Nitric Oxide 86 (2019) 1–11

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Cross-talk between the Ras GTPase and the Hog1 survival pathways in response to nitrosative stress in *Paracoccidioides brasiliensis*

Palloma Mendes Conceição[a], Alison Felipe Alencar Chaves[b], Marina Valente Navarro[b], Daniele Gonçalves Castilho[b], Juliana Cristina P. Calado[b], Ana Eliza Coronel Janu Haniu[b], Patricia Xander[a], Wagner L. Batista[a,b,*]

[a] Department of Pharmaceutical Sciences, Universidade Federal de São Paulo, Campus Diadema, SP, Brazil
[b] Department of Microbiology, Immunology and Parasitology, Escola Paulista de Medicina, Universidade Federal de São Paulo, SP, Brazil

ARTICLE INFO

Keywords:
Paracoccidioides brasiliensis
Nitric oxide
Ras GTPase
Hog1 MAP kinase and nitrosative stress

ABSTRACT

Paracoccidioides brasiliensis is a temperature-dependent dimorphic fungus that cause paracoccidioidomycosis (PCM), the major systemic mycosis in Latin America. The capacity to evade the innate immune response of the host is due to *P. brasiliensis* ability to respond and to survive the nitrosative stress caused by phagocytic cells. However, the regulation of signal transduction pathways associated to nitrosative stress response are poorly understood. Ras GTPase play an important role in the various cellular events in many fungi. Ras, in its activated form (Ras-GTP), interacts with effector proteins and can initiate a kinase cascade. In this report, we investigated the role of Ras GTPase in *P. brasiliensis* after *in vitro* stimulus with nitric oxide (NO). We observed that low concentrations of NO induced cell proliferation in *P. brasiliensis*, while high concentrations promoted decrease in fungal viability, and both events were reversed in the presence of a NO scavenger. We observed that high levels of NO induced Ras activation and its *S*-nitrosylation. Additionally, we showed that Ras modulated the expression of antioxidant genes in response to nitrosative stress. We find that the Hog1 MAP kinase contributed to nitrosative stress response in *P. brasiliensis* in a Ras-dependent manner. Taken together, our data demonstrate the relationship between Ras-GTPase and Hog1 MAPK pathway allowing for the *P. brasiliensis* adaptation to nitrosative stress.

1. Introduction

Paracoccidioidomycosis (PCM) is the predominant systemic mycosis in Latin America with a mortality rate of 25% [1]. Approximately 80% of all the reported cases appear in Brazil, although Colombia, Venezuela, Argentina, Ecuador, and Peru have reported a substantial number of cases [2]. The disease is caused by fungi that belong to the genus *Paracoccidioides*, and the pathogenic switching of members of this genus is triggered by temperature changes. Once the host has inhaled infective propagules and the fungus reaches the lower respiratory tract, the immune system of the host needs to limit the infection before the disease outcome. Innate immune cells are specialized to produce a set of chemical species, reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and nitric oxide (NO). Thus, the outcome of the infection relies on the ability of the fungus to survive the ROS and RNS produced by the host [3].

It has been demonstrated that NO produced by the host in response to the infection is responsible for controlling fungal proliferation and for avoiding an exacerbated inflammatory response, since the fungal burden in the lungs and liver was higher in mice that do not express inducible nitric oxide synthase (iNOS) compared to wild-type mice [4]. On the other hand, if the host is not able to produce enough RNS to kill the fungus, the host could benefit from the lower levels of RNS produced during disease onset. This fact was demonstrated by the ability of *P. brasiliensis* to survive and proliferate when exposed to sub-toxic levels of NO, which occurred in a Ras-dependent manner [5].

Ras GTPases are a family of functionally conserved small proteins that switch between GTP-bound (active form) and GDP-bound (inactive form) conformations. Ras is involved in signal transduction pathways connecting the events from many cell surface receptors to intracellular processes [6]. Depending on the cellular context, Ras activation can stimulate the cell division cycle, morphogenesis, differentiation, or apoptosis [6]. Ras proteins upstream of the cAMP-PKA or MAPK

* Corresponding author. Department of Pharmaceutical Sciences, Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, Rua São Nicolau no 210, CEP: 09913-030, Diadema, Brazil.
E-mail address: batista@unifesp.br (W.L. Batista).

https://doi.org/10.1016/j.niox.2019.02.002
Received 7 October 2018; Received in revised form 10 January 2019; Accepted 12 February 2019
Available online 14 February 2019
1089-8603/

(mitogen-activated protein kinase) pathway are involved in the regulation of filamentation and/or virulence in *Cryptococcus neoformans* [7] and *Candida albicans* [8,9], cellular aggregation [10], cell wall integrity [11] and other phenotypes. *In P. brasiliensis*, two Ras isoforms (Ras1 and Ras2) were characterized with important roles during fungal dimorphism, thermal stress, and in parasite-host interactions [12].

Signaling pathways that control the redox stress response in *P. brasiliensis* are poorly known, but in other dimorphic fungi, the involvement of MAPK is an important factor in this process [13–15]. For example, when alveolar macrophages are challenged with *Aspergillus* or *Cryptococcus* conidia, the macrophages are able to produce RNS levels as high as 50 μM, and the fungi are efficiently phagocytosed [16]. However, even when the host is able to produce high concentrations of NO, the fungi still possess mechanisms to adapt to nitrosative stress and cause disease. This is because fungi have an effective enzymatic repertoire to counteract the RNS produced by the host, which functions as a virulence factor [17,18].

Currently, different research groups have sought to study the mechanisms that regulate the response of fungi to oxidative stress [19–21], but despite the importance of RNS in the death of the fungi, as well as the signaling process, little progress has been made even though this field has become a topic of interest [18,22]. In this study, we highlight the cross-talk between Ras GTPase and Hog1 MAPK activation in the context of nitrosative stress in the human pathogenic fungus *P. brasiliensis*.

2. Materials and methods

2.1. Materials

The following reagents were purchased from Merck-Millipore-Calbiochem (Darmstadt, Germany): the antibodies anti-pan-Ras and anti-Hog1, and the inhibitors FPT inhibitor III (Ras inhibitor) and SB202190 (Hog-1 inhibitor). Glutathione-Sepharose® beads were obtained from GE Healthcare (Chicago, IL, USA). The anti-phospho-Hog-1 antibody was from Santa Cruz Biotechnology (Dallas, TX, USA). Appropriate secondary antibodies conjugated to horseradish peroxidase were from KPL (Gaithersburg, MD, USA). The Western blotting developing reagents (Super Signal® System) were obtained from Pierce (Rockford, IL, USA).

2.2. Fungal strain and growth conditions

We used *P. brasiliensis* isolate Pb18 in our experiments. Unless otherwise mentioned, yeast extract-peptone-dextrose modified medium (mYPD) (0.5% yeast extract, 1% casein peptone and 0.5% glucose, pH 6.7) was used to cultivate the yeast cells, which was cultured at 37 °C. The CFU count was performed in supplemented BHI plates (Becton Dickinson Company) containing 4% fetal calf serum, 5% spent medium, ampicillin (100 IU/mL) and streptomycin (100 μg/mL).

2.3. NO stimulation

In experiments involving nitrosative stress, *P. brasiliensis* cells were cultivated in modified YPD for 5 days at 37 °C. Yeast cells (1×10^5) were seeded in a 6-well culture plate and subjected to a 24 h period of starvation with RPMI media to reduce or stop fungal growth until the treatment was started. This strategy was used to verify the role of the NO stimulus on fungal growth and cell signaling. Yeast cells were exposed to different concentrations of $NaNO_2$ in mildly acidic culture media, pH 5.6, for 5 h at 37 °C; under these conditions, $NaNO_2$ releases NO [5,23–25]. Yeast cells were washed and incubated for 24 h at 37 °C with shaking in fresh culture media. Finally, 100 μL of yeast cultures was plated on supplemented BHI plates for 7 days at 37 °C. The experiment was repeated three times. Cell proliferation or cytotoxicity were evaluated by fungal counts after 4 days. Yeast cell suspension aliquots (100 μL) were stained with equal volumes of Trypan Blue vital dye and counted in a Neubauer chamber. Viable cells were not stained by the vital dye.

2.4. Animal experiments and ethics statement

Male BALB/c mice (6- to 8-week-old) were maintained under specific pathogen-free conditions at a temperature of 23–24 °C with a light/dark cycle of 12 h and provided with food and water *ad libitum*. Animal experimentation was approved by the Ethics Committee on the use of animals at the Federal University of São Paulo (CEP 8888301117). The animals were handled according to the Brazilian National Council for Animal Experimentation Control (CONCEA) guidelines.

Paracoccidioides yeast cells (1×10^6) were pretreated with FPT inhibitor III (Ras inhibitor) at 50 μM for 24 h at 37 °C in RPMI media. Then, the yeast cells were recovered by centrifugation at 10,000 × *g* for 5 min. After treatment with the Ras inhibitor, the yeast cells (1×10^6 cells in 40 μL of saline solution) were used to infect male BALB/c. Two groups of mice (n = 6 animals each) were used. Briefly, the mice were anesthetized by intraperitoneal injection with 50 μL of a solution containing 40% ketamine and 40% xylazine. After approximately 15 min, their trachea was exposed and injected with 1×10^6 viable Pb18 yeast cells. The incisions were sutured with 4–0 silk. After 30 days of infection, the animals were euthanized, the lungs were excised, and the numbers of viable microorganisms in the lungs were determined by enumerating the CFUs [26].

2.5. Determination of NO levels

NO generation was evaluated by the use of the cell permeable specific fluorescent NO indicator 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF2-DA). Yeast cells (1×10^6) were preincubated with 5 μM DAF2-DA dissolved in RPMI media for 30 min at 37 °C in the dark. Once deacetylated, DAF2-DA remains free in the cytoplasm to react with NO and generate the fluorescent benzotriazole DAF2. Yeast cells were incubated with 0.25 or 1 mM NO_2, at pH 5.6, and analyzed in a FACSCalibur flow cytometer (Becton Dickinson Co., Franlin Lajes, NJ, USA). The excitation wavelength was set at 495 nm, and the emission wavelength was set at 515 nm. The results are expressed as DAF-2-derived fluorescence (mean fluorescence intensity). Unlabeled samples were used as blanks. The experiments were performed in triplicate.

