Current Genetics (2020) 66:1135–1153
https://doi.org/10.1007/s00294-020-01099-3

ORIGINAL ARTICLE

Contribution of the mitogen-activated protein kinase Hog1 to the halotolerance of the marine yeast *Debaryomyces hansenii*

Norma Silvia Sánchez[1] · Martha Calahorra[1] · James González[2] · Tatiana Defosse[3] · Nicolas Papon[3] · Antonio Peña[1] · Roberto Coria[1]

Received: 5 June 2020 / Revised: 10 July 2020 / Accepted: 17 July 2020 / Published online: 27 July 2020
© Springer-Verlag GmbH Germany, part of Springer Nature 2020

Abstract
Halotolerant species are adapted to dealing continually with hyperosmotic environments, having evolved strategies that are uncommon in other organisms. The HOG pathway is the master system that regulates the cellular adaptation under these conditions; nevertheless, apart from the importance of *Debaryomyces hansenii* as an organism representative of the halotolerant class, its HOG1 pathway has been poorly studied, due to the difficulty of applying conventional recombinant DNA technology. Here we describe for the first time the phenotypic characterisation of a null *HOG1* mutant of *D. hansenii*. *Dhhog1Δ* strain was found moderately resistant to 1 M NaCl and sensitive to higher concentrations. Under hyperosmotic shock, DhHog1 fully upregulated transcription of *DhSTL1* and partially upregulated that of *DhGPD1*. High osmotic stress lead to long-term inner glycerol accumulation that was partially dependent on DhHog1. These observations indicated that the HOG pathway is required for survival under high external osmolarity but dispensable under low and mid-osmotic conditions. It was also found that DhHog1 can regulate response to alkali stress during hyperosmotic conditions and that it plays a role in oxidative and endoplasmic reticulum stress. Taken together, these results provide new insight into the contribution of this MAPK in halotolerance of this yeast.

Keywords HOG pathway · Glycerol · Transcription regulation · Stl1 symporter · Gpd1 · Alkali-halotolerant · Oxidative stress · ER stress

Introduction

Debaryomyces hansenii (anamorph: *Candida famata*) is a halotolerant yeast able to grow in the presence of high NaCl concentrations. This yeast can tolerate as much as 4 M of salt, while the growth of the budding yeast *Saccharomyces cerevisiae* is completely inhibited with 1.7 M NaCl (Onishi 1963; Prista et al. 2016). Originally isolated from seawater (Norkrans 1966), *D. hansenii* can also be found in salty and fermented foods with high osmolarity (Gori et al. 2012; Ramos et al. 2017). Importantly and due to its ability to inhibit fungal growth, this yeast species can be employed in the prevention of food contamination (Medina-Córdova et al. 2018).

The halotolerant behaviour of *D. hansenii* is the result of several properties acting in concert. These include its particular potassium and sodium homeostasis (Norkrans 1966, 1968), its membrane composition (sterol-to-phospholipid ratio) (Turk et al. 2007), the presence of glycerol and alkali-metal-cation transporters (Prista et al. 1997, 2016; Lages

Communicated by M. Kupiec.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00294-020-01099-3) contains supplementary material, which is available to authorized users.

✉ Norma Silvia Sánchez
nsanchez@ifc.unam.mx

✉ Roberto Coria
rcoria@ifc.unam.mx

[1] Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 México City, CDMX, México

[2] Departamento de Biología Celular, Facultad de Ciencias, Universidad Nacional Autónoma de México. Ciudad Universitaria, 04510 México City, CDMX, México

[3] Groupe d'Etude des Interactions Hôte-Pathogène, GEIHP, EA 3142, SFR ICAT 4208, UNIV Angers, UNIV Brest, Institut de Biologie en Santé, IRIS, CHU Angers, Angers, France

et al. 1999; Ramos et al. 2011; Martínez et al. 2011; Michán et al. 2013), the resistance of some enzymes to salt stress or expression induced by high salt concentrations (Alba-Lois et al. 2004; Sharma et al. 2005; Guerrero et al. 2005; Minhas et al. 2012; Chawla et al. 2017), and energy obtaining pathways that are optimised in the presence of salts (Sánchez et al. 2006, 2008; Calahorra et al. 2009; Cabrera-Orefice et al. 2014a, b) among others.

Highly-active glycerol uptake for the maintenance of an adequate intracellular concentration is one of the main processes that characterises halotolerant species (Lages et al. 1999). Indeed, in *D. hansenii*, as in many yeast species, glycerol has been found to be a preferential compatible solute (Gustafsson and Norkrans 1976; André et al. 1988; Larsson et al. 1990); however, *D. hansenii* is also able to efficiently produce trehalose or arabinitol as compatible solutes under high salt concentration (Adler and Gustafsson 1980; Blomberg and Adler 1992; González-Hernández et al. 2005). The intracellular level of glycerol depends on its synthesis, utilisation and transport, responding to various stress solutes besides NaCl, i.e. KCl, sucrose or Na_2SO_4 (André et al. 1988; Neves et al. 1997). The main metabolic pathways (Adler et al. 1985; André et al. 1988) and the transport systems (Lages et al. 1999; Neves et al. 2004; Klein et al. 2017) involved in glycerol metabolism in *D. hansenii* are similar to those present in *S. cerevisiae*, with the exception of the occurrence of a *D. hansenii* Na^+/glycerol symporter that can also use potassium ions to internalise glycerol (Lucas et al. 1990).

Upon hyperosmotic stress, glycerol synthesis in *S. cerevisiae* as well as in other eukaryotic cells is mainly driven by the HOG1 (High Osmolarity Glycerol) pathway. The exposure to hyperosmotic stress triggers this MAPK pathway, which comprises two sub-pathways: the SHO1 and the SLN1 branches. Each one, by means of a cascade of progressive phosphorylations, activates the Hog1 protein, which internalises into the nucleus and triggers the transcription of genes needed to cope with the osmotic stress, especially those encoding enzymes for the synthesis and accumulation of glycerol (de Nadal et al. 2002; Saito and Posas 2012; Hohmann 2015).

DhGPD1, the gene encoding glycerol-3-phosphate dehydrogenase, which catalyses the first step in glycerol synthesis, was identified, isolated and heterologously expressed in a *gpd1Δ* strain of *S. cerevisiae*. It was found that hyperosmotic stress activated the transcription of *DhGPD1* gene, restoring glycerol production in the *Scgpd1Δ* mutant, responding to the induction exerted by the native ScHog1 (Thomé and Trench 1999; Thomé 2004, 2005). In addition, the regulation of *DhGPD1* and *DhGPP2* genes was analysed in comparison to that of their orthologous in *S. cerevisiae* grown under different NaCl concentrations, and it was confirmed that these genes are upregulated upon hyperosmotic stress and that they play a significant role in the NaCl tolerance of *D. hansenii* (Gori et al. 2005).

Saline tolerance is also achieved by the activity of several ion transporters located at the plasma membrane of yeast cells, including uniporters such as Trk1, antiporters such as Nha1 and the Na^+ -ATPase Ena1 (Ariño et al. 2019). It has been determined that osmotic stress induced by high sodium concentration, increases the transcriptional activity of the *ENA1* gene in *S. cerevisiae* in a Hog1 dependent manner (Marquez and Serrano 1996). Transcription of the orthologues *ENA1* and *ENA2* genes of *D. hansenii* is also increased in high salt concentrations and at least for *ENA2* with high pH (Almagro et al. 2001).

At the present time, the HOG1 pathway of *D. hansenii* has yet to be described. The only reports published include the cloning and sequencing of the *DhHOG1* gene and its heterologous expression in a *S. cerevisiae hog1Δ* mutant (Bansal and Mondal 2000) and a study of the cellular localisation of DhHog1 after hyperosmotic treatment (Sharma et al. 2005). In the former report, the expressed *D. hansenii* gene conferred to the *S. cerevisiae hog1Δ* mutant the ability to grow under hyperosmotic stress and to induce glycerol overproduction. In the latter report, it was observed that phosphorylated DhHog1 had a delayed entry into the *D. hansenii* nucleus and was exported back in the cytoplasm after exposure to severe saline stress in a phosphorylated state. In this study, it was also shown that UV light and oxidative stress additionally induce DhHog1 phosphorylation.

D. hansenii belongs to the monophyletic clade that ambiguously translates CTG into Ser and Leu (Tekaia et al. 2000; Fitzpatrick et al. 2006; Papon et al. 2014). Besides the existence of some reports describing the DNA transformation of *D. hansenii* (Ricaurte and Govind 1999; Voronovsky et al. 2002; Minhas et al. 2009), gene modification using the conventional tools of molecular biology has been challenging in this yeast (Gerami-Nejad et al. 2009). It has been argued that this may be due to its robust cell wall (Gezelius and Norkrans 1970), or because it has a highly-efficient Non-Homologous End Joining (NHEJ) repair system, which does not allow deletion or modification of a gene of interest.

Given the important role that the HOG1 pathway plays in the halotolerance behavior, in the present study we report for the first time its inactivation by deletion of the *DhHOG1* gene in *D. hansenii*. This was achieved using a suitable plasmid, specially constructed for genetic manipulation of the CTG clade yeast (Defosse et al. 2018). The phenotypical and physiological characteristics of this mutant (*Dhhog1Δ*) were investigated so that we might be able to understand the role of Hog1 in *D. hansenii's* halotolerance behaviour as well as to other stresses. We found that DhHog1 was required for survival to high external osmolarity by regulating the expression of genes required for glycerol accumulation and

it also has a role in the response to alkali, oxidative and endoplasmic reticulum stresses.

Materials and methods

Strains, plasmids and culture media

D. hansenii Y7426 was kindly donated by the US Department of Agriculture, Peoria, Illinois, USA. *Dhhog1Δ* (*hog1Δ::SAT1-yeYFP1*) is isogenic to strain Y7426 and its construction is depicted in Fig. 3a. *S. cerevisiae* BY4742 (*MATα, his3-Δ1, leu2-Δ0, lys2-Δ0, ura3-Δ0*) and its isogenic *hog1Δ* strain (*MATα, his3-Δ1, leu2-Δ0, lys2-Δ0, ura3-Δ0, hog1Δ::kanMX*) were obtained from the Yeast Knockout Collection.

Yeast cells were routinely grown at 28 °C in YPD (1% yeast extract, 2% peptone, 2% glucose). For mutant selection and maintenance, YPD was supplemented with 150 µg/ml nourseothricin (clonNAT) (Werner BioAgents GmbH, Germany). Modifications to the media and temperatures are indicated in figure legends of each experiment.

Escherichia coli DH5α strain was used to propagate plasmids. Bacteria were grown at 37 °C in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 100 µg/ml ampicillin for plasmid selection.

The pAYCU244 vector (P_{DhTEF1}-*SAT1*-T_{MgPGK1}-P_{MgACT1}-*yeYFP*-T_{MgTRP1}), especially adapted to *D. hansenii* (Defosse et al. 2018) was used for yeast transformation. The pGEM-T Easy vector (Promega ™) was used for cloning and sequencing of PCR products.

Growth assays

Growth curves were determined in YPD medium supplemented with the indicated NaCl concentrations using 250 ml nephelometric flasks. Cultures were incubated at 28 °C with shaking at 250 rpm. OD readings were obtained every hour using a Klett-Summerson colorimeter. Klett units were converted to OD_{540} by multiplying by 0.002. Equations for linear regressions were calculated at the exponential growth interval in each condition. The relative growth was then calculated as the slope value in each condition in proportion to the slope value of the zero-NaCl condition in each species.

Growth on YPD plates supplemented by the indicated NaCl concentrations (or the indicated oxidative and ER stress agents) was performed by spotting tenfold serial dilutions. Cells were grown overnight in YPD liquid medium and the OD_{600} was adjusted to 1.0. Dilution series were prepared in YPD and aliquots were spotted onto agar plates. Plates were incubated at 28 °C for 48 h and photographed. When required, incubation temperature and time were modified as indicated.

Gene cloning and disruption

The *DhHOG1* gene was amplified by PCR using primers *DhHog1F* and *DhHog1R* (Table S1) and genomic *D. hansenii* DNA as a template. The PCR product was cloned into the pGEM-T Easy Vector.

The *D. hansenii hog1Δ* mutant was constructed by homologous recombination using a PCR amplicon containing the *SAT1-yeYFP* cassette and HOG1 recombinant ends (Fig. 3a). The PCR amplicon was synthesised using the hybrid oligonucleotides *Dhhog1Δp244hyb_F* and *R* (Table S1) and the pAYCU244 plasmid as a template.

DhHog1-yeYFP fusion

DhHOG1 tagging with the yeast enhanced Yellow Fluorescent Protein *(yeYFP)* sequence was achieved by successive PCR reactions using plasmid pAYCU244 as starting template (Fig. 3a). A PCR reaction was designed to fuse the *yeYFP* ORF in frame with the 3′ end of *DhHOG1*, and to eliminate the *HOG1* stop codon at the same time. A separate PCR reaction was carried out to fuse the *SAT1* terminator region with the *DhHOG1* 3′ UTR. These two PCR products were fused in a third PCR reaction that yielded an amplicon of 2899 bp containing 120 bp of the *DhHOG1* 3′ ORF, followed by the *yeYFP* gene (with the *TRP1* terminator), the *SAT1* gene (flanked by the *TEF1* promoter and the *PGK* terminator) and 120 bp of the *DhHOG1* 3′ UTR. The primers used for the PCR reactions are listed in Table S1. The PCR product was sequenced and used for yeast transformation.

