:12-9.

11. Kim H, Jacobson EL, Jacobson MK. Synthesis and degradation of cyclic ADP-ribose by NAD glycohydrolases. Science 1993;261:1330-3.

12. Bang S, Lee D, Kim H, Park J, Bahn YS. Global transcriptome analysis of eukaryotic genes affected by gromwell extract. J Sci Food Agric 2014;94:445-52.

13. Perfect JR, Ketabchi N, Cox GM, Ingram CW, Beiser CL. Karyotyping of *Cryptococcus neoformans* as an epidemiological tool. J Clin Microbiol 1993;31:3305-9.

14. Kim MS, Kim SY, Yoon JK, Lee YW, Bahn YS. An efficient gene-disruption method in *Cryptococcus neoformans* by double-joist PCR with NAT-split markers. Biochem Biophys Res Commun 2009;390:983-8.

15. Davidson RC, Cruz MC, Sia RA, Allen B, Alsquaugh JA, Heitman J. Gene disruption by biolistic transformation in serotype D strains of *Cryptococcus neoformans*. Fungal Genet Biol 2000;29:38-48.

16. Liang Q, Zhou B. Copper and manganese induce yeast apoptosis via different pathways. Mol Biol Cell 2007;18:4741-9.

17. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem 2005;12:1161-208.

18. Berger NA. Poly(ADP-ribose) in the cellular response to DNA damage. Radiat Res 1985;101:4-15.

19. Schreiber V, Dantzer F, Ame JC, de Murcia G. Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol 2006;7:517-28.

20. Ferreira GF, Baltazar Lde M, Santos JR, Monteiro AS, Fraga LA, Resende-Stoianoff MA, Santos DA. The role of oxidative and nitrosative bursts caused by azoles and amphotericin B against the fungal pathogen *Cryptococcus gattii*. J Antimicrob Chemother 2013;68:1801-11.

21. Lamy-Freund MT, Ferreira VF, Schreier S. Mechanism of inactivation of the polyene antibiotic amphotericin B. Evidence for radical formation in the process of autooxidation. J Antibiot (Tokyo) 1985;38:753-7.

22. Giaever G, Chu AM, Ni L, Connelly C, Riles L, Véroneau S, Dow S, Lucau-Danila A, Anderson K, André B, et al. Functional profiling of the *Saccharomyces cerevisiae* genome. Nature 2002;418:387-91.

23. Svensson JP, Pesudo LQ, Fry RC, Adeleye YA, Carmichael P, Samson LD. Genomic phenotyping of the essential and non-essential yeast genome detects novel pathways for alkylation resistance. BMC Syst Biol 2011;5:157.

24. Shimizu M, Masuo S, Fujita T, Doi Y, Kamimura Y, Takaya N. Hydrolase controls cellular NAD+ and sirtuin, and secondary metabolites. Mol Cell Biol 2012;32:3743-55.

25. Barkauskaite E, Jankevicius G, Ladurner AG, Abel I, Timinszky G. The recognition and removal of cellular poly(ADP-ribose) signals. FEBS J 2013;280:3491-507.

26. Koh DW, Lawler AM, Poitras MF, Sasaki M, Wattaer S, Nehls MC, Stöger T, Poirier GG, Dawson VL, Dawson TM. Failure to degrade poly(ADP-ribose) causes increased sensitivity to cytotoxicity and early embryonic lethality. Proc Natl Acad Sci U S A 2004;101:17699-704.

27. Tong L, Denu JM. Function and metabolism of sirtuin metabolite O-acetyl-ADP-ribose. Biochim Biophys Acta 2010;1804:1617-25.

28. Kruszewski M, Szumiel I. Sirtuins (histone deacetylases III) in the cellular response to DNA damage: facts and hypotheses. DNA Repair (Amst) 2005;4:1306-13.

29. Anderson RM, Bitterman KJ, Wood JG, Medvedik O, Sinclair DA. Nicotinamide and PNC1 govern lifespan extension by calorie restriction in *Saccharomyces cerevisiae*. Nature 2003;423:181-5.

30. Bitterman KJ, Anderson RM, Cohen HY, Latorre-Estives M, Sinclair DA. Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J Biol Chem 2002;277:45099-107.

31. Semighini CP, Savoldi M, Goldman GH, Harris SD. Functional characterization of the putative *Aspergillus nidulans* poly(ADP-ribose) polymerase homolog PrpA. Genetics 2006;173:87-98.

32. Kothe GO, Kitamura M, Masutani M, Selker EU, Inoue H. PARP is involved in replicative aging in *Neurospora crassa*. Fungal Genet Biol 2010;47:297-309.