2.6. Spot test

The nitrosative sensitivity of the yeast strains was determined using the standard spot test technique. Briefly, yeast cultures in an early stationary phase of growth (2×10^6 cells/mL) were obtained after 4–5 days of growth in mYPD media at 37 °C, subjected to a 24-h period of starvation with RPMI medium. Yeast cells were preincubated with Hog1 inhibitor (10 μM) and incubated with 4 mM NO for 5 h. The yeast cultures were serially diluted (1:10 at each step) in a sterile saline solution (0.9% NaCl), and 30 μL of each suspension was plated on mYPD. The plates were photographed after 7 days of growth at 37 °C. The results shown are representative of three independent experiments.

2.7. Ras activity assay

Ras activation was determined using the RBD (Byr2)-GST fusion protein [5], which binds tightly to the GTP-associated form of Ras. After stimulus, the yeast cells were collected by centrifugation, washed 3 times, and disrupted with glass beads in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% (w/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 10% (w/v) glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium vanadate, and one tablet of protease

inhibitor (Roche Diagnostic, Mannheim, Germany)) in 5 mL of extraction medium. The solubilized extract was centrifuged at 14,000 × *g* for 15 min, and the supernatant was used in pull-down assays. The protein content of the cell extract was determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA). A protein sample (1 mg) was incubated with glutathione-Sepharose beads associated with RBD (Byr2)-GST for 3 h with gentle rocking. The samples were spun at 7200 × *g* for 10–20 s, and the resin was washed three times with lysis/binding/wash buffer (7200 × *g* for 30 s). The final pull-down was assayed by Western blot probed with mouse monoclonal anti-pan-Ras antibody. The remaining lysate was probed with the same antibody to determine the levels of total and endogenous Ras. The ratio between the Ras signal intensity bound to RBD (Byr2)-GST beads and that obtained from the total Ras, determined by densitometry, is proportional to the level of Ras activity [27,28].

2.8. Pull-down assay

For pull-down studies, the lysates were incubated with the RBD (Byr2)-GST probe overnight at 4 °C followed by an additional 2 h incubation at 4 °C with protein glutathione Sepharose beads. The beads were centrifuged for 3 min at 10,000 × *g*, and the supernatant was discarded. The beads were washed three times in PBS, resuspended in 2X gel loading buffer, and boiled for 5 min prior to separation using SDS–polyacrylamide gel electrophoresis. The separated proteins were transferred onto nitrocellulose membranes. After transfer, the membranes were preincubated with a 5% solution of blocking buffer for 1 h followed by overnight incubation with the anti-Ras primary antibody. After incubation with the appropriate HRP-conjugated secondary antibodies, the blots were developed using the Super Signal system (Thermo-Pierce, Rockford, IL) and digitally registered on UVITEC Imaging Systems (Warwickshire, UK).

2.9. Western blotting analysis

P. brasiliensis yeast cells were starved for 24 h in RPMI media and stimulated with 4 mM NO for increasing periods at 37 °C. Where indicated, FTP inhibitor III was added 24 h prior to the stimulation of the cells with NO. Lysates were obtained by incubation with lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 0.25% sodium deoxycholate, 1% NP40, 1 mM NaF, 1 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 1 mM PMSF) for 30 min on ice. The samples were centrifuged at 10,000 × *g* for 10 min, and the protein concentration in the supernatant was determined by Bradford assay. The protein content was adjusted, so that each sample contained an equal concentration of protein (50 μg/mL). Total cell lysates (50 μg/mL) were resolved in 10% SDS–PAGE gels and transferred onto nitrocellulose membranes. The blots were probed using specific antibodies against Ras and Hog-1. After incubation with the appropriate HRP-conjugated secondary antibodies, the blots were developed using the Super Signal system and digitally registered on UVITEC Imaging Systems (Warwickshire, UK).

2.10. Detection of Ras S-nitrosylation

The biotin switch technique (BST) was performed to detect Ras *S*-nitrosylation as described by Forrester et al. [29]. To detect the Ras *S*-nitrosylation in *P. brasiliensis* after NO treatment, the yeast cells were cultured in RPMI media for 24 h (starvation), treated with NO for increasing time periods, and then disrupted with glass beads in buffer containing 25 mM HEPES, 50 mM NaCl, 0.1 mM EDTA, 1% NP-40, 0.5 mM PMSF, and protease inhibitors (Roche Diagnostic, Mannheim, Germany), pH 7.4. The cell debris was removed by centrifugation, and samples (1–0.6 mg protein extract) were diluted to 1.8 mL with HEN buffer (100 mM HEPES, 1 mM EDTA, and 0.1 mM neocuproine, pH 8.0); SDS and MMTS were added to final concentrations of 2.5 and 0.1%, respectively. Following frequent vortexing and incubation at 50 °C in the dark for 20 min, the lysates were precipitated with 3 vol of acetone at −20 °C for 1 h. The proteins were centrifuged at 2000 × *g* for 15 min, and the protein pellet was gently washed with 70% acetone four times. The pellets were suspended in 240 μL of HENS (HEN buffer with 1% SDS). The samples were then incubated with 30 μL of biotin-HPDP (2.5 mg/mL) in the presence or absence of 20 mM ascorbate at room temperature in the dark for 1 h. After acetone precipitation, the proteins were resuspended in 250 μL of HENS, followed by the addition of 750 μL of neutralization buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100, pH 7.5). Fifty microliters of streptavidin-agarose beads (prewashed) were added to each sample and incubated overnight at 4 °C. The beads were washed with washing buffer (neutralization buffer with 600 mM NaCl) four times. To detect the *S*-nitrosylated proteins, 50 μL of 2 × SDS sample buffer was added to the beads and tested by immunoblotting with anti-Ras antibody.

Paracoccidioides RNA isolation and Real-Time quantitative (RT-qPCR).

Paracoccidioides yeast cells grown to exponential phase in RPMI media were collected via centrifugation (2000 × *g*), washed 4 times and resuspended in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was released from the yeast cells mechanically by beating with 0.5-mm-diameter glass beads for 10 min in the presence of the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was purified using $CHCl_3$ extraction followed by alcohol precipitation of the aqueous phase. Reverse transcription was performed using RevertAid Premium Reverse Transcriptase (Thermo Scientific, Bremen, GA, USA), 5 μg of total RNA and 22-mer oligo-dT primers. Quantitative PCR assays were assembled using a SYBR-Green-based PCR master mix (Applied Biosystems) with diluted reverse-transcribed templates (1:200 final) and a 0.5 μM concentration of each gene-specific primer (Table 1). Amplification was performed in a StepOne Plus (Applied Biosystems Inc., Carlsbad, CA, USA) using the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The dissociation curve was determined with an additional cycle of 95 °C (15 s), 60 °C (20 s) and 95 °C (15 s). DNA contamination was evaluated via PCR amplification of the *PbGP43* gene (Accession No. U26160). The negative controls lacked DNA or RNA. The relative expression ratio (experimental/control) was determined based on the 2-ΔΔCt method [30] after normalization to the level of the β-tubulin (*β-TUB*) and *18S* transcripts.

Table 1
Oligonucleotides used in qPCR.

Target[a] (ID)	Sense	Sequence (5'→3')
PbCAT (PADG_00225)	Fw	CTACCACGACAAGAAGACTAC
	Rev	CGGATATGAGAACAGCGACTT
TRX1 (PADG_05504)	Fw	AGCCATCCCTAGTCGTCATA
	Rev	GTTCGGGTAGGATTCGGAAA
TRX2 PADG_03161)	Fw	GACACGGACGAACAGCTAAT
	Rev	GGAGGTGATCTGTAGCGAATG
SOD1 (PADG_01755)	Fw	ACCACCAAACCTACGTAAACTC
	Rev	GGGATTTGATATCGGCCTTCTC
SOD2 (PADG_07418)	Fw	TCAACCCGTTCGGCAAAT
	Rev	CCTGAGCGTCAGTGGTAATG
GR (PABG_06068)	Fw	GTTGAGACGCACATGTTTATCC
	Rev	GTGACGCCCATATCCTCATAC
AOX (PADG_03747)	Fw	CAGGGTGTCTTCTTCAACTCTT
	Rev	CGTCATAACAGCCTCCTCTTC
YHB (PADG_03872)	Fw	GCGTTTCTGGAGTTCATGTTTC
	Rev	CGTCACTGATGGGAGTGTAAG
PCNA (PADG_11,679)	Fw	TTCCACCATATCACCTGCGACAGT
	Rev	ACGTGCGAATTGTCCATAGCTTGC
β-TUB (PADG_00128)	Fw	GTGGACCAGGTGATCGATGT
	Rev	ACCCTGGAGGCAGTCACA
18S (PADG_12,090)	Fw	CGGAGAGAGGGAGCCTGAGAA
	Rev	GGGATTGGGTAATTTGCGC

[a] Accession numbers from *Paracoccidioides brasiliensis* genes http://www.fungidb.org/.

2.11. Mass spectrometry

Proteomic analysis was performed using an LTQ-Orbitrap Velos (Thermo Scientific) instrument operating with a nanoelectrospray source (nano-ESI). Positive ionization and data dependent acquisition after high-energy collisional dissociation (HCD) were set up. Chromatographic separation was performed using an EASY-nLC system utilizing C18 capillary columns. MS/MS spectra were analyzed using PEAKS Studio (v. 5.3, Bioinformatics Solutions Inc.). Mass tolerances were set to 1.2 Da for MS and 0.6 Da for MS/MS spectra. One missed cleavage site was allowed for trypsin, and cysteine carbamidomethylation (+57 Da) was applied as a fixed modification. As variable modifications, methionine oxidation (+16 Da) and the phosphorylation of serine, tyrosine, and threonine residues (+79 Da) were set up. The protein list was annotated using Blast2GO, and association networks were constructed using the STRING database [31].

2.12. Statistical analysis

All the results are presented as the mean ± standard deviation (SD). The overall significance was determined by Student's *t*-test. Differences between the groups were considered statistically significant at $p < 0.05$.

3. Results

Nitric oxide modulates the growth and death of the fungus P. brasiliensis in a dose-dependent manner.