Yeast transformation

D. hansenii cells were grown in 10 ml YPD for 24 h. 1 ml of this culture was used to inoculate 50 ml of YPD and allowed to grow for 24 h. Cells were harvested by centrifugation (4500*g*, 10 min, RT), suspended in 6 ml of 50 mM phosphate buffer pH 7.5 to which fresh 25 mM DTT was added, and incubated at 30 °C for 15 min. After centrifugation (4000*g*, 10 min, 4 °C), cells were washed twice with 20 ml ice-cold water, once with 20 ml ice-cold 1 M sorbitol, and finally harvested by centrifugation (4000*g*, 10 min, 4 °C). Cells were resuspended in 3 ml ice-cold 1 M sorbitol. 1 µg DNA (suspended in 10 µl water) was added to a 200 µl cell suspension and transferred to a 0.2 cm gap width electroporation cuvette. A BioRad gene pulser was used for electroporation with an electric pulse of 2.3 kV, 25 µF and 200 ohms. The cells were immediately washed out from the cuvette with 1 ml YPD. This cell suspension was used to inoculate 10 ml of YPD and incubated for 24 h (28 °C, 150 rpm) to allow cell regeneration. 100 µl were plated onto YPD supplemented with 150 µg/ml clonNAT and incubated at 28 °C until colonies appeared (2–3 days). Transformants were

transferred to fresh YPD plus 150 µg/ml of clonNAT and analysed for DNA integration.

Phosphorylation assays and immunoblotting

Western blots were performed as reported by Vázquez-Ibarra et al. (2018). Cells grown overnight in YPD were collected, washed, resuspended in fresh medium and treated with different concentrations of NaCl for different periods of time (as indicated in each figure). Cells were fixed with 85% trichloroacetic acid for 10 min at room temperature (RT) and washed twice with 1 ml water. Cells were lysed with SB-DTT buffer and glass beads as previously described (Velázquez-Zavala et al. 2015). Protein samples (20 µl of cell-free extracts for *D. hansenii* and 3 to 5 µl for *S. cerevisiae*) were resolved by SDS/PAGE and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Edo. de México, México). Immunoblotting was performed using anti-Hog1 (1:1000 y-215; Santa Cruz Biotechnology, Dallas, TX, USA) for total Hog1 detection, and anti-phospho-p38 (1:1000; Cell Signaling Technology, Danvers, MA, USA) for phospho-Hog1 detection. Reconstituted goat anti-rabbit HRP antibody from Invitrogen, G21234 (0.1 mg/ml in 50% glycerol-PBS, 1:10,000) was used as a secondary antibody. Immobilon™ Western Chemiluminescent HRP substrate (Millipore, MA., USA) was used in detect the chemiluminescence. All western blots were repeated three times and representative blots are shown in figures.

Fluorescence microscopy

Cells containing the integrative SAT1-yeYPF cassette into the *DhHOG1* locus were grown overnight in YPD plus 150 µg/ml clonNAT. Cells were harvested and washed with water and observed with an LSM 710-Zeiss microscope with the Zen black 2.3 software. Images were obtained by confocal microscopy in the double photon mode. yeYFP was detected at 514–527 nm, excitation and emission wavelengths respectively.

The DhHog1-yeYFP fusion was detected in cells grown overnight in YPD, collected and suspended at $OD_{600}=1.0$. Cells were then centrifuged and resuspended in fresh YPD plus 2 M NaCl and incubated at 28 °C. 1 ml samples were taken at different times and fixed with 150 ul 37% formaldehyde for 15 min. Fixed cells were pelleted and washed three times with 1 ml PBS. For nucleus staining, DAPI (4′,6-diamidino-2-pheylindole) was added at a final concentration of 90 nM, incubated for 30 min in the dark at RT, washed three times with PBS and suspended in 100 ul PBS. yeYFP was visualised using a NIKON epifluorescence microscope with a green filter. Images were taken and processed using QCapture Pro (version 6.0) software.

Measurements of glycerol production

Overnight cultures were used to prepare 2.0 OD_{600} suspensions in YPD. For each experimental sample, 10 ml of the suspension were centrifuged and re-suspended in 5 ml of fresh YPD supplemented or not with the indicated NaCl concentrations and incubated for 4 h in a rotator at 28 °C. For determination of external glycerol, 1 ml of each suspension was centrifuged at 1625*g* and the supernatant kept on ice. For total glycerol, 1 ml of each suspension was boiled for 15 min in capped tubes, placed on ice for 10 min, vortexed for 2 min and centrifuged; glycerol content was determined in 10 µl of each supernatant with the enzymatic Glycerol kit (Boehringer Mannheim/R-Biopharm) and adapted to be performed in 96 well plates. A POLARstar Omega microplate reader was used for measurement of absorbance at 340 nm. Glycerol content was normalised to the yeast dry weight of each sample. The internal glycerol was calculated by means of the subtraction of the external glycerol value from the total glycerol value in each condition. Data shown are average values ± SD. Every sample was measured in duplicate from 3 biological replicates.

RNA extraction

Overnight cultures of wild type *D. hansenii* and *Dhhog1Δ* mutant were adjusted to 0.5 OD_{600} in 100 ml YPD media and incubated for 3 h at 28 °C with shaking. 50 ml of each culture was centrifuged (5 min, 1750*g*) and resuspended in 10 ml of YPD supplemented or not with 2 M NaCl and incubated for 60 min under the same conditions. After incubation, cells were centrifuged and resuspended in 1 ml of AE buffer (50 mM sodium acetate and 10 mM EDTA) to perform the RNA extraction protocol according to Schmitt et al. (1990). RNA integrity was verified by electrophoresis in a 1% denaturing agarose gel.

Analysis of gene expression

Total RNA was digested with DNaseI (RQ1 RNase-Free DNase, Promega) to remove any contaminating genomic DNA. cDNA synthesis reactions were performed using the ImProm-II™ Reverse Transcription System (Promega kit). Quantitative real-time PCR (qRT-PCR) was performed using the standard curve method with specific primers for the genes *DhGPD1* (ID: 2903610), *DhSTL1* (ID: 2902951) and *DhACT1* (ID: 2901278), encoding putative NAD^+-dependent glycerol-3-phosphate dehydrogenase, the glycerol/H^+ symporter of the plasma membrane and actin, respectively. Oligonucleotides were initially screened for the absence of dimers formation and cross-hybridisation. Oligonucleotide primer pairs with 100% of amplification efficiencies were used (Table S1). Control reactions without

reverse transcriptase were performed. qRT-PCR analysis was performed using a Rotor-Gene Q (Qiagen) machine. The detection dye used was SYBR Green (2 × SYBR Select Mastermix from Applied Biosystems). qRT-PCR was carried out as follows: 95 °C for 5 min (1 cycle), 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 20 s (35 cycles). Transcripts were normalised relative to the *DhACT1* transcript quantities. Relative expression levels (fold induction) were evaluated with respect to wild type cells (control) by the standard curve method. Data shown are the mean values ± SD of four biological replicates.

Results

Determination of the halotolerance level of *D. hansenii*

To determine the maximum NaCl tolerance of *D. hansenii*, we established growth curves in YPD medium supplemented with different salt concentrations. To get a clear idea of the salt resistance of *D. hansenii*, this and the following experiments were conducted in comparison to the halosensitive *S. cerevisiae* strain. Due to the preferred respiratory metabolism of *D. hansenii* (Sánchez et al. 2008), the growing parameters were obtained in well-aerated flasks, to ensure optimal O_2 availability. We detected that as the NaCl concentration increased, the growth velocity of both strains decreased (Fig. 1); however, 1 M NaCl only showed a moderate effect on the *D. hansenii* growth (Fig. 1a) in contrast to *S. cerevisiae*, which at such concentration had a severe growth defect (Fig. 1b). This is more clearly observed when comparing the relative growth values (obtained with the slope of the linear regression) (Fig. 1, inserts). The *S. cerevisiae* growth was reduced by 70% at 1 M NaCl, while the same NaCl concentration decreased growth by only 20% in *D. hansenii*. At higher NaCl concentrations, *S. cerevisiae* did not grow, while *D. hansenii* was still able to divide. 50% reduction in *D. hansenii* growth velocity was detected with a NaCl concentration as high as 1.5 M. Although 2 M NaCl displayed a significant effect on the growth properties of *D. hansenii*, it accumulated biomass and reached the stationary phase in twice the time it took the culture with no salt (not shown). These observations indicate that *D. hansenii* can grow adequately in NaCl concentrations that are harmful to *S. cerevisiae* and that under these growth conditions it can tolerate as much as 2 M NaCl.

Kinetics of DhHog1 phosphorylation upon NaCl treatment

In *S. cerevisiae*, hyperosmotic stress leads to the activation of the HOG pathway through transient phosphorylation of

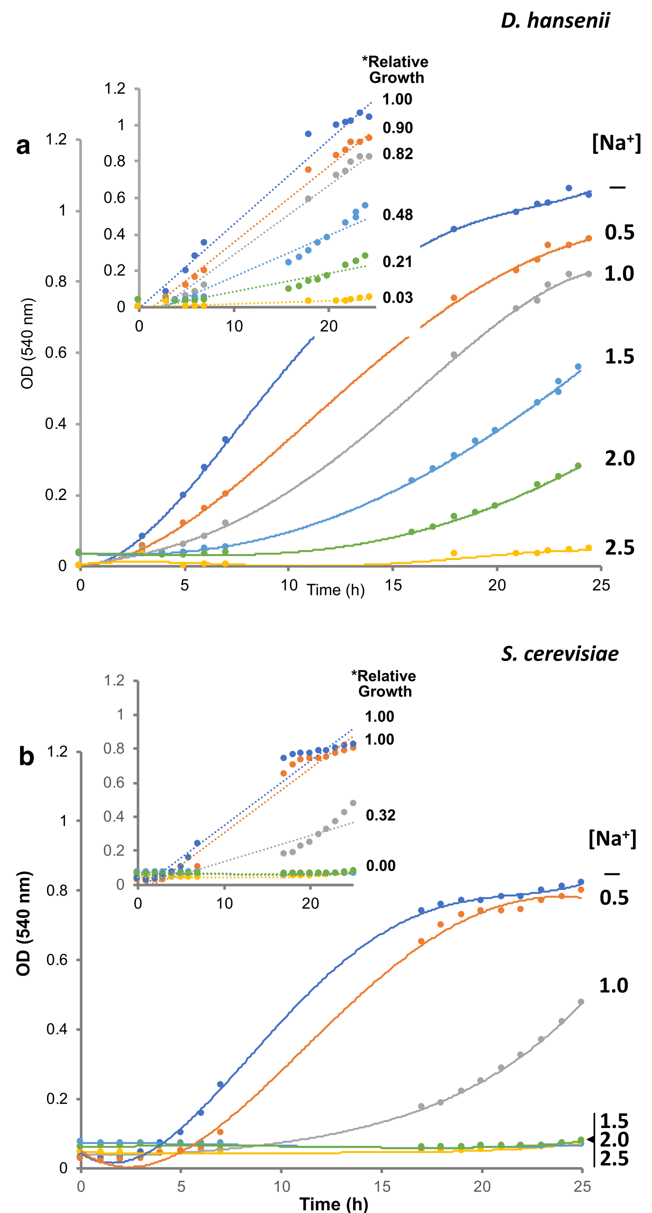

Fig. 1 Growth curves of *Debaryomyces hansenii* (**a**) and *Saccharomyces cerevisiae* (**b**) under different NaCl concentrations. Cultures were grown in YPD with the indicated NaCl concentrations, at 28 °C, in a rotary shaker at 250 rpm. The initial inoculum with freshly harvested overnight YPD cells, was adjusted to 0.1 OD_{600}. OD readings were taken every hour in a Klett- Summerson colorimeter. Linear regressions were adjusted, and the relative growth was calculated with the slope values normalised to the condition with zero NaCl. A representative graph of 3 different biological replicas is shown

the Hog1 MAPK. The time-lapse of phosphorylated Hog1 depends on the severity of the hyperosmotic stimulus, for instance ranging from 40 to 60 min under moderate stress conditions (0.4 to 0.6 M NaCl) (Van Wuytswinkel et al. 2000; Vázquez-Ibarra et al. 2018). To analyse the contribution of Hog1 to the salt tolerance of *D. hansenii*, we

determined the phosphorylation kinetics of DhHog1 in NaCl concentrations where the cell growth was either unaffected or compromised. DhHog1 phosphorylation was monitored with an anti-phospho-p38 antibody that detects the dually phosphorylated form. When *D. hansenii* cells were exposed to increasing NaCl concentrations for 10 min, DhHog1 was barely phosphorylated at 0.3 M, but this gradually increased until a maximum plateau was reached at 1–2 M (Fig. 2a). Although DhHog1 phosphorylation dropped significantly at concentrations higher than 2 M it was still detected at 3 M NaCl (Fig. S1). In contrast, ScHog1 reached its maximum at 0.5 M NaCl and then abruptly declined until being undetected at 2 M NaCl. A time-course assay was subsequently performed using the NaCl concentration at which the peak of phosphorylation was detected in the previous experiment. In this assay, the level of phosphorylated DhHog1 increased with time reaching its maximum at 60 min and then decreased gradually (Fig. 2b), but it was still detected after 180 min exposure to NaCl. In *S. cerevisiae,* the time-lapse of phospho-Hog1 under these conditions was much more transient than that observed in *D. hansenii*. Compared to ScHog1, the right shift of phosphorylated DhHog1 abundance in both the dose-dependent response and the time course indicates that DhHog1 is phosphorylated at higher NaCl concentrations than ScHog1 and for longer periods. This observation is in agreement with the salt tolerance level that *D. hansenii* showed in the cell growth assay and suggests that Hog1 can be required to some extent to induce resistance to high salt concentration.

