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

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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]; Ddp1p 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 plays a key role in modulating intracellular concentrations of ADPr or OAADPr [9]. ADPr is produced by the enzymatic cleavage of the glycosidic bond of nicotinamide adenine dinucleotide (NAD⁺) by NAD⁺ glycohydrolases, ADPr transferases, poly(ADP-ribose) polymerases (PARPs), and cyclic ADPr synthases [10, 11]. OAADPr is generated by sirtuins, which catalyze NAD⁺-dependent lysine deacetylation. Both ADPr and OAADPr are known to act as secondary messengers and play diverse biological roles in yeast and mammals. In *S. cerevisiae*, ADPr or OAADPr counteract the increased levels of reactive oxygen species (ROS) through the inhibition of complex I of the electron transport chain in mitochondria and re-routing of glucose metabolism from the glycolytic pathway to the pentose phosphate pathway, which generates NADPH to detoxify ROS [9]. Therefore, the deletion of the Nudix hydrolase gene, *YSA1*, increases intracellular concentrations of ADPr and OAADPr, which

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result in enhanced cellular resistance to oxidative stress (e.g., H_2O_2) 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

Table 1. Strains used in this study

Name	Genotype	Parent	Reference
H99	<i>MATα</i>		[32]
YSB2544	<i>MATα ysa1Δ::NAT-STM#177</i>	H99	This study
YSB2545	<i>MATα ysa1Δ::NAT-STM#177</i>	H99	This study
YSB2546	<i>MATα ysa1Δ::NAT-STM#177</i>	H99	This study

Fig. 1. Discovery and disruption of a Nudix hydrolase gene, YSA1, in *Cryptococcus neoformans*. A, Phylogenetic analysis of Nudix hydrolase proteins in fungi. The phylogenetic tree was generated using Phylodendron Phylogenetic Tree Printer software (<http://iubio.bio.indiana.edu/treeapp/treeprint-form.html>). The scale bar indicates an evolutionary distance of 0.1. Species names are abbreviated as follows: S.kud, *Saccharomyces kudriavzevii*; S.cer, *Saccharomyces cerevisiae*; C.gla, *Candida glabrata*; A.fum, *Aspergillus fumigatus*; A.cla, *Aspergillus clavatus*; A.fla, *Aspergillus flauus*; A.ory, *Aspergillus oryzae*; A.ter, *Aspergillus terreus*; A.nig, *Aspergillus niger*; A.nid, *Aspergillus nidulans*; N.cra, *Neosartorya fischeri*; C.ort, *Candida orthosporus*; C.alb, *Candida albicans*; S.pom, *Schizosaccharomyces pombe*; C.cin, *Coprinopsis cinerea*; U.may, *Ustilago maydis*; C.neo, *Cryptococcus neoformans*; P.gra, *Puccinia graminis*; P.tri, *Puccinia tritricina*; P.str, *Puccinia striiformis*; B, Conserved Nudix hydrolase domains of Ysa1 proteins in fungi and humans. Species names are abbreviated as follows: C.n, *C. neoformans*; S.c, *S. cerevisiae*; S.p, *S. pombe*; S.k, *S. kudriavzevii*; C.a, *C. albicans*; H.s, *Homo sapiens*. C, The YSA1 gene disruption strategy. X; XbaI restriction site; D, Verification of the *ysa1Δ* mutants. The correct genotypes of the *ysa1Δ* mutants were verified by Southern blot analysis using genomic DNA digested with the restriction enzyme XbaI and the YSA1-specific probe denoted in panel (C).

(YP) medium for inducing glucose starvation.

Construction of the *ysa1Δ* mutants. *YSA1* gene disruption cassettes were generated by using the nourseothricin acetyltransferase (*NAT*)-split marker-based double joint (DJ) polymerase chain reaction (PCR) method as previously described [14, 15] with some modifications. In the first round of PCR, the 3'- and 5'-flanking regions of *YSA1* 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, *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, 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 *ysa1Δ* mutants (YSB2544, YSB2545, and YSB2546) were verified by Southern blot analysis, using a *YSA1*-specific probe that was PCR-amplified with the primer pair B5567/B5755. Each primer sequence was described in Table 2.