We have previously shown that different levels of NO (sub-toxic or toxic concentrations) can induce distinct responses within *P. brasiliensis* [5]. Initially, we investigated the intracellular NO levels in *P. brasiliensis* yeast treated with 0.25 and 1 mM NO_2 for 5 h at 37 °C (in mYPD pH 5.6). In both conditions tested, we detected the presence of intracellular NO, and the peak of the 1 mM NO showed 4 times more NO than the control (Fig. 1A). To better understand this type of stimulus in *P. brasiliensis*, logarithmically growing yeast cells were cultured in RPMI media for 24 h and subsequently treated with different concentrations of NO (0.25 or 1 mM) *in vitro* for 5 h at 37 °C in pH 5.6 in the presence or absence of the NO scavenger cPTIO (30 mM, Sigma-Aldrich, St. Louis, MO, USA). We observed a decrease in cellular viability in yeast cells preincubated for 5 h with higher concentrations of NO (1 mM) (Fig. 1B). Yeast cells preincubated with low concentrations of NO (0.25 mM) responded with significant cell proliferation ($3.2 \pm 0.29 \times 10^5$ CFU) compared to unstimulated controls ($1.8 \pm 0.16 \times 10^5$ CFU) (Fig. 1B). These data confirm our previous results [5]. However, the treatment of *P. brasiliensis* yeast cells with low levels of NO (0.25 mM) in the presence of the NO scavenger cPTIO (30 μM) inhibited fungal proliferation, while the treatment of yeasts cells with 1 mM NO in the presence of cPTIO inhibited cellular death (Fig. 1B). These data demonstrate the involvement of NO in both modulating cell proliferation (at sub-toxic concentrations) and cell death (at toxic concentrations).

To confirm the participation of sub-toxic concentrations of NO in the process of cell proliferation, we evaluated the expression of the PCNA (proliferating cell nuclear antigen) gene, a marker of cell proliferation [32]. Thus, mRNA samples were prepared from yeast treated with low concentrations of NO in the presence or absence of the cPTIO, and RT-qPCR was performed. We observed a strong increase in the PCNA mRNA levels of *P. brasiliensis* after treatment with 0.25 mM NO. On the other hand, the PCNA expression in *P. brasiliensis* yeast cells stimulated with low concentrations of NO and preincubated with cPTIO, was similar to that observed in the controls without stimuli (Fig. 1C). These data confirm our previous observations that NO and Ras are able to stimulate cell proliferation in *P. brasiliensis*.

Ras GTPase proteins are activated by several stimuli in response to stress, including heat shock and fungal virulence [8,33]. Thus, we evaluated Ras activation in response to nitrosative stress. We used the

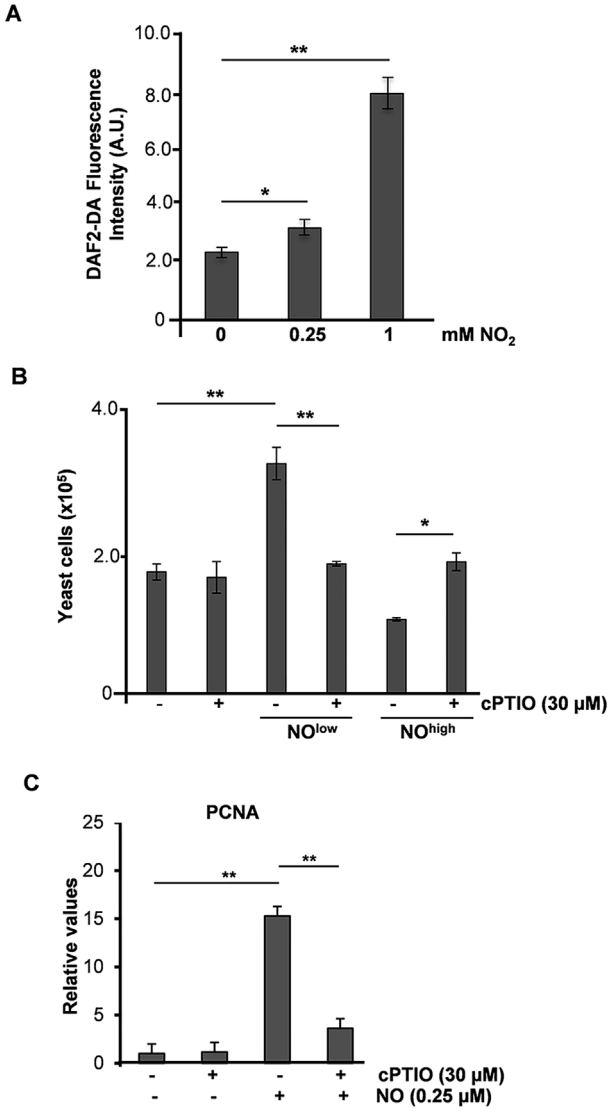

Fig. 1. (A) Measurement of intracelular levels of NO in *Paracoccidioides* treated with NO_2. Yeast cells were treated with two different concentrations of NO_2 (0.25 or 1 mM) at 37 °C, in the presence of PBS or DAF-2DA (5 μM) and analyzed in flow cytometer. The results are expressed as mean fluorescence intensity of DAF-2A. (B) Pb18 yeast cells were seeded in a 6-well culture plate subjected to a 24-h period of starvation with RPMI medium, pretreated with or without 30 μM cPTIO (NO scavanger) and stimulated with 0.25 or 1 mM NO_2 at pH 5.6 for 5 h at 37 °C. Treated cells were cultured in the mYPD medium (n = 4 each point) and after 4 days the fungal growth was determined by counting in a Neubauer chamber. (C) Relative levels of PCNA mRNAs determined by real-time qPCR in *P. brasiliensis* yeast cells. Pb18 yeast cells were pretreated with or without 30 μM cPTIO and stimulated with 0.25 or 1 mM NO_2 at pH 5.6 for 5 h at 37 °C. Gene specific-primer was used for this reaction, and the change in transcriptional levels was calculated by the ΔΔCt method with two housekeepers (*β-TUB* and *18S*). Statistically significant samples are indicated (*$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$). The results are representative of three independent experiments.

RBD (Byr2)-GST probe (produced in a previous study [5]) to assess the ability of high concentrations (toxic) of NO to activate Ras. Ras activity was determined in *P. brasiliensis* yeasts exposed to 1, 2 or 4 mM NO for 60 min. We observed that high concentrations of NO induced Ras activation. Under these conditions, the peak activation was observed with 4 mM NO (Fig. 2A). Thus, to verify the kinetics of activation of Ras in nitrosative stress, a concentration of 4 mM of NO was used for increasing periods of time. Early Ras activation was observed after 15 min

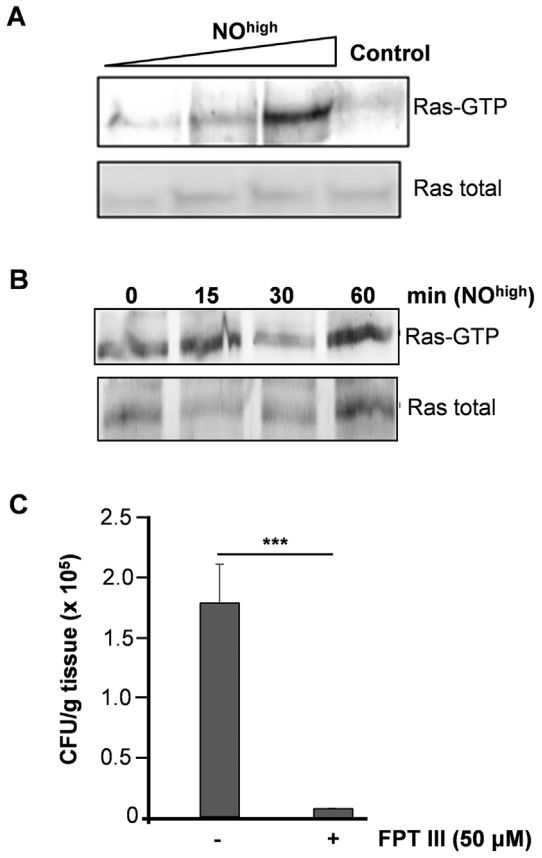

Fig. 2. Ras is activated by high concentrations of NO and participates in the virulence of *P. brasiliensis*. (A) Pb18 yeast cells were cultivated in modified YPD for 5 days at 37 °C, subjected to a 24-h period of starvation with RPMI medium. Yeast cells were incubated with different concentration of NO (1, 2 and 4 mM – in mYPD, pH 5.6) at 37 °C. After cell lysis, Ras activation was determined using the RBD (Byr2)-GST fusion protein, which binds with high affinity to the GTP-associated form of Ras. Ras-GTP (active) and total Ras (50 µg total protein) were assayed by western blots probed with a mouse monoclonal anti-Ras antibody. (B) Yeast cells were incubated with 4 mM NO (in mYPD pH 5,6) at 37 °C for increasing periods. Ras activity assay was performed same as (A). Western blots are representative of three independent experiments performed. (C) Colony-forming units (CFU) of lungs from infected BALB/c mice with *P. brasiliensis* after 30 days post infection. Groups of mice (6 animals each) included only infected (Control) or infected with yeast pretreated with FPT inhibitor III. Data were analyzed using Student's t-test. Error bars correspond to the standard deviation of measurements performed in triplicate, and asterisks show significant differences (***$p < 0.001$).

of stimulation with NO (Fig. 2B). Maximal Ras activation was observed after 60 min of incubation (Fig. 2B). In longer times (up to 5 h) the Ras activation was not observed (data not show). Additionally, the fungal protein extract was incubated with glutathione-Sepharose beads alone (negative control) and analyzed by Western blot, but no reaction was observed (data not shown). The protein loading of the samples used in the Ras activity assay observed by Coomassie blue staining of the SDS-PAGE gel were fairly homogeneous. Therefore, nitrosative stress induced by NO promoted guanine nucleotide exchanges in the critical cellular signaling Ras in *P. brasiliensis*.

Ras of P. brasiliensis is important for the response of fungi that survived in the host.

Ras pathways are involved in the regulation of virulence in *Cryptococcus neoformans* [7] and *Candida albicans* [8,9]. To verify the importance of Ras in the survival response of the fungus in the host, a pathogenicity test of *P. brasiliensis* was performed before and after treatment with the Ras inhibitor (FPT inhibitor III). BALB/c mice were inoculated intratracheally with yeast cells preincubated in the presence or absence (control) of the Ras inhibitor. In this experiment, all the infected animals were euthanized after 30 days of infection; the lungs were isolated and macerated, and the material was loaded onto supplemented BHI plates. After 10 days, the plates were analyzed and the CFUs counted (Fig. 2C). Animals infected with yeast cells plus inhibitor showed significant reductions in the fungal cell numbers in their lungs, as demonstrated by the reduced CFUs/g of tissue recovered from this group compared to that recovered from the positive control (untreated infected group) ($p < 0.05$) (Fig. 2C). Therefore, these data indicate that Ras GTPase is required for the pathogenicity of *P. brasiliensis*.