Construction of the DhHOG1 null mutant

These previous observations suggest that the Hog1 MAPK may play an important role in the halotolerance mechanism in *D. hansenii*. The *DhHOG1* gene was cloned and sequenced (Bansal and Mondal 2000) and shown to encode a protein highly similar to Hog1 from a variety of species, including non-conventional yeast species (Fig. S2). The deduced DhHog1 protein is predicted to be 387 amino acid residues in length and shares 86% identity with ScHog1. It displays a conserved phosphorylatable TGY motif within the activation loop, and the two Pbs2 binding domains: the common docking (CD) domain and the PBD-2 domain (Murakami et al. 2008). The C-terminus of DhHog1 shows extensive differences in length and sequence compared to that of ScHog1; however, DhHog1 conserves the region that is involved in the regulation of ScHog1 autophosphorylation (Maayan et al. 2012). Interestingly, alignment shown in Fig. S2 indicates that species related to the CTG clade display globally a reduced Hog1 sequence in length concerning their C-terminus. This could reflect that, as previously observed in *Saccharomycotina* two-component systems (Hérivaux et al. 2018), some clade-specific evolution paths in stress response cell circuitries have likely intervened in budding yeasts.

To assess the contribution of DhHog1 to the halotolerance of *D. hansenii*, we designed a strategy for the construction of a *HOG1*-deleted strain. Conventional tools for gene replacement and inactivation are not useful in *D. hansenii*, since this yeast belongs to the CTG clade which ambiguously translates the CUG codon into Ser or Leu (Tekaia et al. 2000). In this work the pAYCU244 plasmid (Fig. 3a) was used, which was previously optimised for genetic engineering the *D. hansenii* codon usage (Defosse et al. 2018). A cassette containing the *SAT1* gene (which confers nourseothricin resistance) and the *yeYFP* reporter gene (yeast-enhanced Yellow Fluorescent Protein) was used to replace the *DhHOG1* locus by homologous recombination (Fig. 3a). Only 9 clones (out of 624 nourseothricin-resistant clones) that were sensitive to 2 M NaCl were isolated. These 9 clones were subjected to a series of PCR reactions to explore the structure of their *HOG1* loci (Fig. 3b). Three clones appeared to have integrated the *SAT1-yeYFP* cassette in the right locus (Clones 2, 5 and 8), however, clones 5 and 8 presented also the wild type *HOG1* amplicon. Only in clone 2 was the *SAT1-yeYFP* cassette correctly integrated by a double-crossing over in the *HOG1* locus, suggesting that this was indeed a *bona fide HOG1* null mutant. This was further confirmed with two more observations: clone 2 showed a high expression of the *yeYFP* reporter (Fig. 3c) and it did not show cross reaction with the anti-Hog1 antibody nor with the anti-p38 antibody after treatment with 1 M NaCl (Fig. 3d). Taken together, the previous observations suggest that double-crossing over is not a frequent event in *D. hansenii* and that most integrations occur ectopically as previously observed in other unconventional yeast species (Schorsch et al. 2009; Kretzschmar et al. 2013; Oguro et al. 2017).

DhHog1 is required for halotolerance in *D. hansenii*

The participation and contribution of Hog1 in *D. hansenii* halotolerance were determined by spotting tenfold serial dilutions onto YPD plates containing increasing NaCl concentrations. In this assay, the wild-type (*WT*) *D. hansenii* strain grew adequately under all NaCl concentrations tested (Fig. 4) which is in agreement with the growth kinetics depicted in Fig. 1. Compared to the *Schog1Δ* mutant, which is highly sensitive to mid-NaCl concentrations, the growth of the Dhhog1 mutant on 0.5 M NaCl was indistinguishable from that of the *WT* strain. However, inactivation of DhHog1 caused a strong reduction of growth at 1 M and higher NaCl concentrations. The effect of sorbitol, which imposes a different sort of osmotic stress than NaCl, was also tested. Growth of *WT D. hansenii* cells on sorbitol was similar to that on NaCl, however, the *Dhhog1Δ* mutant was very sensitive to high

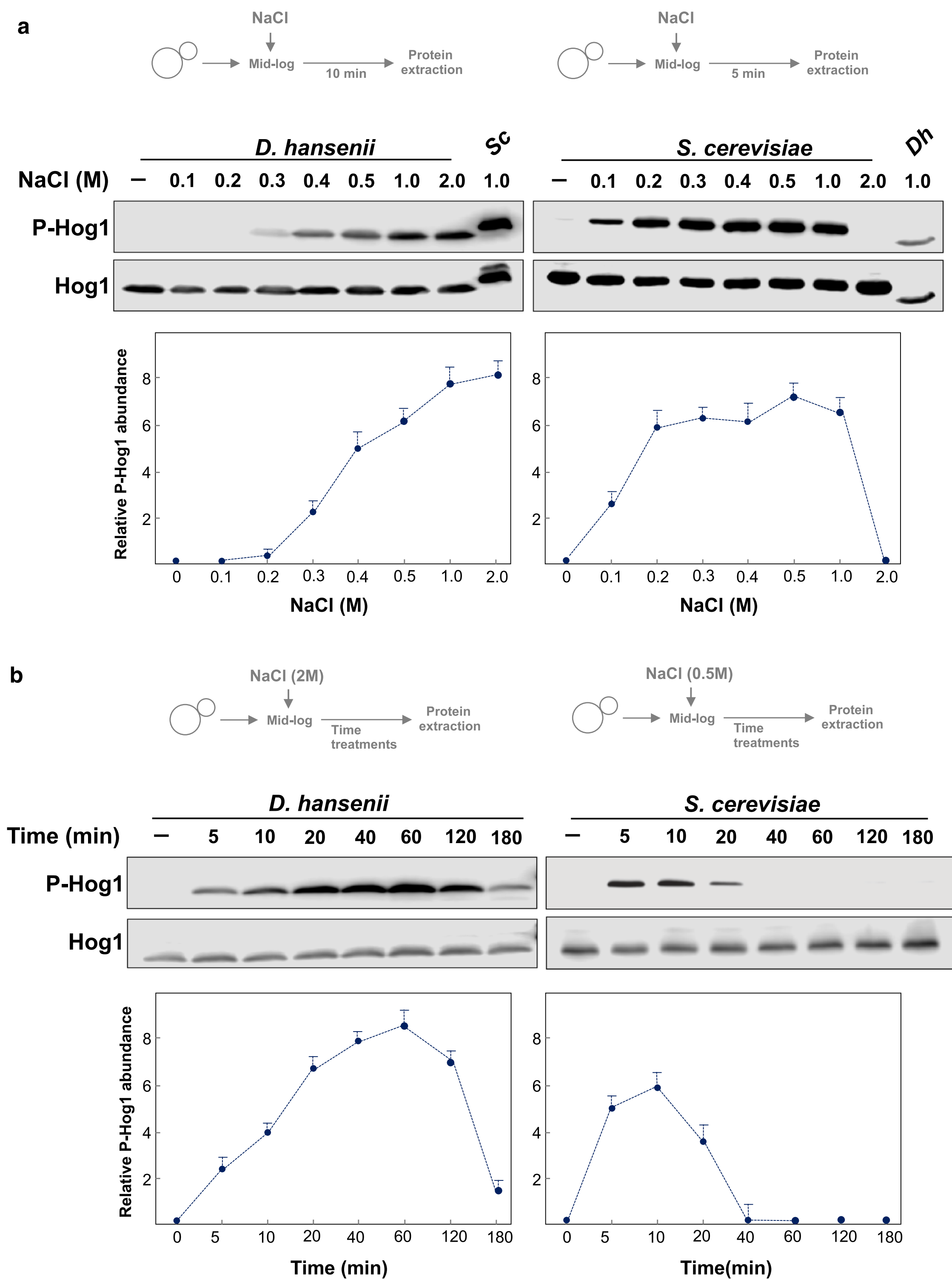

Fig. 2 Dose-dependent and time-course Hog1 phosphorylation in *D. hansenii* and *S. cerevisiae*. Cells were grown in YPD to mid-log phase and treated with NaCl. **a** Dose–response. Cells were treated with the indicated NaCl concentrations for 10 min (*D. hansenii*) or 5 min (*S. cerevisiae*). Protein extracts from the opposite strain treated with the indicated NaCl concentration (10 min for Sc and 5 min for Dh) were included in each blot. Extended NaCl dosage to 3 M is shown in Fig S1. **b** Time course. Cells were treated with 2 M (*D. hansenii*) or 0.5 M (*S. cerevisiae*) NaCl for the indicated times. Relative phospho-Hog1 abundance was determined by densitometry of three independent blots. Values represent the quotient of phospho-Hog1 divided by total Hog1 and multiplied by 10. Bars represent the standard error. Total Hog1 was detected with anti-Hog1 antibody and phosphorylated Hog1 was detected in the same stripped membrane with an anti-phospho p38 antibody. Representative blot images are presented

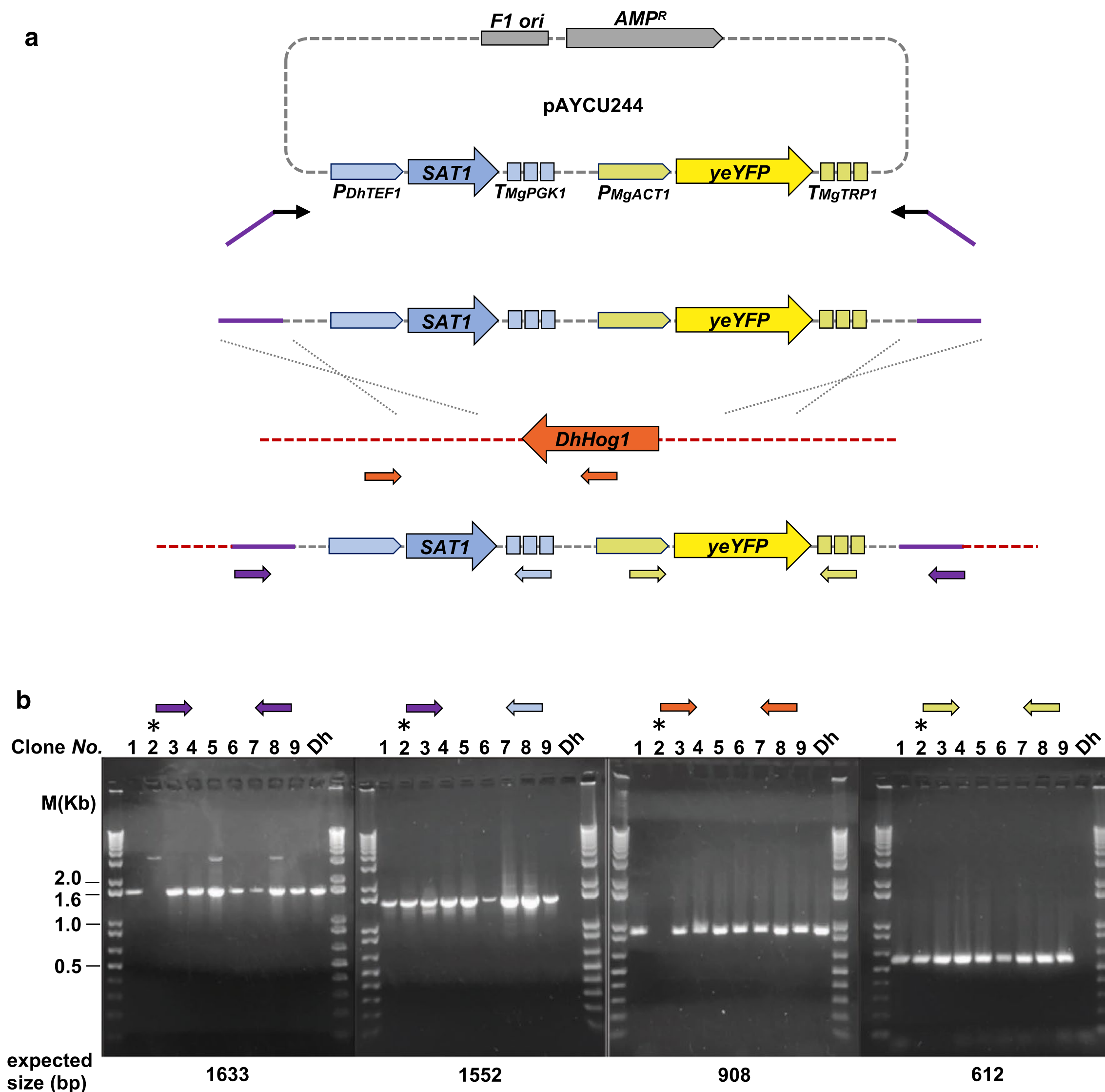

Fig. 3 **a** pAYCU244 plasmid map and DhHOG1 deletion strategy. Only relevant genes and regulatory sequences of pAYCU244 (Defosse et al. 2018) are depicted. The *SAT1* gene confers resistance to nourseothricin and the *yeYFP* gene encodes the yeast-enhanced yellow fluorescent protein. Primers to generate the *SAT1-yeYFP* cassette flanked by 40 bp recombinant tails (purple) are indicated with small black arrows. Cassette integration and *DhHOG1* gene replacement are indicated with tiny dotted grey lines. Small colored arrows indicate primers used to verify gene replacement. **b** PCR analysis of putative recombinant clones. Genomic DNA was used as a template. Coloured arrows (same code as **a**) on top of gels indicate the primers used in each reaction. Dh denotes PCR products from the *WT* strain. Expected PCR products size (bp) is indicated. Asterisk indicates clone with the right integration. **c** Microscopic images of *D. hansenii* WT strain (control) and clone No. 2. Fresh cells grown in YPD or YPD + nourseothricin (clone No. 2) were observed by confocal microscopy in the double photon mode with an LSM 710-Zeiss microscope. yeYFP was observed at 514–527 nm, excitation and emission wavelengths respectively. Control images were visualised in brightfield mode. **d** Hog1 and phospho-Hog1 detection in putative recombinant clones by western-blotting. Cells grown to mid-log phase were incubated for 10 min in 1 M NaCl and protein extracts were prepared. Total Hog1 was detected with an anti-Hog1 antibody and phospho-Hog1 was detected in the same stripped membrane with an anti-p38 antibody. *D. hansenii* (Dh) and *S. cerevisiae* (Sc) protein extracts from cells treated in the same conditions were loaded in the same gel