Phenotypic analysis. For antifungal drug sensitivity and stress response tests, wild-type and mutant strains were incubated in YPD medium for 16 hr at 30°C, 10-fold serially diluted ($1\sim 10^4$ dilutions) in dH₂O, and spotted onto solid YPD medium containing indicated concentrations (Figs. 2~4) of stress-inducers and antifungal drugs. Stress-inducers included oxidizing agents (hydrogen peroxide, *tert*-butyl hydroperoxide, menadione, and diamide), 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, 04718, 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.broadinstitute.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 (GxxxxxxExxxxxxxREUxEEExGU, 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

Table 2. Primers used in this study

Name	Sequence	Description
B79	TGTGGATGCTGGCGGAGGATA	Screening primer
B1026	GTAAAACGACGGCCAGTGAGC	M13 forward primer
B1027	CAGGAAACAGCTATGACCATG	M13 reverse primer
B1454	AAGGTGTTCCCCGACGACGAATCG	<i>NAT</i> split marker primer 1
B1455	AACTCCGTCGCGAGCCCCATCAAC	<i>NAT</i> split marker primer 2
B5567	TGTGCTTCTTGCTGTCTGC	Primer for the 5' flanking region of <i>YSA1</i>
B5568	TCACTGGCCGTCGTTTTACAAGGTTCTCAGGCTTCTGG	Primer for the 5' flanking region of <i>YSA1</i>
B5569	CATGGTCATAGCTGTTTCTGACTATGCCAGGAGAGGTTTC	Primer for the 3' flanking region of <i>YSA1</i>
B5570	TGAACGAGTGGTAATGTTGC	Primer for the 3' flanking region of <i>YSA1</i>
B5566	TGGCTTCGTTTCATAGGTG	Screening primer of <i>YSA1</i>
B5755	ATGACCGTTGAAGCGACAG	Primer for the <i>YSA1</i> probe (with B5567)

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

Fig. 2. Ysa1 was dispensable for resistance to reactive oxygen species-generating oxidizing agents and reducing agents, but promoted resistance to a thiol-specific oxidant diamide. *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 ($1\sim 10^4$ dilutions), and spotted (3 μ L of dilution) onto YPD agar containing the indicated concentration of hydrogen peroxide or menadione (A), $CuSO_4$ (B), diamide (C), and dithiothreitol (D). Cells were incubated at 30°C for 3 or 4 days and photographed.

three independent *ysa1Δ* 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 *C. neoformans* (data not shown).

Ysa1 was dispensable in *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 *S. cerevisiae* is its ability to control intracellular ROS levels. Accordingly, *S. cerevisiae ysa1Δ* mutants show increased resistance to H_2O_2 [9]. Therefore, we first measured the H_2O_2 sensitivity of *C. neoformans ysa1Δ* mutants. Unexpectedly, *ysa1Δ* mutants were as resistant to H_2O_2 as the wild-type strain (Fig. 2A). In response to organic peroxides, such as *tert*-butyl hydroperoxide, the *ysa1Δ* 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 *ysa1Δ* 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, *S. cerevisiae ysa1Δ* mutants also exhibit enhanced resistance to $CuSO_4$ [9]. Therefore, we also measured copper ion sensitivity in the *C. neoformans ysa1Δ* mutants. Similarly, the *ysa1Δ* mutants showed wild-type levels of resistance to $CuSO_4$ (Fig. 2B).

Next, we tested the cellular susceptibility of the *ysa1Δ* 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 *ysa1Δ* mutants exhibited enhanced sensitivity to diamide (Fig. 2C), suggesting that Ysa1 promotes cellular resistance to diamide in *C. neoformans*. Diamide is able to induce abnormal disulfide bond formation and perturb normal protein structure. Therefore, we examined whether the *ysa1Δ* mutants also exhibit enhanced sensitivity to DTT, which is a reducing agent that also perturbs protein structure by breaking disulfide bonds. Unlike diamide, the *ysa1Δ* mutants showed wild-type levels of sensitivity to DTT (Fig. 2D). In conclusion, Ysa1 appears to play a specific role in diamide resistance in *C. neoformans*.

Ysa1 was dispensable for genotoxic stress and other environmental stress responses in *C. neoformans*.

Based on a large-scale survey of *S. cerevisiae* phenotypes, *S. cerevisiae ysa1Δ* 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 *C. neoformans*, however, *ysa1* mutants exhibited wild-type levels of resistance to UV radiation and genotoxic

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 ($1\sim 10^4$ dilutions), and spotted (3 μ L 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 μ g/mL), or CdSO₄ (32.5 μ M) (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.

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 an azole 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

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 ($1\sim 10^4$ dilutions), and spotted (3 μ L 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.

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) and azole drugs (fluconazole, itraconazole, and ketoconazole). Interestingly, the *ysa1* 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*.

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 (H_2O_2 and $CuSO_4$) 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-ribosyl)ation 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 PARGs, generating a free ADPr. Mice deleted of all PARG 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-ribosyl)ation 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-ribosyl)ation 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*.

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