3.1. High concentrations of NO promote Ras S-nitrosylation in P. brasiliensis

S-nitrosylation is a posttranslational modification (PTM), which occurs from the covalent attachment of NO to a cysteine residue to form an *S*-nitrosothiol (SNO) [34]. The small GTPase Ras was one of the earliest described regulatory targets of *S*-nitrosylation [35]. In a previous study, we demonstrated that Ras1 of *P. brasiliensis* shows a putative *S*-nitrosylation site localized in Cys123 [5]. Thus, we evaluated whether Ras would be nitrosylated under high concentrations of NO. After analysis using the Biotin Switch Technique (BST) assay, we found that yeast cells stimulated with high concentrations of NO (4 mM) for 60 min exhibited a rapid increase in the *S*-nitrosylation levels in Ras (Fig. 3). These findings suggested that high levels of NO induce Ras activation through its *S*-nitrosylation.

3.2. Ras modulates the expression of genes involved with redox regulation

We evaluated if Ras modulates the expression of genes related to the response to redox stress. For this, we treated the fungus with a high concentration of NO (4 mM) in the presence and absence of a Ras inhibitor (FPT inhibitor III). In yeast cells treated with NO, a significantly high expression of *SOD1*, *SOD2*, *TRX1*, *TRX2*, *GR*, *CAT*, *AOX* and *YHB* genes was found (Fig. 4). FPT inhibitor III exerted its inhibitory effects on *SOD1*, *SOD2*, *TRX1*, *TRX2*, *GR* and *YHB* expression (Fig. 4A, B, C, D, E e F). This indicates that the induction of the expression of these genes requires full activation of the Ras pathway in *P. brasiliensis*. In Fig. 4, we observed that the genes showed increased expression. The induction of these genes ranged between 2- to 5-fold after treatment with NO (4 mM) (Fig. 4A, B, C, D, E e F). However, when we treated the samples with a Ras inhibitor, the expression decreased. Under these conditions, we did not observe significant changes in the mRNA expression levels of the *PbCAT* gene (data not shown). On the other hand, *AOX* expression was increased after stimulation with high concentrations of NO (Fig. 4G). However, the participation of Ras in the regulation of *AOX* expression was negative. These data demonstrate that Ras modulates some genes involved in the response to nitrosative stress.

3.3. Isolation of Ras protein complexes from P. brasiliensis after nitrosative stress

We used mass spectrometry to analyze proteins that associate with

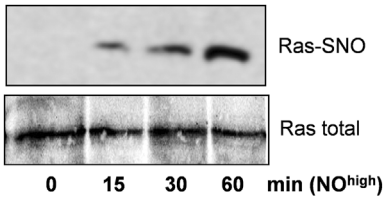

Fig. 3. High concentrations of NO induce *S*-nitrosylation of Ras. Pb18 yeast cells were incubated with 4 mM NO at 37 °C for increasing periods. Cell extracts were analyzed by the biotin-switch technique (see the Materials and Methods sections for details), and western blots were probed with anti-Ras antibody.

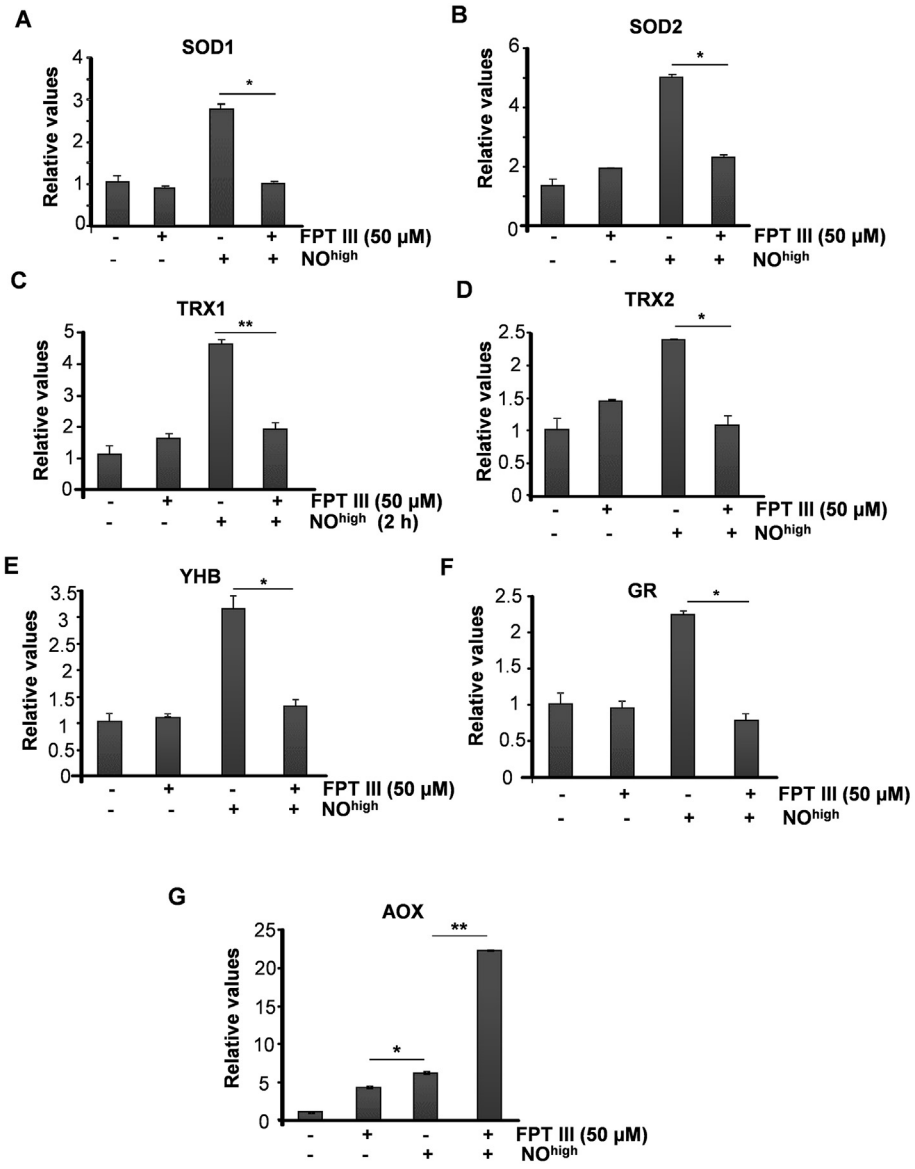

Fig. 4. Relative expression of the protein-encoding genes of redox response in *P. brasiliensis* yeast cells. Quantitative RT-PCR of RNA isolated from Pb18 grown in mYPD for 5 days. Yeast cells were treated with high concentrations of NO (4 mM) in the presence or absence of the Ras inhibitor (FPT III 50 μM). Gene-specific primers of (A) *SOD1*, (B) *SOD2*, (C) *TRX1*, (D) *TRX2*, (E) *YHB*, (F) *GR* and (G) *AOX* were used for each reaction. The change in transcriptional levels was calculated by the ΔΔCt method with two housekeepers (*β-TUB* and *18S*). Data are presented as fold changes in gene expression levels in the sample of interest normalised to the housekeepers. Error bars represent ± SD of n = 3 by Student's *t*-test (∗$p < 0.05$; ∗∗$p < 0.01$ and ∗∗∗$p < 0.001$).

Ras in *P. brasiliensis* yeast cells treated with NO. Ras complexes were isolated using the RBD (Byr2)-GST assay that detects the activated form of Ras, and the associated proteins were excised and digested with trypsin. The resulting peptides were analyzed using LC-tandem mass spectrometry (MS/MS) and assigned to specific proteins with the Mascot search engine. The mass spectrometry analysis identified both Byr2-RBD (Ras Binding Domain) fragment peptides (167 peptides) and the Ras protein (20 peptides), thus confirming that the probe can interact with Ras. In addition, in the group of peptides of Byr2 (designated Ste11), both peptides that are part of the probe used in the assay and other regions of the protein were identified (Fig. 5A). These data indicate that under this condition, the native Byr2 may be part of the redox signaling pathways triggered by Ras.

As shown in Table 2, 11 protein kinases were identified in association with Ras. All the kinases identified were previously reported to interact with Ras from *S. cerevisiae* as identified by the iGPS algorithm [36]. Among the kinases identified, we detected the MAP kinases Ste7 (Byr1) and Hog1, which act upstream of the cascade involving Byr2 (Ste11). Thus, we hypothesize that this signaling pathway would be involved in the response of *P. brasiliensis* triggered by NO activation induced by Ras.