sorbitol concentrations. Close examination of plates with osmo-equivalent concentrations of NaCl and sorbitol (*e.g.* 1.0 M NaCl vs 1.5 M sorbitol) (Almagro et al. 2000), show a more pronounced requirement of DhHog1 to deal with high sorbitol concentrations. These observations indicate that DhHog1 is needed for optimal growth at high osmotic conditions, which is consistent with the observed phosphorylation peak which occurred at 2 M NaCl, and that it is required for halotolerance in *D. hansenii*.

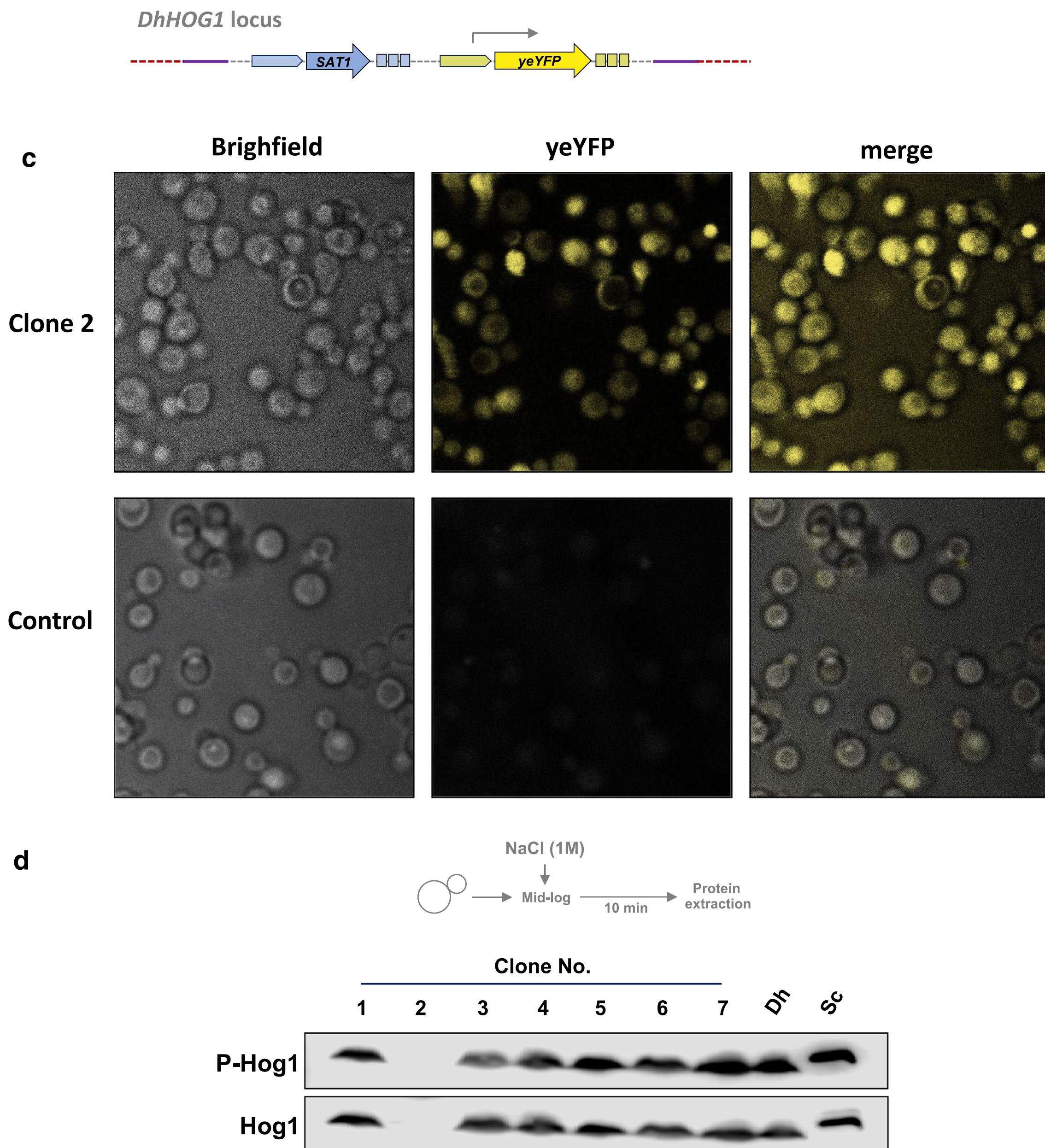

Fig. 3 (continued)

DhHog1 is partially translocated to the nucleus after hyperosmotic treatment

In *S. cerevisiae*, phosphorylation induces translocation of Hog1 from the cytoplasm to the nucleus, and this is achieved in *WT* cells after 5 min of exposure to hyperosmotic stress (Ferrigno et al. 1998). To determine whether DhHog1 would translocate into the nucleus after 2 M NaCl exposure, DhHog1 was tagged with the yeYFP protein. A similar strategy as for the construction of the *HOG1* null mutant was designed except that the *yeYFP* gene was cloned in frame into the 3′ end of *DhHOG1*. Integration of the *HOG1-yeYFP-SAT* cassette into the *DhHOG1* locus was also done by homologous recombination. With this strategy, it was detected that DhHog1 is in the cytoplasm under isosmotic conditions, but after different incubation times under hyperosmotic shock (2 M NaCl), a fraction of the yeYFP signal was detected also into the nucleus (Fig. 5). In our experiments we observed that DhHog1 was evenly distributed throughout the cytoplasm and nucleus from 10 to 180 min (Fig. S3) after the hyperosmotic

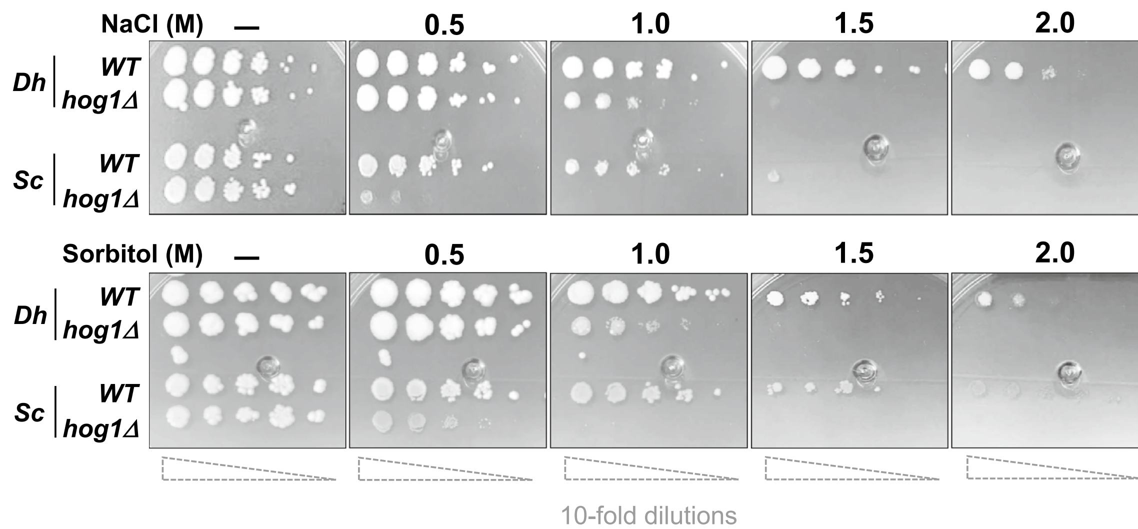

Fig. 4 Effect of NaCl and Sorbitol dosage in cell growth. The indicated strains were grown to mid-log phase and adjusted to $OD_{600} = 1.0$. Aliquots of tenfold serial dilution were spotted on YPD or YPD containing the indicated NaCl or Sorbitol concentrations. Plates were incubated for 72 h at 28 °C and photographed. *DhHog1Δ* corresponds to clone 2 of Fig. 3

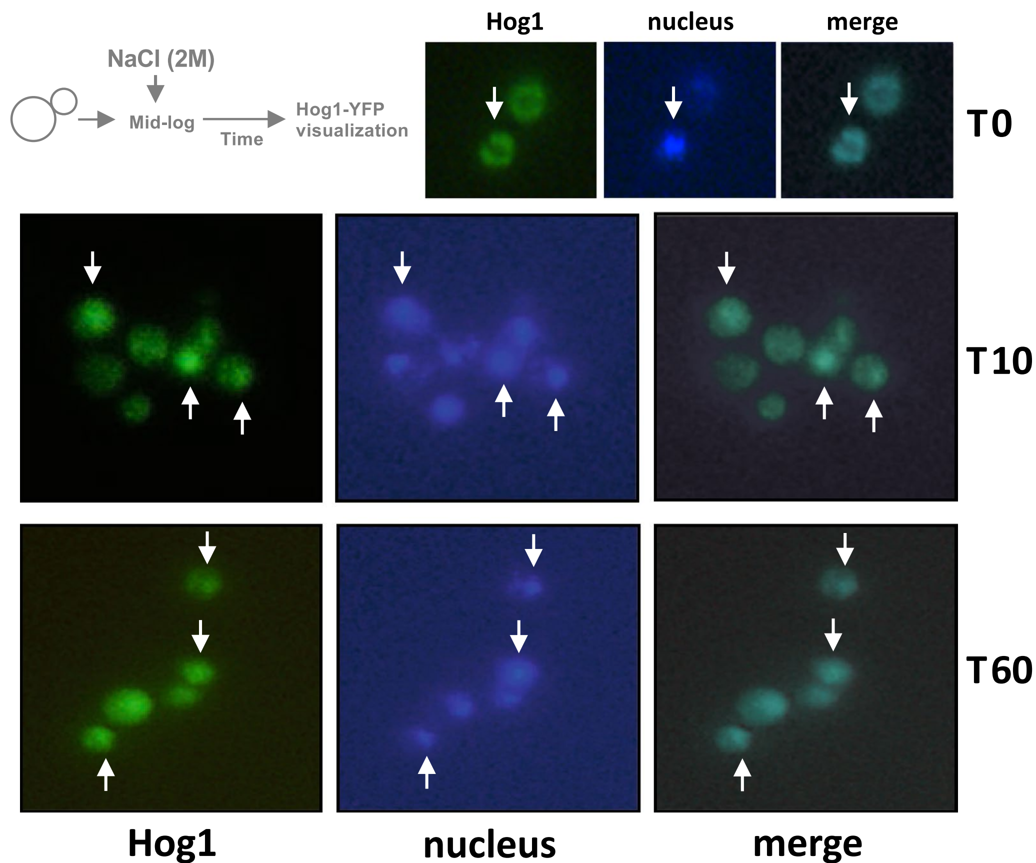

Fig. 5 DhHog1-yeYFP localisation after hyperosmotic treatment. Cells carrying the integrated *DhHOG1-yeYFP* hybrid gene were grown to mid-log phase and treated with or without (T0) 2 M NaCl for 10 min (T10) or 60 min (T60). Representative images of cells showing DhHog1-yeYFP and DAPI (nucleus) are shown. Images were acquired by epifluorescence microscopy. An extended time course (from 5 to 180 min) is shown in Fig S3

treatment, although by 180 min the amount of phospho-DhHog1 had decreased significantly (Fig. 2b).