3.4. Cross-talk between Ras and Hog1 pathways in P. brasiliensis

To determine whether the exposure of yeast cells to high levels of NO activated the Hog1 protein, we analyzed the activation of Hog1 by Western blotting with an antibody specific for the phosphorylated form of Hog1. As shown in Fig. 5B, high levels of NO (4 mM) induce a rapid phosphorylation of Hog1 when compared with the controls. Thus, we investigated whether Ras interacts with Hog1 after stimulation with high concentrations of NO in *P. brasiliensis*. For this, the RBD (Byr2)-GST probe was used to evaluate the interaction of Ras-GTP and Hog1. This assay was conducted in *P. brasiliensis* lysates using the RBD (Byr2)-GST probe followed by antibodies against Hog1. When *P. brasiliensis* was stimulated with high concentrations of NO, we observed that Hog1 interacts with the activated form of Ras (GTP-Ras) and that this interaction occurred biphasically with peaks at 30 and 60 min after stimulation with 4 mM NO (Fig. 5C). We spotted serially diluted cell suspensions onto YPD plates, incubated the plates at 37 °C for 7 days and assessed the cellular survival under nitrosative stress. We observed that the yeast cells treated with both Hog1 inhibitor and the control, exhibited comparable levels of cellular survival (Fig. 5D). However, the survival was diminished in the yeast treated with high levels of NO. We also examined the cellular survival in cells treated with high levels of

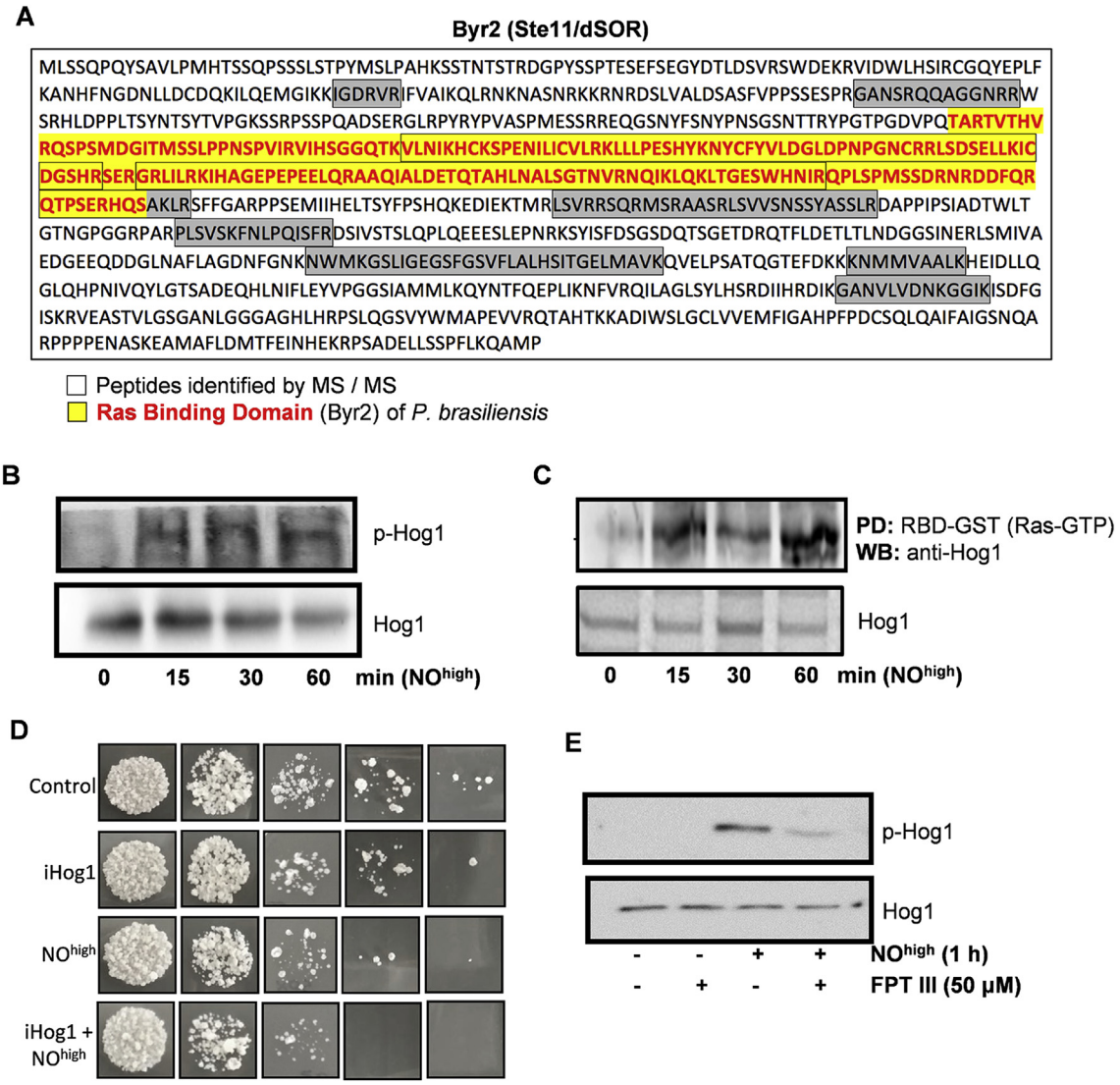

Fig. 5. Cross-talk between Ras and Hog1 pathways in *P. brasiliensis*. (A) Sequence deduced from the protein Byr2 (Ser/Thr kinase - dual specificity mitogen-activated protein kinase dSOR1) (PADG_02230) of *P. brasiliensis*. The boxes indicate fragments obtained by mass spectrometry for the Byr2 protein from the samples incubated with the RBD (Byr2)-GST probe. The Ras Binding Domain (RBD) region of the protein is indicated in yellow. (B) Yeast cells were incubated with 4 mM NO (in mYPD pH 5,6) at 37 °C for increasing periods. Hog1 phosphorylation and total Hog1 (50 µg total protein) levels were assayed by western blots probed with a mouse monoclonal anti-phospho-Hog1 (Thr 171 and Tyr 173) and anti-Hog1 antibodies, respectively. (C) *P. brasiliensis* yeast cells were stimulated with 4 mM up to 60 min at 37 °C. After pulldown assay with RBD (Byr2)-GST probe, association with Ras was detected using an anti-Hog1 antibody. (D) Yeast cells were pre-incubated for 24 h with 50 µM FPT III before stimulation with 4 mM for 60 min at 37 °C. Hog1 phosphorylation and total Hog1 (50 µg total protein) levels were assayed by western blots probed with a mouse monoclonal anti-phospho-Hog1 (Thr 171 and Tyr 173) and anti-Hog1 antibodies, respectively. (E) Spot assay under nitrosative stress before and after treatment with Hog1 inhibitor (10 µM). A total of 2×10^6 *P. brasiliensis* yeast cells were cultivated in modified YPD for 5 days at 37 °C, subjected to a 24-h period of starvation with RPMI medium. Yeast cells were preincubated with Hog1 inhibitor (10 µM) and incubated with 4 mM NO for 5 h. Cells were spotted on mYPD and allowed to grow for 7 days. The results shown are representative of three independent experiments.

NO plus a Hog1 inhibitor, which reduced growth when compared with the sample treated with NO (Fig. 5D). These results showed that the inhibition of Hog1 decreased cellular survival under nitrosative stress, suggesting that the Hog1 activation impedes cell death under nitrosative stress. Finally, we evaluated whether FTP inhibitor III exposure inhibits the Hog1 phosphorylation. We observed Hog1 activation after treatment with NO, but the Ras inhibitor caused a decreased in the phosphorylation of Hog1 upon NO exposure (Fig. 5E). Thus, all these data indicate that the Ras and Hog-1 pathways may cross-talk in response to nitrosative stress in *P. brasiliensis*.

4. Discussion

Phagocytic cells generate potent ROS and RNS, which are toxic to most fungal pathogens [37]. However, some fungi have an efficient response that detoxifies these chemicals and repairs the molecular damage that they cause. This response helps fungal pathogens to survive their initial contact with the host immune system, which is predicted to be an important mechanism of virulence and is crucial for establishing disease [22,38]. NO homeostasis, based on the balance between NO synthesis and degradation, is important for regulating its physiological functions, since an excess of NO causes nitrosative stress due to the high reactivity of NO and NO-derived compounds [39]. In general, NO has toxic effects at higher concentrations. The cytotoxic effects caused by excessive levels of NO occur through various mechanisms, such as an increase in metal toxicity [40] and the promotion of glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-targeted proteolysis by metacaspase through the *S*-nitrosylation of GAPDH [41,42]. On the other hand,

Table 2
Kinases identified by LC-MS/MS.

ID[a]	Protein	Peptides	Coverage
PADG_05489	Ras1	20	17.69%
PADG_02153	Hog1	7	19.41%
PADG_02230	Byr2	28	27.33%
PADG_05980	Byr1	12	42.64%
PADG_00597	Sck1	7	7.30%
PADG_01178	STE20/YSK	14	20.35%
PADG_01368	Gad 8	11	18.16%
PADG_02485	CMGC/CK2	4	23.44%
PADG_02880	Kin 1	24	28.05%
PADG_04970	CKI	9	23.57%
PADG_07240	Tim 23	20	20.20%
PADG_07652	CAMK1-CMK	13	25.21%

[a] Accession numbers from *Paracoccidioides brasiliensis* genes http://www.fungidb.org/.

fungi contain several NO detoxification systems, including NO dioxygenase (NOD) and *S*-nitrosoglutathione reductase (GSNOR) [43]. In this study, we examined the response of *P. brasiliensis* yeast cells to nitrosative stress. In our attempt to understand the biological pathways that may be targets for NO, since this free radical shows signaling properties [44], we found that the Ras GTPase and Hog1 pathways play central roles in the responses to nitrosative stress.

Several studies have reported the strong involvement of the Ras protein in response to stress, differentiation and fungal growth after different stimuli [33,45,46], including oxidative stress [47], but the relationship of Ras activation to the nitrosative stress response in fungi has not yet been demonstrated. In mammalian cells, RNS are known to be involved in Ras-dependent cell proliferation [48,49]. Previous data from our group demonstrated that low levels of NO induce cell proliferation of *P. brasiliensis* in a Ras-dependent manner [5]. We developed a specific probe to detect Ras activation (Ras-GTP form) under stimulus with sub-toxic concentrations of NO, and this activation may induce the PKA pathway.

Using the same probe, we demonstrated that a high concentration of NO induces the activation of Ras and consequently the expression of different genes involved in the redox response. Ras can be activated by NO due to *S*-nitrosylation [5,35,49–53]. *S*-nitrosylation is a reversible posttranslational modification derived from the interaction of NO with the thiol group of specific cysteines [54]. Redox regulation of Ras GTPases occurs in a redox-active cysteine (X) present in a conserved NKXD motif [55]. In *P. brasiliensis*, there is a putative site of *S*-nitrosylation in Ras1, Cys123 [5]. Several studies have shown that NO reacts with Ras through Cys 118 (Cys nitrosylable in mammalian cells) to promote nucleotide exchange and, consequently, Ras activation [56–58]. Thus, NO can increase Ras downstream signaling through the mitogen-activated kinase pathway [35]. The ability of Ras to induce different pathways is widely known. Previously, we showed that Ras participates in cell proliferation stimulated by low concentrations of NO [5], but in this study, we observed that high concentrations are also able to activate Ras. In addition, we detected that Ras was *S*-nitrosylated in both conditions, but the levels of Ras *S*-nitrosylation observed in this study were higher. These phenomena, apparently contradictory, can be explained by the dual role that NO exerts in cell signaling [59,60]. NO is a gaseous messenger that affects various biological functions, either at low concentrations as a signal transducer in many physiological processes or at high concentrations as a cytotoxic defense mechanism against pathogens that can also have a role in signaling processes [47]. In this sense, we propose that the Ras-dependent response to nitrosative stress occurs concomitantly with the inhibition of the PKA pathway, as hypothesized by Brown et al. [22], thus releasing the pathway to trigger the stress response genes.