DhHog1 regulates transcription of DhGPD1 and DhSTL1 under hyperosmotic stress

Hyperosmotic stress induces changes in the transcription of several genes required for short- and long-term responses. To determine whether *D. hansenii* Hog1 has transcriptional activity on canonical osmo-regulated genes, expression of *DhGPD1* (encoding the NAD^+-dependent glycerol-3-phosphate dehydrogenase) and *DhSTL1* (encoding the glycerol-proton symporter of the plasma membrane) under hyperosmotic stress in both *WT* and *Dhhog1Δ* strains was measured. High osmotic shock led to a strong induction of transcription in the *WT* strain, in which both genes were upregulated about tenfold under 2 M NaCl (Fig. 6). In the *Dhhog1Δ* mutant, the expression of *DhGPD1* and *DhSTL1* dropped under osmotic stress twofold and fourfold respectively. Interestingly, while *DhSTL1* expression was fully dependent on DhHog1 under stress, around 50% of the *DhGPD1* expression did not depend on DhHog1 in high NaCl concentration.

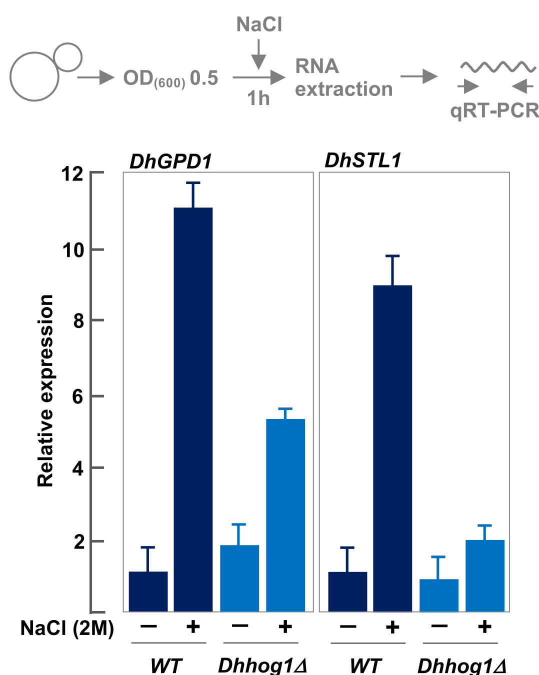

Fig. 6 Effect of hyperosmotic stress and DhHog1 inactivation on gene expression. *WT* and *Dhhog1Δ* cells were grown in YPD to OD = 0.5 and treated or not treated with 2 M NaCl for 1 h. Total RNA was obtained and subjected to qRT-PCR analysis. Relative transcript levels of *DhGPD1* or *DhSTL1* were obtained from the mean value of three independent experiments (± SD) normalised to the transcript levels of the *DhACT1* gene

DhHog1 partially contributes to glycerol production upon hyperosmotic stress

In *S. cerevisiae*, Hog1 regulates glycerol concentration through two mechanisms: rapid accumulation due to direct modulation of metabolic enzymatic activity, and a long-term transcriptional induction of genes required for glycerol production and accumulation (Schaber et al. 2012). We studied the contribution of DhHog1 to long-term glycerol production and extracellular accumulation, which could explain the cellular adaptation to high salt environments. Cells were incubated with increasing NaCl concentrations and total and external glycerol were measured. The hyperosmotic treatment induced a gradual increase in total glycerol concentration, which reached its maximum at 1.5–2 M NaCl (Fig. 7). The amount of glycerol at these NaCl concentrations was about threefold higher compared to the untreated cells. At 2.5 M NaCl the amount of glycerol decreased, most probably due to pleiotropic defects in cell physiology and viability. The total glycerol accumulation in the *Dhhog1Δ* mutant followed similar kinetics compared to the WT strain, except that the maximum accumulation in 2 M NaCl was twofold higher than the condition with no stress and this value represents 60% of the maximum amount of glycerol accumulated in the *WT* strain (Fig. 7). The internal glycerol followed the same kinetics as the total glycerol in both, the *WT* strain and the *Dhhog1Δ* strain (Fig. 7, insert). These observations indicate that the contribution of DhHog1 to total glycerol accumulation is about 40% under hyperosmotic conditions. This result is in line with the previous observation through which it was detected that the expression of *DhGPD1* has a component that is independent of DhHog1 under hyperosmotic conditions. In contrast to total glycerol, the amount of external glycerol in the *WT* strain remained constant until 1.5 M NaCl, and then dropped moderately at 2.0 and at 2.5 M (Fig. 7). A similar kinetics for external glycerol under the NaCl dose curve was detected in the *Dhhog1Δ* mutant, indicating that DhHog1 does not contribute to the regulation of glycerol flux through the membrane under these conditions.

Involvement of DhHog1 in the response to a variety of stressors

To date, it is clear that HOG pathways have pleiotropic roles in a variety of stressful conditions (Hernández-Elvira et al. 2019). Such is the case of the *Schizosaccharomyces pombe* Sty1 pathway, which is analogous to the *S. cerevisiae* HOG pathway. In *S. pombe*, Sty1 participates in a variety of stresses such as osmotic stress, heat shock, oxidative stress, UV light, nitrogen starvation and some other stress conditions (Hohmann 2002). To assess whether DhHog1 plays a role in stress conditions other

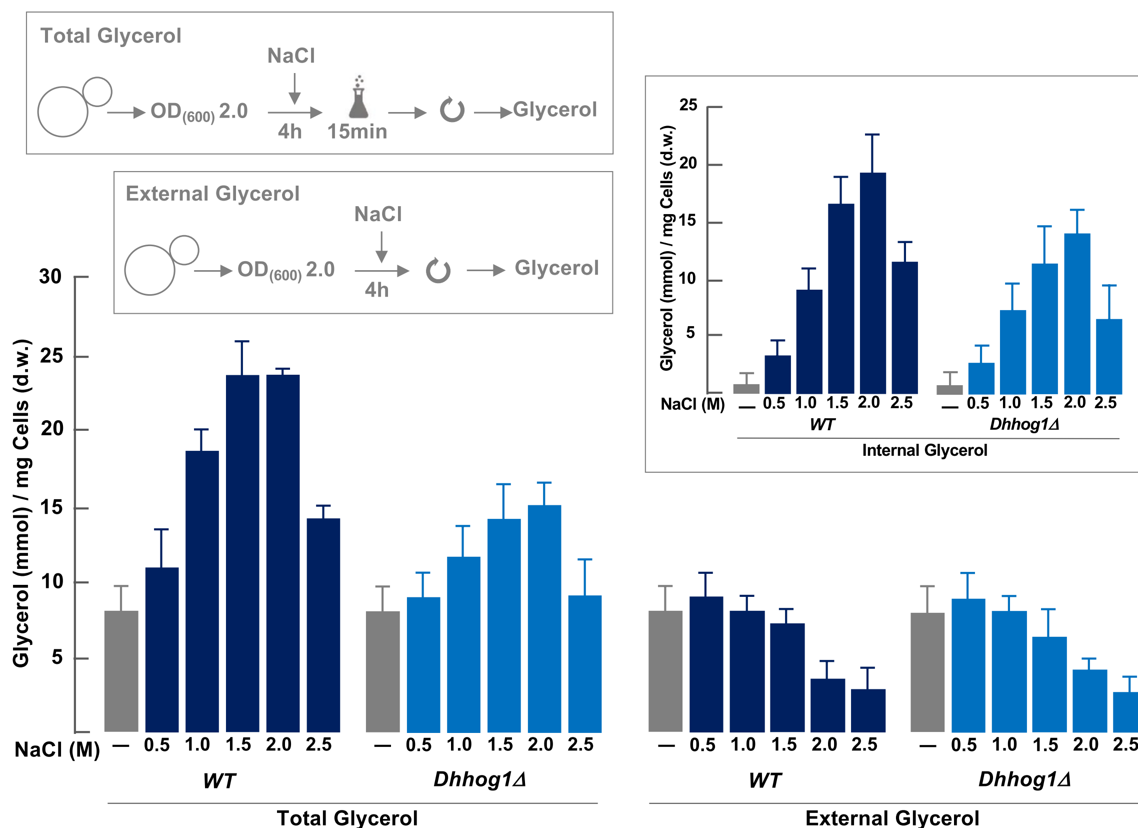

Fig. 7 Effect of hyperosmotic stress and DhHog1 inactivation on glycerol accumulation. *WT* and *Dhhog1Δ* cells were grown in YPD to OD = 2.0 and treated or not treated with the indicated NaCl concentrations for 4 h. Cell suspension was divided into two samples; one was boiled for 15 min and used for total glycerol determination; the second was centrifuged and the supernatant was used for external glycerol determination. Internal glycerol (insert) was calculated by means of the subtraction of external glycerol values from total glycerol values. Glycerol content is the mean value (± SD) of three independent experiments and referred to yeast dry weight

than hyperosmotic stress, we determined cell growth by spotting serial dilutions of cell suspension onto plates containing agents that induce oxidative stress, endoplasmic reticulum (ER) stress, and alkaline stress, and also under cold and heat stress. Under conditions of oxidative stress induced with either hydrogen peroxide (H_2O_2) or t-butyl-hydroperoxide (t-BHP) we were able to make two general observations: First, compared to *S. cerevisiae*, *D. hansenii* was highly sensitive to H_2O_2, but surprisingly resistant to stress induced with t-BHP (Fig. 8a). Secondly, the *Dhhog1Δ* mutant was moderately sensitive to oxidative stress induced with H_2O_2 but not with t-BHP, while the *Schog1Δ* mutant was resistant to both agents (Fig. 8a). These observations suggest that DhHog1 may participate in the response to oxidative stress which is in agreement with the DhHog1 phosphorylation induced with H_2O_2 (Sharma et al. 2005). Under ER stress conditions induced with the antibiotic tunicamycin (Tn) (Torres-Quiroz et al. 2010), it was found that the *Dhhog1Δ* mutant was moderately sensitive to Tn, at a concentration where the *Schog1Δ* mutant shows severe growth impairment (Fig. 8a).

Regarding the stress imposed by pH, the alkali-tolerant nature of *D. hansenii* is known. It can grow reasonably well at a pH of 8.0, a condition where *S. cerevisiae* is highly sensitive (Fig. 8b; Sánchez et al. 2018). Accordingly, the ability of the *Dhhog1Δ* mutant to grow on plates buffered at pH 8.0 compared with neutral and acidic pH (7.0 and 6.0 respectively) was tested. First, as expected, it was found that the *WT* strain of *D. hansenii* grew adequately in the three conditions, while the growth of *S. cerevisiae* at pH 8.0 was impaired (Fig. 8b). Interestingly, the *Dhhog1Δ* mutant showed an unexpected high sensitivity to pH 8.0 when combined with 1 M NaCl, which is a mid-stress condition for *D. hansenii* (Fig. 8b). The effect of hyperosmotic stress on the *Dhhog1Δ* mutant was not observed at pH 6.0 and 7.0. This indicates that under high salt concentrations, Hog1 is required to cope with alkali stress in *D. hansenii*.

Finally, it was explored whether DhHog1 is involved in response to cold stress and heat stress. *D. hansenii* Hog1 has no apparent role in this sort of stress (Fig. S4). However, it was detected that D. *hansenii* is cold-resistant, based on its ability to grow at 16 °C in short periods and at 4 °C in long