In this study, using the RBD (Byr2)-GST pull-down assay associated with mass spectrometry, we also detected some kinases interacting with Ras-GTP under NO treatment. Among the kinases identified by mass spectrometry, we highlight Ste11 (designated Byr2 in *S. pombe*), Ste7 (Byr1) and Hog1. Interestingly, these kinases are part of a signaling pathway involved in the response to different stress types [33]. Hog1 is

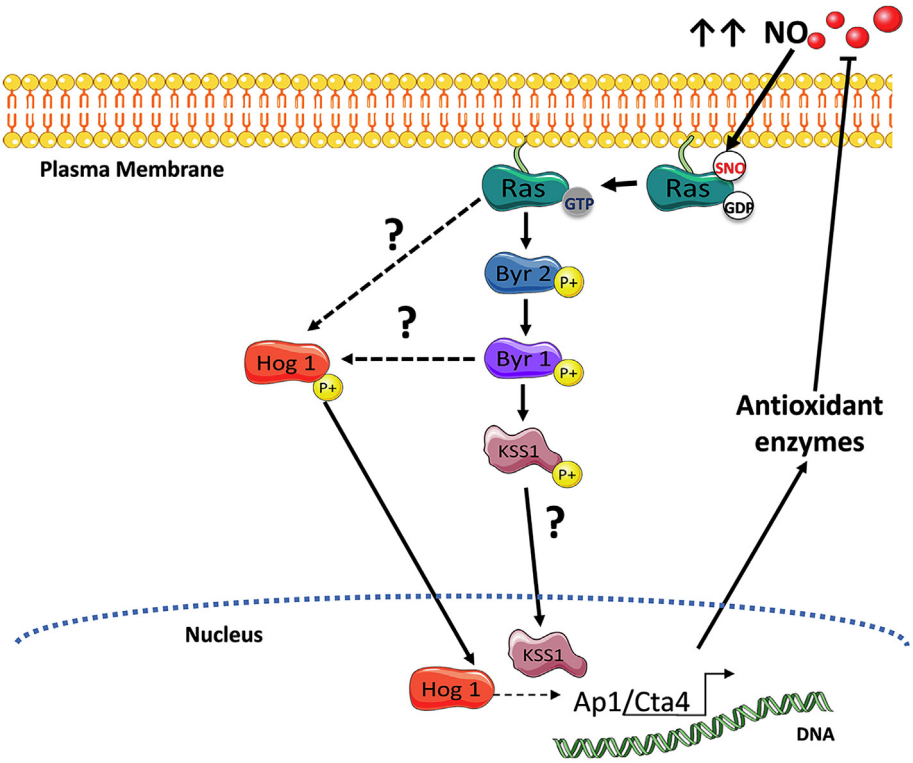

Fig. 6. A model of the cellular response to nitrosative stress in *P. brasiliensis*. NO induces the Ras activation by *S*-nitrosylation, which leads to mitogen-activated protein kinase-dependent phosphorylation (Byr2-Byr1-Hog1), and consequently induces the expression of antioxidant genes.

a component of a highly conserved MAP kinase pathway involved classically in osmo-adaptation in other yeasts [61,62]. In *C. albicans*, this MAP kinase (MAPK) is activated by the MAP kinase (MAPKK) Pbs2 (designated Ste7 in *S. cerevisiae*), which in turn is activated by a single MAP kinase kinase (MAPKKK), Ste11 [33,63,64]. In addition, the Hog1 pathway is also known as an important defense mechanism against oxidative stress [65,66]. Our data revealed the activation mechanism for Hog1 during NO-induced nitrosative stress in *P. brasiliensis* (Fig. 6). Recently, Herrero-de-Dios et al. [67] also showed that Hog1 MAPK contributes to nitrosative stress resistance in *C. albicans*.

Mechanisms to detoxify RNS are broadly conserved, since they are found in organisms ranging from bacteria to humans [68]. Fungi consume NO primarily by a flavohaemoglobin, nitric oxide dioxygenase (Yhb1), which is present in the cytosol and mitochondrial matrix and indirectly participates in the oxidative stress [69]. As expected, the communication between signaling pathways, which respond to oxidative or nitrosative stress, seems to share some components [22]. MAPK are ubiquitous components in the response to almost any stresses imposed on cells [21,70,71]. We observed that after Ras inhibition, some genes involved in oxidative (e.g., *TRX*, *SOD,* and *GR*) and nitrosative (*YHB1*) stress responses decreased their expression after treatment with high concentrations of NO. The response of *C. albicans* to oxidative stress is primarily mediated by Cap1 (AP-1 like) and Hog1 signaling [20,72–74], while the resistance to nitrosative stress is dependent on the transcription factor Cta4 [75]. In *Sporothrix* spp., it was also observed that the signaling pathway to oxidative stress is conserved and that Cap1 also participates in this response [76]. Thus, our data suggest that nitrosative stress activates cross-talk between the Ras-Hog1 pathway that probably mediates the activation of AP-1 and Cta4 transcription factors in *P. brasiliensis* (Fig. 6).

Cross-talk between the Ras and Hog1 pathways has been confirmed in *S. cerevisiae*, *C. albicans* and *Beauveria bassiana* [77–83]. However, in these different studies, it was verified that the types of responses, activation or inhibition of Ras, varied according to the stimulus. Ras pathway signaling is a critical virulence determinant in pathogenic fungi [8,84], and therefore, this pathway has been explored as a potential target for novel antifungal therapies [84]. Finally, based on all this evidence, our findings provide the first insight into the significance of Ras-GTPase and Hog1 MAPK pathways for the *P. brasiliensis* adaptation to nitrosative stress.

Funding

This work was supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo/Brazil (FAPESP) (grants numbers 2014/52,730–7 and 2017/04592–0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico/Brazil (CNPq) (grant number 478,023/2013–8).

References

[1] G.D. Brown, D.W. Denning, N.A.R. Gow, S.M. Levitz, M.G. Netea, T.C. White, Hidden killers: human fungal infections, Sci. Transl. Med. 4 (2012) 165rv13, https://doi.org/10.1126/scitranslmed.3004404.

[2] R. Martinez, New trends in paracoccidioidomycosis epidemiology, J. Fungi. 3 (2017) 1, https://doi.org/10.3390/jof3010001.

[3] V.L.G. Calich, T.A. da Costa, M. Felonato, C. Arruda, S. Bernardino, F.V. Loures, L.R.R. Ribeiro, R. de Cássia Valente-Ferreira, A. Pina, Innate immunity to *Paracoccidioides brasiliensis* infection, Mycopathologia 165 (2008) 223–236, https://doi.org/10.1007/s11046-007-9048-1.

[4] M. Livonesi, M. Rossi, J. Desouto, A. Campanelli, R. Desousa, C. Maffei, B. Ferreira, R. Martinez, J. Da Silva, Inducible nitric oxide synthase-deficient mice show exacerbated inflammatory process and high production of both Th1 and Th2 cytokines during paracoccidioidomycosis, Microb. Infect. 11 (2009) 123–132, https://doi.org/10.1016/j.micinf.2008.10.015.

[5] A.E.C.J. Haniu, J.T. Maricato, P.P.M. Mathias, D.G. Castilho, R.B. Miguel, H.P. Monteiro, R. Puccia, W.L. Batista, Low concentrations of hydrogen peroxide or nitrite induced of *Paracoccidioides brasiliensis* cell proliferation in a Ras-dependent manner, PLoS One 8 (2013) e69590, , https://doi.org/10.1371/journal.pone.0069590.

[6] A.E. Karnoub, R.A. Weinberg, Ras oncogenes: split personalities, Nat. Rev. Mol. Cell Biol. 9 (2008) 517–531, https://doi.org/10.1038/nrm2438.

[7] J.A. Alspaugh, L.M. Cavallo, J.R. Perfect, J. Heitman, RAS1 regulates filamentation, mating and growth at high temperature of *Cryptococcus neoformans*, Mol. Microbiol. 36 (2000) 352–365.

[8] D.A. Hogan, P. Sundstrom, The Ras/cAMP/PKA signaling pathway and virulence in *Candida albicans*, Future Microbiol. 4 (2009) 1263–1270, https://doi.org/10.2217/fmb.09.106.

[9] E. Leberer, D. Harcus, D. Dignard, L. Johnson, S. Ushinsky, D.Y. Thomas, K. Schröppel, Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus *Candida albicans*, Mol. Microbiol. 42 (2001) 673–687.

[10] P. Bolourani, G.B. Spiegelman, G. Weeks, Delineation of the roles played by RasG and RasC in cAMP-dependent signal transduction during the early development of *Dictyostelium discoideum*, Mol. Biol. Cell 17 (2006) 4543–4550, https://doi.org/10.1091/mbc.e05-11-1019.

[11] J.R. Fortwendel, K.K. Fuller, T.J. Ptephens, W.C. Bacon, D.S. Askew, J.C. Rhodes, *Aspergillus fumigatus* RasA regulates asexual development and cell wall integrity, Eukaryot. Cell 7 (2008) 1530–1539, https://doi.org/10.1128/EC.00080-08.

[12] L. Fernandes, H.C. Paes, A.H. Tavares, S.S. Silva, A. Dantas, C.M.A. Soares, F.A.G. Torres, M.S.S. Felipe, Transcriptional profile of ras1 and ras2 and the potential role of farnesylation in the dimorphism of the human pathogen *Paracoccidioides brasiliensis*, FEMS Yeast Res. 8 (2008) 300, https://doi.org/10.1111/j.1567-1364.2007.00317.x.

[13] J. Chow, M. Notaro, A. Prabhakar, S. Free, P. Cullen, Impact of fungal MAPK pathway targets on the cell wall, J. Fungi. 4 (2018) 93, https://doi.org/10.3390/jof4030093.

[14] C.A. Chavel, L.M. Caccamise, B. Li, P.J. Cullen, Global regulation of a differentiation MAPK pathway in yeast, Genetics 198 (2014) 1309–1328, https://doi.org/10.1534/genetics.114.168252.

[15] A. Winkler, C. Arkind, C.P. Mattison, A. Burkholder, K. Knoche, I. Ota, Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress, Eukaryot. Cell 1 (2002) 163–173.

[16] N.T. Gross, K. Nessa, P. Camner, C. Jarstrand, Production of nitric oxide by rat alveolar macrophages stimulated by *Cryptococcus neoformans* or *Aspergillus fumigatus*, Med. Mycol. 37 (1999) 151–157, https://doi.org/10.1046/j.1365-280X.1999.00209.x.

[17] M. De Jesús-Berríos, L. Liu, J.C. Nussbaum, G.M. Cox, J.S. Stamler, J. Heitman, Enzymes that counteract nitrosative stress promote fungal virulence, Curr. Biol. 13 (2003) 1963–1968, https://doi.org/10.1016/j.cub.2003.10.029.

[18] A. Tillmann, N.A.R. Gow, A.J.P. Brown, Nitric oxide and nitrosative stress tolerance in yeast, Biochem. Soc. Trans. 39 (2011) 219–223, https://doi.org/10.1042/BST0390219.

[19] A. da S. Dantas, A. Day, M. Ikeh, I. Kos, B. Achan, J. Quinn, Oxidative stress responses in the human fungal pathogen, *Candida albicans*, Biomolecules 5 (2015) 142–165, https://doi.org/10.3390/biom5010142.

[20] C. Komalapriya, D. Kaloriti, A.T. Tillmann, Z. Yin, C. Herrero-de-Dios, M.D. Jacobsen, R.C. Belmonte, G. Cameron, K. Haynes, C. Grebogi, A.P.S. de Moura, N.A.R. Gow, M. Thiel, J. Quinn, A.J.P. Brown, M.C. Romano, Integrative model of oxidative stress adaptation in the fungal pathogen *Candida albicans*, PLoS One 10 (2015) e0137750, , https://doi.org/10.1371/journal.pone.0137750.

[21] C.H. de Dios, E. Román, R.A. Monge, J. Pla, The role of MAPK signal transduction pathways in the response to oxidative stress in the fungal pathogen *Candida albicans*: implications in virulence, Curr. Protein Pept. Sci. 11 (2010) 693–703.

[22] A.J.P. Brown, K. Haynes, J. Quinn, Nitrosative and oxidative stress responses in fungal pathogenicity, Curr. Opin. Microbiol. 12 (2009) 384–391, https://doi.org/10.1016/j.mib.2009.06.007.

[23] C. Nathan, M.U. Shiloh, Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens, Proc. Natl. Acad. Sci. U. S. A 97 (2000) 8841–8848.

[24] D. Schnappinger, S. Ehrt, M.I. Voskuil, Y. Liu, J.A. Mangan, I.M. Monahan, G. Dolganov, B. Efron, P.D. Butcher, C. Nathan, G.K. Schoolnik, Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment, J. Exp. Med. 198 (2003) 693–704, https://doi.org/10.1084/jem.20030846.

[25] K.Y. Rhee, H. Erdjument-Bromage, P. Tempst, C.F. Nathan, S-nitroso proteome of Mycobacterium tuberculosis: enzymes of intermediary metabolism and antioxidant defense, Proc. Natl. Acad. Sci. U. S. A 102 (2005) 467–472, https://doi.org/10.1073/pnas.0406133102.

[26] P. Xander, A.F. Vigna, L.D.S. Feitosa, L. Pugliese, A.M. Bailão, C.M. de A. Soares, R.A. Mortara, M. Mariano, J.D. Lopes, A surface 75-kDa protein with acid phosphatase activity recognized by monoclonal antibodies that inhibit *Paracoccidioides brasiliensis* growth, Microb. Infect. 9 (2007) 1484–1492, https://doi.org/10.1016/j.micinf.2007.08.001.

[27] M. van Triest, J. de Rooij, J.L. Bos, Measurement of GTP-bound Ras-like GTPases by activation-specific probes, Methods Enzymol. 333 (2001) 343–348.

[28] S. Colombo, D. Ronchetti, J.M. Thevelein, J. Winderickx, E. Martegani, Activation state of the Ras2 protein and glucose-induced signaling in *Saccharomyces cerevisiae*, J. Biol. Chem. 279 (2004) 46715–46722, https://doi.org/10.1074/jbc.M405136200.

[29] M.T. Forrester, M.W. Foster, M. Benhar, J.S. Stamler, Detection of protein S-nitrosylation with the biotin-switch technique, Free Radic. Biol. Med. 46 (2009) 119–126, https://doi.org/10.1016/j.freeradbiomed.2008.09.034.

[30] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (2008) 1101–1108.