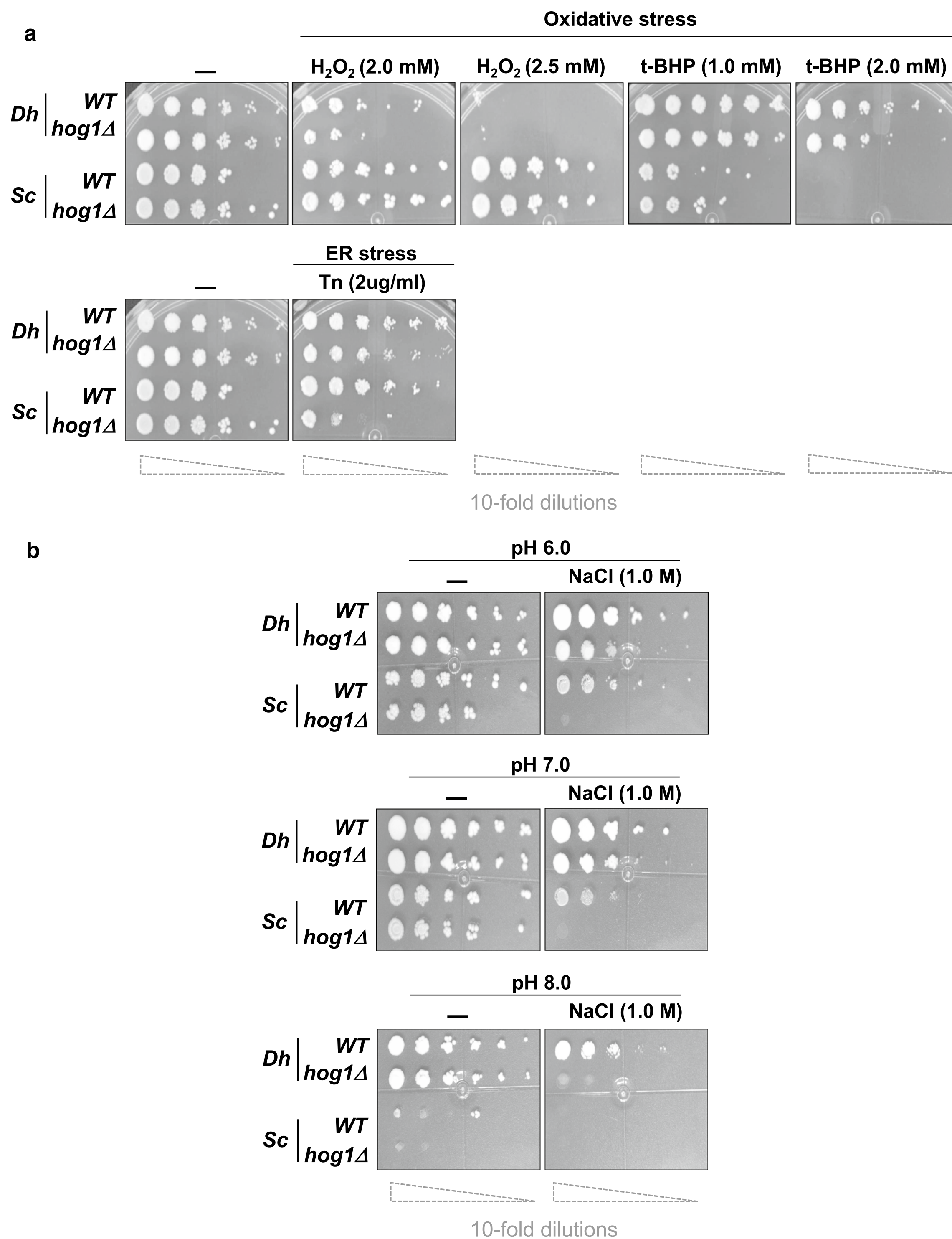

Fig. 8 **a** Effect of oxidative and ER stress on cell growth. The indicated strains were grown to mid-log phase and adjusted to $OD_{600}=1.0$. Aliquots of tenfold serial dilution were spotted on YPD or YPD containing the indicated concentrations of hydrogen peroxide (H_2O_2), t-butyl-hydroperoxide (t-BHP) or tunicamycin (Tn). Plates were incubated for 72 h at 28 °C and photographed. Oxidative and ER stress assays were performed with the same cultures at the same time, so the control plate has been duplicated for better comparison. **b** Effect of alkali and hyperosmotic stress in cell growth. The indicated strains were grown and platted as indicated in **a**. The pH of YPD medium was adjusted to the indicated values prior to sterilisation. 50 mM MES-TEA, 50 mM HEPES-TEA or 50 mM TAPS-TEA were used to buffer at pH 6.0, 7.0 and 8.0, respectively. Plates were incubated for 72 h at 28 °C and photographed

periods (which is not observed in *S. cerevisiae*) and that it is sensitive to heat stress, due to its inability to grow at 33 °C or higher temperatures, at which *S. cerevisiae* still grows adequately.

Discussion

D. hansenii is one of the most halotolerant and osmotolerant yeasts, which under some conditions, it can grow even in concentrations as high as 4.0 M NaCl (Onishi 1963; Norkrans 1966). Given this characteristic, *D. hansenii* has high biotechnological potential in the food industry, which makes it of high interest to understand the molecular mechanisms that contribute to its halotolerant behaviour. Although some studies have addressed the participation of the HOG pathway in the response to hyperosmotic stress (Sharma and Mondal 2005; Sharma et al. 2005), some conclusions have been obtained by inter-species gene expression. Here we report for the first time the phenotypic characterization of a null *HOG1* mutant in *D. hansenii* that allowed us to unequivocally determine the contribution of this MAPK to the halotolerance of this yeast. We have also given an accurate description of the activation kinetics of DhHog1, its transcriptional activity in canonical osmo-responsive genes, its participation in long-term glycerol production, and its involvement under different stress conditions.

Our observations indicate that *WT D. hansenii* can grow in NaCl concentrations ≥ 2 M, which is consistent with the previous classification of this species as a yeast belonging to the 3 M salt-stress class (Lages et al. 1999), and that deletion of *DhHOG1* induces sensitivity to that range of concentrations. Although at a slower rate than the *WT* strain, a mutant devoid of DhHog1 was still able to grow in 1 M NaCl, a concentration that imposes a strong negative effect on *S. cerevisiae* as detected in this and other works (Blomberg 1997; Rodríguez-González et al. 2017).

Our data indicate that strong Hog1 phosphorylation depends on high external osmolarity and that in low osmolarity, where ScHog1 is phosphorylated at a high level, DhHog1 is not activated. Accordingly, high expression of *DhSTL1* and *DhGPD1* was detected at 2 M NaCl and the long-term glycerol production peaked at this concentration. These observations indicate that the *D. hansenii* HOG pathway is set to be activated with high external osmolarity and that in low and mid-osmotic conditions, other protection systems should be active. A similar situation is present in the extreme halotolerant yeast *Hortaea werneckii*, where its two redundant paralogous Hog1A and Hog1B are fully active at salinities ≥ 3 M, but not at moderate NaCl concentrations (Kejžar et al. 2015). The fact that the HOG pathway can be activated at high NaCl concentrations is supported by the ability of *D. hansenii* to accumulate a large amount of Na^+ inside the cell, which is a trait that prompted some authors to describe it as a 'sodium includer' (Norkrans and Kylin 1969; Prista et al. 1997; Prista and Loureiro-Dias 2007).

In *S. cerevisiae*, phosphorylation is necessary to allow translocation of Hog1 from the cytoplasm to the nucleus, and this is achieved in wild-type cells after 5 min of exposure to hyperosmotic stress (Ferrigno et al. 1998). Nuclear localisation under this condition is transient and retro-translocation to cytoplasm occurs through the action of protein phosphatases, including nuclear Tyr-phosphatase Ptp2 (Mattison and Ota 2000). Thus, translocation to the nucleus of DhHog1 was detected within 10 min after hyperosmotic stress was imposed with 2 M NaCl and it was still detected inside the nucleus after 180 min of treatment. This behaviour is consistent with the phosphorylation time course obtained with 2 M NaCl. Interestingly, DhHog1 was also detected in the cytoplasm throughout the entire time course. These observations show some differences with previous studies in which DhHog1 was visualised by indirect immunostaining (Sharma et al. 2005). In those experiments, the nuclear accumulation of DhHog1 was delayed 90 min after hyperosmotic shock, and then re-entry into the cytoplasm occurred 150 min later. The discrepancy with our observations could be explained by differences in the detection level between indirect immunofluorescence and detection of the hybrid DhHog1-yeYFP, and/or differences in strain's genetic backgrounds. Although we do not know whether cytoplasmic DhHog1 is active in our conditions, its long nuclear retention not only matches with its phosphorylation status but also its role under extreme hyperosmotic conditions in which sustained long activity is expected. Although a decline in phosphorylation occurred in long periods, DhHog1 was still present in the nucleus. Disregarding an effect of increased protein turnover, this observation suggests that a mechanism of downregulation, mediated perhaps by nuclear phosphatases (Mattison and Ota 2000) may be present in *D. hansenii*. Interestingly a study of comparative genomics of the HOG signalling system predicts lack of Ptp2 phosphatase in *D. hansenii* (Krantz et al. 2006), suggesting that Ptp3 (which is present) may take both roles.

The peak in glycerol accumulation at 2 M NaCl correlates very well with the high expression of *DhGPD1* and *DhSTL1* detected at this concentration. While expression of *DhSTL1* was fully dependent on DhHog1, the DhHog1-dependent expression of *DhGPD1* was 60%. The high expression of *DhSTL1* is strongly correlated with the high glycerol/H^+ symporter activity that has been determined in *D. hansenii* (Pereira et al. 2014). The expression of both *DhGPD1* and *DhSTL1* in *D. hansenii*, followed a pattern of behaviour similar to that found in *S. cerevisiae*. *STL1* is an osmo-responsive gene whose expression completely depends on Hog1 (O'Rourke and Herskowitz 2004), while *GPD1* not only depends on Hog1 under hyperosmotic stress but also

on other signalling systems (Rep et al. 1999). It is worth considering that under hyperosmotic stress, *GPD1* can also be transcriptionally regulated by a Cyc8/Tup1-SUMOylation-dependent des-repression mechanism (Nadel et al. 2019), thus several mechanisms can be acting in concert to achieve a tight control of *DhGPD1* expression. Our observations indicate that a significant expression of *DhGPD1* was induced by osmostress in the *Dhhog1Δ* mutant, suggesting that other pathways may be participating or that a fraction of *DhGPD1* expression is controlled by a general stress mechanism in *D. hansenii*. It would be interesting to determine whether the stress caused specifically by Na^+ ions in *D. hansenii* can activate the Ca^{++}-Calcineurin pathway and to what extent this may regulate the response to hyperosmotic stress.

In *S. cerevisiae*, Hog1 regulates glycerol concentration through two distinct mechanisms. One is a rapid accumulation of glycerol which includes the closure of the Fps1 glycerol channel to diminish glycerol efflux (Lee et al. 2013) and the increase of glycerol synthesis by upregulation of both *GPD1* (Westfall et al. 2008), and *PFK2* (which encodes 6-phosphofructo-2-kinase) (Dihazi et al. 2004). The second is the transcriptional regulation of a large set of genes required for glycerol synthesis and accumulation (O'Rourke and Herskowitz 2004). Here we found that DhHog1 contributes to long term responses. Indeed, the amount of accumulated glycerol gradually increased with the increase in osmolarity until reaching a plateau at 1.5–2 M NaCl, in both, total and internal glycerol. This long-term glycerol production was just partially dependent on Hog1 since the maximum accumulation dropped just 1.5 fold in the *Dhhog1Δ*. The DhHog1-independent glycerol accumulation has not only the transcriptional component as discussed above, but also the increase in enzymatic activity directly exerted by NaCl (Adler et al. 1985; André et al. 1991). In fact, glycerol production can be enhanced by increased activity of Gpd1 and dihydroxyacetone kinase (Dak1,2) promoted by NaCl, which appears to modulate the specific activity of these enzymes. Interestingly, *D. hansenii* Gpd1 displays 10-folds more specific activity in the presence of NaCl than that of *S. cerevisiae* (Adler et al. 1985; André et al. 1991; Thomé 2005). Although the role of glycerol as a suitable compatible osmolyte in *D. hansenii* has been determined (Adler and Gustafsson 1980; Lages et al. 1999; Gori et al. 2005), it is important to consider that other polyols like arabinitol and trehalose may also contribute to the osmo-protection through a combined interplay (Adler and Gustafsson 1980; González-Hernández et al. 2005). The increase in total and internal glycerol accumulation and the contribution of Hog1 in *D. hansenii* coincide with those of *Kluyveromyces lactis*, where glycerol increases fivefold under hyperosmotic stress and KlHog1 contributes 50% to this increase (Rodríguez-González et al. 2017). Given the halo-sensitive nature of *K. lactis*, the common feature between both species is that they share highly respiratory metabolisms.

Regarding the amount of external glycerol under increasing NaCl concentrations, we found that this did not follow the same kinetics as total and internal glycerol. In fact, although no significant differences were detected, external glycerol showed a tendency to diminish as the concentration of NaCl rose. One likely explanation was that this was due to increased glycerol uptake mediated by high Stl1 symporter activity. We have disregarded this possibility since the profile of the mutant strain, where expression of *DhSTL1* is negligible, mirrored that of the wild type strain. However, it is important to mention that *D. hansenii* can accumulate glycerol against a strong concentration gradient (Alder et al. 1985). Whether this is due only to the DhStl1 activity and/or to other transport systems remains to be determined. Another possibility could be that glycerol efflux is a low capacity process in *D. hansenii*, due to the lack of a canonical aquaglyceroporin (Fps1 in *S. cerevisiae*) capable of releasing glycerol under hyperosmotic stress (Petterson et al. 2005; Sabir et al. 2016). It is worth noting, however, that *D. hansenii* contains a gene that is orthologous to the *S. cerevisiae* aquaporins Aqy1 and Aqy2, which are orthodox water channels that apparently may transport not only water but also solutes (Petterson et al. 2005). Orthodox aquaporins have been poorly studied in yeast species. In *S. cerevisiae*, functional expression of aquaporins is strongly dependent on the strain. When expressed, aquaporins appear to play a role in freeze tolerance, but Aqy1 seems to be specific for sporulation, while Aqy2 is expressed in vegetative cells but is downregulated by hyperosmotic stress in a Hog1-dependent manner (Ahmadpour et al. 2014). The genome of *D. hansenii* appears to encode only one aquaporin (which is slightly more related to ScAqy2) (Sabir et al. 2016). Whether this putative DhAqy2 participates in the regulation of glycerol efflux under hyperosmotic stress in *D. hansenii*, it is necessary to speculate that its regulation should largely differ from that of *S. cerevisiae*. It would be interesting to determine whether DhAqy2 has a role in the cold tolerance of *D. hansenii* detected in this work. Finally, the total and external glycerol reduction observed in concentrations over 2 M NaCl, which coincide with the drop in DhHog1 phosphorylation, cannot be explained as a regulatory mechanism exerted by DhHog1, but rather by an altered metabolic condition promoted by the hyperosmotic stress.