[31] D. Szklarczyk, J.H. Morris, H. Cook, M. Kuhn, S. Wyder, M. Simonovic, A. Santos, N.T. Doncheva, A. Roth, P. Bork, L.J. Jensen, C. von Mering, The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible, Nucleic Acids Res. 45 (2017) D362–D368, https://doi.org/10.1093/nar/gkw937.
[32] R. Bologna-Molina, A. Mosqueda-Taylor, N. Molina-Frechero, A.-D. Mori-Estevez, G. Sánchez-Acuña, Comparison of the value of PCNA and Ki-67 as markers of cell proliferation in ameloblastic tumors, Med. Oral Patol. Oral Cir. Bucal 18 (2013) e174–e179.
[33] F. Tamanoi, Ras signaling in yeast, Genes Cancer 2 (2011) 210–215, https://doi.org/10.1177/1947601911407322.
[34] D.T. Hess, J.S. Stamler, Regulation by *S*-nitrosylation of protein post-translational modification, J. Biol. Chem. 287 (2012) 4411–4418, https://doi.org/10.1074/jbc.R111.285742.
[35] H.M. Lander, J.S. Ogiste, S.F. Pearce, R. Levi, A. Novogrodsky, Nitric oxide-stimulated guanine nucleotide exchange on p21ras, J. Biol. Chem. 270 (1995) 7017–7020.
[36] C. Song, M. Ye, Z. Liu, H. Cheng, X. Jiang, G. Han, Z. Songyang, Y. Tan, H. Wang, J. Ren, Y. Xue, H. Zou, Systematic analysis of protein phosphorylation networks from phosphoproteomic data, Mol. Cell. Proteomics 11 (2012) 1070–1083, https://doi.org/10.1074/mcp.M111.012625.
[37] C. Bogdan, M. Röllinghoff, A. Diefenbach, Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity, Curr. Opin. Immunol. 12 (2000) 64–76.
[38] T.A. Missall, J.K. Lodge, J.E. McEwen, Mechanisms of resistance to oxidative and nitrosative stress: implications for fungal survival in mammalian hosts, Eukaryot. Cell 3 (2004) 835–846, https://doi.org/10.1128/EC.3.4.835-846.2004.
[39] L.A. Ridnour, D.D. Thomas, D. Mancardi, M.G. Espey, K.M. Miranda, N. Paolocci, M. Feelisch, J. Fukuto, D.A. Wink, The chemistry of nitrosative stress induced by nitric oxide and reactive nitrogen oxide species. Putting perspective on stressful biological situations, Biol. Chem. 385 (2004) 1–10, https://doi.org/10.1515/BC.2004.001.
[40] K.T. Chiang, M. Shinyashiki, C.H. Switzer, J.S. Valentine, E.B. Gralla, D.J. Thiele, J.M. Fukuto, Effects of Nitric oxide on the copper-responsive transcription factor Ace 1 in *Saccharomyces cerevisiae*: cytotoxic and cytoprotective actions of Nitric Oxide, Arch. Biochem. Biophys. 377 (2000) 296–303, https://doi.org/10.1006/abbi.2000.1785.
[41] A. Silva, B. Almeida, B. Sampaio-Marques, M.I.R. Reis, S. Ohlmeier, F. Rodrigues, A. do Vale, P. Ludovico, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a specific substrate of yeast metacaspase, Biochim. Biophys. Acta Mol. Cell Res. 1813 (2011) 2044–2049, https://doi.org/10.1016/j.bbamcr.2011.09.010.
[42] B. Almeida, S. Buttner, S. Ohlmeier, A. Silva, A. Mesquita, B. Sampaio-Marques, N.S. Osorio, A. Kollau, B. Mayer, C. Leao, J. Laranjinha, F. Rodrigues, F. Madeo, P. Ludovico, NO-mediated apoptosis in yeast, J. Cell Sci. 120 (2007) 3279–3288, https://doi.org/10.1242/jcs.010926.
[43] R.I. Astuti, R. Nasuno, H. Takagi, Nitric oxide signalling in yeast, Adv. Microb. Physiol. (2018) 29–63, https://doi.org/10.1016/bs.ampbs.2018.01.003.
[44] H.P. Monteiro, A. Stern, Redox modulation of tyrosine phosphorylation-dependent signal transduction pathways, Free Radic. Biol. Med. 21 (1996) 323–333.
[45] P.J. Cullen, G.F. Sprague, The regulation of filamentous growth in yeast, Genetics 190 (2012) 23–49, https://doi.org/10.1534/genetics.111.127456.
[46] W.J. Belden, L.F. Larrondo, A.C. Froehlich, M. Shi, C.-H. Chen, J.J. Loros, J.C. Dunlap, The band mutation in Neurospora crassa is a dominant allele of ras-1 implicating RAS signaling in circadian output, Genes Dev. 21 (2007) 1494–1505, https://doi.org/10.1101/gad.1551707.
[47] M.A. de la Torre-Ruiz, N. Pujol, V. Sundaran, Coping with oxidative stress. The yeast model, Curr. Drug Targets 16 (2015) 2–12.
[48] A. Villalobo, Nitric oxide and cell proliferation, FEBS J. 273 (2006) 2329–2344, https://doi.org/10.1111/j.1742-4658.2006.05250.x.
[49] W.L. Batista, F.T. Ogata, M.F. Curcio, R.B. Miguel, R.J. Arai, A.L. Matsuo, M.S. Moraes, A. Stern, H.P. Monteiro, S-nitrosoglutathione and endothelial nitric oxide synthase-derived nitric oxide regulate compartmentalized ras S-nitrosylation and stimulate cell proliferation, Antioxidants Redox Signal. 18 (2013) 221–238, https://doi.org/10.1089/ars.2011.4455.
[50] I.M. Ahearn, K. Haigis, D. Bar-Sagi, M.R. Philips, Regulating the regulator: post-translational modification of RAS, Nat. Rev. Mol. Cell Biol. 13 (2012) 39–51, https://doi.org/10.1038/nrm3255.
[51] H.P. Monteiro, P.E. Costa, A.K.C.A. Reis, A. Stern, Nitric oxide: protein tyrosine phosphorylation and protein S-nitrosylation in cancer, Biomed. J. 38 (2015) 380–388, https://doi.org/10.4103/2319-4170.158624.
[52] A. Martínez-Ruiz, Post-Translational nitric oxide-dependent modifications in immune system, Redox Biol 5 (2015) 418–419, https://doi.org/10.1016/j.redox.2015.09.027.
[53] K.-H. Lim, B.B. Ancrile, D.F. Kashatus, C.M. Counter, Tumour maintenance is mediated by eNOS, Nature 452 (2008) 646–649, https://doi.org/10.1038/nature06778.
[54] A.M. Evangelista, M.J. Kohr, E. Murphy, S. nitrosylation, specificity, occupancy, and interaction with other post-translational modifications, Antioxidants Redox Signal. 19 (2013) 1209–1219, https://doi.org/10.1089/ars.2012.5056.
[55] L. Mitchell, G.A. Hobbs, A. Aghajanian, S.L. Campbell, Redox regulation of Ras and Rho GTPases: mechanism and function, Antioxidants Redox Signal. 18 (2013) 250–258, https://doi.org/10.1089/ars.2012.4687.
[56] M.F. Davis, D. Vigil, S.L. Campbell, Regulation of Ras proteins by reactive nitrogen species, Free Radic. Biol. Med. 51 (2011) 565–575, https://doi.org/10.1016/j.freeradbiomed.2011.05.003.
[57] K.W. Raines, M.G. Bonini, S.L. Campbell, Nitric oxide cell signaling: S-nitrosation of Ras superfamily GTPases, Cardiovasc. Res. 75 (2007) 229–239, https://doi.org/10.1016/j.cardiores.2007.04.013.
[58] J. Heo, K.C. Prutzman, V. Mocanu, S.L. Campbell, Mechanism of free radical nitric oxide-mediated Ras guanine nucleotide dissociation, J. Mol. Biol. 346 (2005) 1423–1440, https://doi.org/10.1016/j.jmb.2004.12.050.
[59] S. Mocellin, V. Bronte, D. Nitti, Nitric oxide, a double edged sword in cancer biology: searching for therapeutic opportunities, Med. Res. Rev. 27 (2007) 317–352, https://doi.org/10.1002/med.20092.
[60] S.S. Ramesh, A. Prasanthi, D.I. Bhat, B.I. Devi, R. Cristopher, M. Philip, Correlation between plasma total nitric oxide levels and cerebral vasospasm and clinical outcome in patients with aneurysmal subarachnoid hemorrhage in Indian population, J. Neurosci. Rural Pract. 5 (2014) S22–S27, https://doi.org/10.4103/0976-3147.145196.
[61] E. Nikolaou, I. Agriafioti, M. Stumpf, J. Quinn, I. Stansfield, A.J. Brown, Phylogenetic diversity of stress signaling pathways in fungi, BMC Evol. Biol. 9 (2009) 44, https://doi.org/10.1186/1471-2148-9-44.
[62] D.A. Smith, B.A. Morgan, J. Quinn, Stress signalling to fungal stress-activated protein kinase pathways, FEMS Microbiol. Lett. 306 (2010) 1–8, https://doi.org/10.1111/j.1574-6968.2010.01937.x.
[63] D.M. Arana, The Pbs2 MAP kinase kinase is essential for the oxidative-stress response in the fungal pathogen *Candida albicans*, Microbiology 151 (2005) 1033–1049, https://doi.org/10.1099/mic.0.27723-0.
[64] J. Cheetham, D.A. Smith, A. da Silva Dantas, K.S. Doris, M.J. Patterson, C.R. Bruce, J. Quinn, A single MAPKKK regulates the Hog1 MAPK pathway in the pathogenic fungus *Candida albicans*, Mol. Biol. Cell 18 (2007) 4603–4614, https://doi.org/10.1091/mbc.e07-06-0581.
[65] R. Alonso-Monge, F. Navarro-García, E. Román, A.I. Negredo, B. Eisman, C. Nombela, J. Pla, The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*, Eukaryot. Cell 2 (2003) 351–361.
[66] L. Staleva, A. Hall, S.J. Orlow, Oxidative stress activates FUS1 and RLM1 transcription in the yeast Saccharomyces cerevisiae in an oxidant-dependent Manner, Mol. Biol. Cell 15 (2004) 5574–5582, https://doi.org/10.1091/mbc.e04-02-0142.
[67] C. Herrero-de-Dios, A.M. Day, A.T. Tillmann, S.L. Kastora, D. Stead, P.S. Salgado, J. Quinn, A.J.P. Brown, Redox regulation, rather than stress-induced phosphorylation, of a Hog1 mitogen-activated protein kinase modulates its nitrosative-stress-specific outputs, mBio 9 (2018) e02229-17, , https://doi.org/10.1128/mBio.02229-17.
[68] L. Liu, A. Hausladen, M. Zeng, L. Que, J. Heitman, J.S. Stamler, A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans, Nature 410 (2001) 490–494, https://doi.org/10.1038/35068596.
[69] N. Cassanova, K.M. O'Brien, B.T. Stahl, T. McClure, R.O. Poyton, Yeast flavohemoglobin, a nitric oxide oxidoreductase, is located in both the cytosol and the mitochondrial matrix: effects of respiration, anoxia, and the mitochondrial genome on its intracellular level and distribution, J. Biol. Chem. 280 (2005) 7645–7653, https://doi.org/10.1074/jbc.M411478200.
[70] A.J.P. Brown, S. Budge, D. Kaloriti, A. Tillmann, M.D. Jacobsen, Z. Yin, I. V Ene, I. Bohovych, D. Sandai, S. Kastora, J. Potrykus, E.R. Ballou, D.S. Childers, S. Shahana, M.D. Leach, Stress adaptation in a pathogenic fungus, J. Exp. Biol. 217 (2014) 144–155, https://doi.org/10.1242/jeb.088930.
[71] K. Shiraishi, T. Hioki, A. Habata, H. Yurimoto, Y. Sakai, Yeast Hog1 proteins are sequestered in stress granules under high-temperature stress, J. Cell Sci. 131 (2017) 209114, https://doi.org/10.1242/jcs.209114 jcs.
[72] D.A. Smith, S. Nicholls, B.A. Morgan, A.J.P. Brown, J. Quinn, A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*, Mol. Biol. Cell 15 (2004) 4179–4190, https://doi.org/10.1091/mbc.E04-03-0181.
[73] X. Zhang, M. De Micheli, S.T. Coleman, D. Sanglard, W.S. Moye-Rowley, Analysis of the oxidative stress regulation of the *Candida albicans* transcription factor, Cap1p., Mol. Microbiol. 36 (2000) 618–629.
[74] I. Kos, M.J. Patterson, S. Znaidi, D. Kaloriti, A. da Silva Dantas, C.M. Herrero-de-Dios, C. D'Enfert, A.J.P. Brown, J. Quinn, Mechanisms underlying the delayed activation of the Cap1 transcription factor in *Candida albicans* following combinatorial oxidative and cationic stress important for phagocytic potency, mBio 7 (2016) e00331, , https://doi.org/10.1128/mBio.00331-16.
[75] W. Chiranand, I. McLeod, H. Zhou, J.J. Lynn, L.A. Vega, H. Myers, J.R. Yates, M.C. Lorenz, M.C. Gustin, CTA4 transcription factor mediates induction of nitrosative stress response in *Candida albicans*, Eukaryot. Cell 7 (2008) 268–278, https://doi.org/10.1128/EC.00240-07.
[76] I. Ortega, M.S. Soares Felipe, A.T.R. Vasconcelos, L.M. Lopes Bezerra, A. Da Silva Dantas, Peroxide sensing and signaling in the *Sporothrix schenckii* complex: an in silico analysis to uncover putative mechanisms regulating the Hog1 and AP-1 like signaling pathways, Med. Mycol. 53 (2015) 51–59, https://doi.org/10.1093/mmy/myu069.
[77] G. Boguslawski, PBS2, a yeast gene encoding a putative protein kinase, interacts with the RAS2 pathway and affects osmotic sensitivity of *Saccharomyces cerevisiae*, J. Gen. Microbiol. 138 (1992) 2425–2432, https://doi.org/10.1099/00221287-138-11-2425.
[78] C. Schüller, J.L. Brewster, M.R. Alexander, M.C. Gustin, H. Ruis, The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* CTT1 gene, EMBO J. 13 (1994) 4382–4389.
[79] J.C. Varela, U.M. Praekelt, P.A. Meacock, R.J. Planta, W.H. Mager, The *Saccharomyces cerevisiae* HSP12 gene is activated by the high-osmolarity glycerol pathway and negatively regulated by protein kinase A, Mol. Cell Biol. 15 (1995) 6232–6245.
[80] A. Deveau, A.E. Piispanen, A.A. Jackson, D.A. Hogan, Farnesol Induces Hydrogen

peroxide resistance in *Candida albicans* yeast by inhibiting the Ras-cyclic AMP signaling pathway, Eukaryot. Cell 9 (2010) 569–577, https://doi.org/10.1128/EC.00321-09.

[81] J. Gutin, A. Sadeh, A. Rahat, A. Aharoni, N. Friedman, Condition-specific genetic interaction maps reveal crosstalk between the cAMP/PKA and the HOG MAPK pathways in the activation of the general stress response, Mol. Syst. Biol. 11 (2015) 829.

[82] J. Liu, Z.-K. Wang, H.-H. Sun, S.-H. Ying, M.-G. Feng, Characterization of the Hog1 MAPK pathway in the entomopathogenic fungus *Beauveria bassiana*, Environ. Microbiol. 19 (2017) 1808–1821, https://doi.org/10.1111/1462-2920.13671.

[83] Y. Guan, D.-Y. Wang, S.-H. Ying, M.-G. Feng, A novel Ras GTPase (Ras 3) regulates conidiation, multi-stress tolerance and virulence by acting upstream of Hog1 signaling pathway in *Beauveria bassiana*, Fungal Genet. Biol. 82 (2015) 85–94, https://doi.org/10.1016/j.fgb.2015.07.002.

[84] Q. Al Abdallah, J.R. Fortwendel, Exploration of *Aspergillus fumigatus* Ras pathways for novel antifungal drug targets, Front. Microbiol. 6 (2015) 128, https://doi.org/10.3389/fmicb.2015.00128.