In addition to its participation in the hyperosmotic stress response, ScHog1 has been shown to have pleiotropic activities in a variety of conditions; for example, it has a role in the endoplasmic reticulum (ER) stress response (Torres-Quiroz et al. 2010; Hernández-Elvira et al. 2019), in the general stress response (de Nadal and Posas 2010) and even in mitophagy (Mao and Klionsky 2011). According to the results reported in this work, DhHog1 also appears

to play a role in response to oxidative stress, which is in agreement with the transcriptional induction, triggered by NaCl, of genes for oxidative protection (Segal-Kischinevzky et al. 2011; Calderón-Torres et al. 2011; Michán et al. 2013; Ramos-Moreno et al. 2019); DhHog1 may also play a role in the response to ER stress induced by tunicamycin, which inhibits protein N-glycosilation. Since this is extranuclear Hog1 activity (Torres-Quiroz et al. 2010; Hernández-Elvira et al. 2019), it was not surprising to detect DhHog1 in the cytoplasm during hyperosmotic stress. Our results also suggest that DhHog1 does not participate in cold stress nor in heat stress. Although we performed a wide screening at a variety of temperatures, we do not discard DhHog1 cross-activation by simultaneous induction of temperature stress and hyperosmotic or oxidative stress.

An interesting finding in this work was the strong sensitivity displayed by the *Dhhog1Δ* mutant to combined hyperosmotic stress and alkali stress. The resistance of *D. hansenii* to alkaline conditions is a property well known since the first isolated strains came from the ocean (pH around 8.0) (Norkrans 1966, 1968), and another strain has been isolated from soy sauce with a pH around 10 (Kurita and Yamazaki 2002). In natural environments, where a myriad of stressful conditions are simultaneously present, the finding that Hog1 is essential to cope with alkali stress in a mid-osmotic condition is quite relevant to the understanding of the physiological characteristics of an alkali-halotolerant yeast. In fact, it has been demonstrated that *D. hansenii* has a high capacity to acidify a medium buffered at pH 8.0 in the presence of high salt concentration (Sánchez et al. 2018). Additionally, *D. hansenii* expresses an alkali-metal-cation/H^+ antiporter of broad spectrum (Velkova and Sychrova 2006; Ramos et al. 2011) and two Na^+-ATPases which are overexpressed in high NaCl concentrations and one of them also in high pH (Almagro et al. 2001). It now, appears that this alkali-resistance is at least in part regulated by the Hog1 MAPK in *D. hansenii*.

Our study points to the scenario where the HOG pathway of *D. hansenii* is activated with high external osmolarity, and that under low and mid-osmotic conditions other protection systems are active. Several studies have identified factors that may play a role in the halotolerance of this yeast, including the increase of ergosterol in the plasma membrane in high salt concentrations (Turk et al. 2007) and the induction of expression and activity of several transporters such as the K^+–Na^+ symporter (Martínez et al. 2011). Thus, several osmoregulatory mechanisms must act in a coordinated fashion to permit an adaptive cellular response to salty environments.

Here we described for the first time the construction of a *HOG1* null mutant by homologous recombination in *D. hansenii*, a technical approach that has been elusive for some time, and most importantly we have determined the threshold of salt concentration that *D. hansenii* may tolerate without Hog1 and the contribution that this MAPK plays in the halotolerance of this species.

Acknowledgements NSS is very grateful to Dr Juan Pablo Pardo, of the UNAM school of Medicine, for fruitful discussions throughout the project. The authors wish to thank Dr Yazmín Ramiro for her help with the Two-Photon Confocal Microscopy. We are also grateful to Dr Laura Ongay, MSc Minerva Mora and Biol Guadalupe Códiz from the Molecular Biology Unit. We would like to recognise Dr Laura Kawasaki and Francisco Padilla for their technical assistance. Dr Mariana Hernández-Elvira helped NSS with some methodology. We are also grateful to Gerardo Coello, Ana María Escalante, Juan Barbosa and Ivette Rosas from the Computing Unit and Manuel Ortínez and Aurey Galván from the maintenance workshop. NSS is a PhD student in the Doctorate Program in Biochemical Science, UNAM. This work was supported by CONACyT (Grant No. CB-238497 to AP) and PAPIIT, DGAPA, UNAM (Grants IN223399 and IN202103 to AP and IN210519 to RC). We also extend our thanks to Patrick Weill for revising the English grammar of the text.

References

Adler L, Gustafsson L (1980) Polyhydric alcohol production and intracellular amino acid pool in relation to halotolerance of the yeast *Debaryomyces hansenii*. Arch Microbiol 124:123–130. https://doi.org/10.1007/BF00427716

Adler L, Blomberg A, Nilsson A (1985) Glycerol metabolism and osmoregulation in the salt-tolerant yeast *Debaryomyces hansenii*. J Bacteriol 162:300–306

Ahmadpour D, Geijer C, Tamás MJ, Lindkvist-Petersson K, Hohmann S (2014) Yeast reveals unexpected roles and regulatory features of aquaporins and aquaglyceroporins. Biochim Biophys Acta 1840:1482–1491. https://doi.org/10.1016/j.bbagen.2013.09.027

Alba-Lois L, Segal C, Rodarte B, Valdés-López V, DeLuna A, Cárdenas R (2004) NADP-glutamate dehydrogenase activity is increased under hyperosmotic conditions in the halotolerant yeast *Debaryomyces hansenii*. Curr Microbiol 48:68–72. https://doi.org/10.1007/s00284-003-4076-7

Almagro A, Prista C, Castro S, Quintas C, Madeira-Lopes A, Ramos J, Loureiro-Dias MC (2000) Effects of salts on *Debaryomyces hansenii* and *Saccharomyces cerevisiae* under stress conditions. Int J Food Microbiol 56:191–197. https://doi.org/10.1016/s0168-1605(00)00220-8

Almagro A, Prista C, Benito B, Loureiro-Dias MC, Ramos J (2001) Cloning and expression of two genes coding for sodium pumps in the salt-tolerant yeast *Debaryomyces Hansenii*. J Bacteriol 183:3251–3255. https://doi.org/10.1128/jb.183.10.3251-3255.2001

André L, Nilsson A, Adler L (1988) The role of glycerol in osmotolerance of the yeast *Debaryomyces hansenii*. Microbiology 134:669–677. https://doi.org/10.1099/00221287-134-3-669

André L, Hemming A, Adler L (1991) Osmoregulation in *Saccharomyces cerevisiae*. Studies on the osmotic induction of glycerol production and glycerol 3-phosphate dehydrogenase (NAD+). FEBS Lett 286:13–17. https://doi.org/10.1016/0014-5793(91)80930-2

Ariño J, Ramos J, Sychrova H (2019) Monovalent cation transporters at the plasma membrane in yeasts. Yeast 36:177–193. https://doi.org/10.1002/yea.3355

Bansal PK, Mondal AK (2000) Isolation and sequence of the HOG1 homologue from *Debaryomyces hansenii* by complementation of the *hog1Δ* strain of *Saccharomyces cerevisiae*. Yeast

16:81–88. https://doi.org/10.1002/(SICI)1097-0061(20000115)16:1<81:AID-YEA510>3.0.CO;2-I

Blomberg A (1997) The osmotic hypersensitivity of the yeast *Saccharomyces cerevisiae* is strain and growth media dependent: Quantitative aspects of the phenomenon. Yeast 13:529–539. https://doi.org/10.1002/(SICI)1097-0061(199705)13:6%3C529:AID-YEA103%3E3.0.CO;2-H

Blomberg A, Adler L (1992) Physiology of osmotolerance in fungi. Adv Microb Physiol 33:145–212. https://doi.org/10.1016/S0065-2911(08)60217-9

Cabrera-Orefice A, Chiquete-Félix N, Espinasa-Jaramillo J, Rosas-Lemus M, Guerrero-Castillo S, Peña A, Uribe-Carbajal S (2014a) The branched mitochondrial respiratory chain from *Debaryomyces hansenii*: Components and supramolecular organization. Biochim Biophys Acta Bioenerg 1837:73–84. https://doi.org/10.1016/j.bbabio.2013.07.011

Cabrera-Orefice A, Guerrero-Castillo S, Díaz-Ruíz R, Uribe-Carvajal S (2014b) Oxidative phosphorylation in *Debaryomyces hansenii*: Physiological uncoupling at different growth phases. Biochimie 102:124–136. https://doi.org/10.1016/j.biochi.2014.03.003

Calahorra M, Sánchez NS, Peña A (2009) Activation of fermentation by salts in *Debaryomyces hansenii*. FEMS Yeast Res 9:1293–1301. https://doi.org/10.1111/j.1567-1364.2009.00556.x

Calderón-Torres M, Castro DE, Montero P, Peña A (2011) DhARO4 induction and tyrosine nitration in response to reactive radicals generated by salt stress in *Debaryomyces hansenii*. Yeast 28:733–746. https://doi.org/10.1002/yea.1903

Chawla S, Kundu D, Randhawa A, Mondal AK (2017) The serine/threonine phosphatase DhSIT4 modulates cell cycle, salt tolerance and cell wall integrity in halo tolerant yeast *Debaryomyces hansenii*. Gene 606:1–9. https://doi.org/10.1016/j.gene.2016.12.022

de Nadal E, Posas F (2010) Multilayerd control of gene expression by stress-activated protein kinases. EMBO J 29:4–13. https://doi.org/10.1038/emboj.2009.346

de Nadal E, Alepuz PM, Posas F (2002) Dealing with osmostress through MAP kinase activation. EMBO Rep 3:735–740. https://doi.org/10.1093/embo-reports/kvf158

Defosse TA, Courdavault V, Coste AT, Clastre M, Dugé de Bernonville T, Godon C, Vandeputte P, Lanoue A, Touzé A, Linder T, Droby S, Rosa CA, Sanglard D, d'Enfert C, Bouchara JP, Giglioli-Guivarc'h N, Papon N (2018) A standardized toolkit for genetic engineering of CTG clade yeasts. J Microbiol Methods 144:152–156. https://doi.org/10.1016/J.MIMET.2017.11.015

Dihazi H, Kessler R, Eschrich K (2004) High osmolarity glycerol (HOG) pathway-induced phosphorylation and activation of 6-phosphofructo-2-kinase are essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress. J Biol Chem 279:23961–23968. https://www.jbc.org/content/279/23/23961

Ferrigno P, Posas F, Koepp D, Saito H, Silver PA (1998) Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin b homologs NMD5 and XPO1. EMBO J 17:5606–5614. https://doi.org/10.1093/emboj/17.19.5606

Fitzpatrick DA, Logue ME, Stajich JE, Butler G (2006) A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. BMC Evol Biol 6:99. https://doi.org/10.1186/1471-2148-6-99

Gerami-Nejad M, Dulmage K, Berman J (2009) Additional cassettes for epitope and fluorescent fusion proteins in *Candida albicans*. Yeast 26:399–406. https://doi.org/10.1002/yea.1674

Gezelius K, Norkrans B (1970) Ultrastructure of *Debaryomyces hansenii*. Archiv Mikrobiol 70:14–25. https://doi.org/10.1007/BF00691057

González-Hernández JC, Jiménez-Estrada M, Peña A (2005) Comparative analysis of trehalose production by *Debaryomyces hansenii* and *Saccharomyces cerevisiae* under saline stress. Extremophiles 9:7–16. https://doi.org/10.1007/s00792-004-0415-2

Gori K, Mortensen HD, Arneborg N, Jespersen L (2005) Expression of the *GPD1* and *GPP2* orthologues and glycerol retention during growth of *Debaryomyces hansenii* at high NaCl concentrations. Yeast 22:1213–1222. https://doi.org/10.1002/yea.1306

Gori K, Sørensen LM, Petersen MA, Jespersen L, Arneborg N (2012) *Debaryomyces hansenii* strains differ in their production of flavor compounds in a cheese-surface model. Microbiologyopen 1:161–168. https://doi.org/10.1002/mbo3.11

Guerrero CA, Aranda C, DeLuna A, Filetici P, Riego L, Anaya VH, González A (2005) Salt-dependent expression of ammonium assimilation genes in the halotolerant yeast, *Debaryomyces hansenii*. Curr Genet 47:163–171. https://doi.org/10.1007/s00294-004-0560-2

Gustafsson L, Norkrans B (1976) On the mechanism of salt tolerance. Arch Microbiol 110:177–183. https://doi.org/10.1007/BF00690226

Hérivaux A, Lavín JL, de Bernonville TD, Vandeputte P, Bouchara JP, Gastebois A, Oguiza JA, Papon N (2018) Progressive loss of hybrid histidine kinase genes during the evolution of budding yeasts (*Saccharomycotina*). Curr Genet 64:841–851. https://doi.org/10.1007/s00294-017-0797-1

Hernández-Elvira M, Martínez-Gómez R, Domínguez-Martin E, Méndez A, Kawasaki L, Ongay-Larios L, Coria R (2019) Tunicamycin sensitivity-suppression by high gene dosage reveals new functions of the yeast Hog1 MAP kinase. Cells 8:710. https://doi.org/10.3390/cells8070710

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66:300–372. https://doi.org/10.1128/mmbr.66.2.300-372.2002

Hohmann S (2015) An integrated view on a eukaryotic osmoregulation system. Curr Genet 61:373–382. https://doi.org/10.1007/s00294-015-0475-0

Kejžar A, Grötli M, Tamás MJ, Plemenitaš A, Lenassi M (2015) HwHog1 kinase activity is crucial for survival of *Hortaea werneckii* in extremely hyperosmolar environments. Fungal Genet Biol 74:45–58. https://doi.org/10.1016/j.fgb.2014.11.004

Klein M, Swinnen S, Thevelein JM, Nevoigt E (2017) Glycerol metabolism and transport in yeast and fungi: established knowledge and ambiguities. Environ Microbiol 19:878–893. https://doi.org/10.1111/1462-2920.13617

Krantz M, Becit E, Hohmann S (2006) Comparative genomics of the HOG-signalling system in fungi. Curr Genet 49:137–157. https://doi.org/10.1007/s00294-005-0038-x

Kretzschmar A, Otto C, Holz M, Werner S, Hübner L, Barth G (2013) Increased homologous integration frequency in *Yarrowia lipolytica* strains defective in non-homologous end-joining. Curr Genet 59:63–72. https://doi.org/10.1007/s00294-013-0389-7

Kurita O, Yamazaki E (2002) Growth under alkaline conditions of the salt-tolerant yeast *Debaryomyces hansenii* IFO10939. Curr Microbiol 45:277–280. https://doi.org/10.1007/s00284-002-3735-4

Lages F, Silva-Graça M, Lucas C (1999) Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species. Microbiology 145:2577–2585. https://doi.org/10.1099/00221287-145-9-2577

Larsson C, Morales C, Gustafsson L, Adler L (1990) Osmoregulation of the salt-tolerant yeast *Debaryomyces hansenii* grown in a chemostat at different salinities. J Bacteriol 172:1769–1774. https://doi.org/10.1128/jb.172.4.1769-1774.1990

Lee J, Reiter W, Dohnal I, Gregori C, Beese-Sims S, Kuchler K, Ammerer G, Levin DE (2013) MAPK Hog1 closes the *S. cerevisiae* glycerol channel Fps1 by phosphorylating and displacing its positive regulators. Genes Dev 27:2590–2601. https://doi.org/10.1101/gad.229310.113

Lucas C, Da Costa M, Van Uden N (1990) Osmoregulatory active sodium-glycerol co-transport in the halotolerant

yeast *Debaryomyces hansenii*. Yeast 6:187–191. https://doi.org/10.1002/yea.320060303

Maayan I, Beenstock J, Marbach I, Tabachnick S, Livnah O, Engelberg D (2012) Osmostress induces autophosphorylation of Hog1 via a C-terminal regulatory region that is conserved in p38a. PLoS ONE 7:e44749. https://doi.org/10.1371/journal.pone.0044749

Mao K, Klionsky DJ (2011) MAPKs regulate mitophagy in *Saccharomyces cerevisiae*. Autophagy 7:1564–1565. https://doi.org/10.4161/auto.7.12.17971

Marquez JA, Serrano R (1996) Multiple transduction pathways regulate the sodium-extrusion gene PMR2/ENA1 during salt stress in yeast. FEBS Lett 382:89–92. https://doi.org/10.1016/0014-5793(96)00157-3

Martínez JL, Sychrova H, Ramos J (2011) Monovalent cations regulate expression and activity of the Hak1 potassium transporter in *Debaryomyces hansenii*. Fungal Genet Biol 48:177–184. https://doi.org/10.1016/J.FGB.2010.06.013

Mattison CP, Ota IM (2000) Two protein tyrosine phosphatases, Ptp2 and Ptp3, modulate the subcellular localization of the Hog1 MAP kinase in yeast. Genes Dev 14:1229–1235

Medina-Córdova N, Rosales-Mendoza S, Hernández-Montiel LG, Angulo C (2018) The potential use of *Debaryomyces hansenii* for the biological control of pathogenic fungi in food. Biol Control 121:216–222. https://doi.org/10.1016/j.biocontrol.2018.03.002

Michán C, Martínez JL, Alvarez MC, Turk M, Sychrova H, Ramos J (2013) Salt and oxidative stress tolerance in *Debaryomyces hansenii* and *Debaryomyces fabryi*. FEMS Yeast Res 13:180–188. https://doi.org/10.1111/1567-1364.12020

Minhas A, Biswas D, Mondal AK (2009) Development of host and vector for high-efficiency transformation and gene disruption in *Debaryomyces hansenii*. FEMS Yeast Res 9:95–102. https://doi.org/10.1111/j.1567-1364.2008.00457.x

Minhas A, Sharma A, Kaur H, Rawal Y, Ganesan K, Mondal AK (2012) Conserved Ser/Arg-rich motif in PPZ orthologs from fungi is important for its role in cation tolerance. J Biol Chem 287:7301–7312. https://doi.org/10.1074/jbc.M111.299438

Murakami Y, Tatebayashi K, Saito H (2008) Two adjacent docking sites in the yeast Hog1 mitogen-activated protein (MAP) kinase differentially interact with the Pbs2 MAP kinase kinase and the Ptp2 protein tyrosine phosphatase. Mol Cell Biol 28:2481–2494. https://doi.org/10.1128/MCB.01817-07

Nadel CM, Mackie TD, Gardner RG (2019) Osmolyte accumulation regulates the sumoylation and inclusion dynamics of the prionogenic Cyc8-Tup1 transcription corepressor. PLoS Genet 15(4):e1008115. https://doi.org/10.1371/journal.pgen.1008115

Neves ML, Oliveira RP, Lucas CM (1997) Metabolic flux response to salt-induced stress in the halotolerant yeast *Debaryomyces hansenii*. Microbiology 143:1133–1139. https://doi.org/10.1099/00221287-143-4-1133

Neves L, Oliveira R, Lucas C (2004) Yeast orthologues associated with glycerol transport and metabolism. FEMS Yeast Res 5:51–62. https://doi.org/10.1016/j.femsyr.2004.06.012

Norkrans B (1966) Studies on marine occurring yeasts: Growth related to pH, NaCl concentration and temperature. Archiv Mikrobiol 54:374–392. https://doi.org/10.1007/BF00406719

Norkrans B (1968) Studies on marine occurring yeasts: Respiration, fermentation and salt tolerance. Archiv Mikrobiol 62:358–372. https://doi.org/10.1007/BF00425641

Norkrans B, Kylin A (1969) Regulation of the potassium to sodium ratio and of the osmotic potential in relation to salt tolerance in yeasts. J Bacteriol 100:836–845

O'Rourke SM, Herskowitz I (2004) Unique and redundant roles for HOG MAPK pathway components as revealed by whole-genome expression analysis. Mol Biol Cell 15:532–542. https://doi.org/10.1091/mbc.e03-07-0521

Oguro Y, Yamazaki H, Ara S, Shida Y, Ogasawara W, Takagi M, Takaku H (2017) Efficient gene targeting in non-homologous end-joining-deficient *Lipomyces starkeyi* strains. Curr Genet 63:751–763. https://doi.org/10.1007/s00294-017-0679-6

Onishi H (1963) Osmophilic yeasts. Adv Food Res 12:53–94. https://doi.org/10.1016/S0065-2628(08)60006-3

Papon N, Courdavault V, Clastre M (2014) Biotechnological potential of the fungal CTG clade species in the synthetic biology era. Trends Biotechnol 32:167–168. https://doi.org/10.1016/j.tibtech.2013.10.006

Pereira I, Madeira A, Prista C, Loureiro-Dias MC, Leandro MJ (2014) Characterization of new Polyol/H+ symporters in *Debaryomyces hansenii*. PLoS ONE 9:e88180. https://doi.org/10.1371/journal.pone.0088180

Petterson N, Filipsson C, Becit E, Brive L, Hohmann S (2005) Aquaporins in yeast and filamentous fungi. Biol Cell 97:487–500. https://doi.org/10.1042/BC20040144

Prista C, Loureiro-Dias MC (2007) Debaryomyces Hansenii, a salt loving spoilage yeast. A portrait of state-of-the-art research at the Technical University of Lisbon. Springer, Dordrecht

Prista C, Almagro A, Loureiro-Dias MC, Ramos J (1997) Physiological basis for the high salt tolerance of *Debaryomyces hansenii*. Appl Environ Microbiol 63:4005–4009

Prista C, Michán C, Miranda IM, Ramos J (2016) The halotolerant *Debaryomyces hansenii*, the cinderella of non-conventional yeasts. Yeast 33:523–533. https://doi.org/10.1002/yea.3177

Ramos J, Ariño J, Sychrova H (2011) Alkali-metal-cation influx and efflux systems in nonconventional yeast species. FEMS Microbiol Lett 317:1–8. https://doi.org/10.1111/j.1574-6968.2011.02214.x

Ramos J, Melero Y, Ramos-Moreno L, Michán C, Cabezas L (2017) *Debaryomyces hansenii* strains from Valle de los pedroches Iberian dry meat products: Isolation, identification, characterization, and selection for starter cultures. J Microbiol Biotechnol 27:1576–1585. https://doi.org/10.4014/jmb.1704.04045

Ramos-Moreno L, Ramos J, Michán C (2019) Overlapping responses between salt and oxidative stress in *Debaryomyces hansenii*. World J Microbiol Biotechnol 35:170. https://doi.org/10.1007/s11274-019-2753-3

Rep M, Albertyn J, Thevelein JM, Prior BA, Hohmann S (1999) Different signalling pathways contribute to the control of *GPDI* gene expression by osmotic stress in *Saccharomyces cerevisiae*. Microbiology 145:715–727. https://doi.org/10.1099/13500872-145-3-715

Ricaurte ML, Govind NS (1999) Construction of plasmid vectors and transformation of the marine yeast *Debaryomyces hansenii*. Mar Biotechnol (NY) 1:15–19. https://doi.org/10.1007/pl00011745

Rodríguez-González M, Kawasaki L, Velazquez-Zavala N, Domınguez-Martın E, Trejo-Medecigo A, Martagon N, Espinoza-Simon E, Vazquez-Ibarra A, Ongay-Larios L, Georgellis D, de Nadal E, Posas F, Coria R (2017) Role of the Sln1-phosphorelay pathway in the response to hyperosmotic stress in the yeast *Kluyveromyces lactis*. Mol Microbiol 104:822–836. https://doi.org/10.1111/mmi.13664

Sabir F, Loureiro-Dias MC, Prista C (2016) Comparative analysis of sequences, polymorphisms and topology of yeasts aquaporins and aquaglyceroporins. FEMS Yeast Res. https://doi.org/10.1093/femsyr/fow025

Saito H, Posas F (2012) Response to hyperosmotic stress. Genetics 192:289–318. https://doi.org/10.1534/genetics.112.140863

Sánchez NS, Calahorra M, Gónzalez-Hernández JC, Peña A (2006) Glycolytic sequence and respiration of *Debaryomyces hansenii* as compared to *Saccharomyces cerevisiae*. Yeast 23:361–374. https://doi.org/10.1002/yea.1360

Sánchez NS, Arreguín R, Calahorra M, Peña A (2008) Effects of salts on aerobic metabolism of *Debaryomyces*

hansenii. FEMS Yeast Res 8:1303–1312. https://doi.org/10.1111/j.1567-1364.2008.00426.x

Sánchez NS, Calahorra M, Ramírez J, Peña A (2018) Salinity and high pH affect energy pathways and growth in *Debaryomyces hansenii*. Fungal Biol 122:977–990. https://doi.org/10.1016/j.funbio.2018.07.002

Schaber J, Baltanas R, Bush A, Klipp E, Colman-Lerner A (2012) Modelling reveals novel roles of two parallel signalling pathways and homeostatic feedbacks in yeast. Mol Syst Biol 8:622. https://doi.org/10.1038/msb.2012.53

Schmitt ME, Brown TA, Trumpower BL (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res 18:3091–3092. https://doi.org/10.1093/nar/18.10.3091

Schorsch C, Köhler T, Boles E (2009) Knockout of the DNA ligase IV homolog gene in the sphingoid base producing yeast *Pichia ciferrii* significantly increases gene targeting efficiency. Curr Genet 55:381–389. https://doi.org/10.1007/s00294-009-0252-z

Segal-Kischinevzky C, Rodarte-Murguía B, Valdés-López V, Mendoza-Hernández G, González A, Alba-Lois L (2011) The euryhaline yeast *Debaryomyces hansenii* has two catalase genes encoding enzymes with differential activity profile. Curr Microbiol 62:933–943. https://doi.org/10.1007/s00284-010-9806-z

Sharma P, Mondal AK (2005) Evidence that C-terminal non-kinase domain of Pbs2p has a role in high osmolarity-induced nuclear localization of Hog1p. Biochem Biophys Res Commun 328:906–913. https://doi.org/10.1016/J.BBRC.2005.01.039

Sharma P, Meena N, Aggarwal M, Mondal AK (2005) *Debaryomyces hansenii*, a highly osmo-tolerant and halo-tolerant yeast, maintains activated Dhog1p in the cytoplasm during its growth under severe osmotic stress. Curr Genet 48:162–170. https://doi.org/10.1007/s00294-005-0010-9

Tekaia F, Blandin G, Malpertuy A, Tekaia F, Blandin G, Malpertuy A, Llorente B, Durrens P, Toffano-Nioche C, Ozier-Kalogeropoulos O, Bon E, Gaillardin C, Aigle M, Bolotin-Fukuhara M, Casarégola S, de Montigny J, Lépingle A, Neuvéglise C, Potier S, Souciet J, Wésolowski-Louvel M, Dujon B (2000) Genomic exploration of the hemiascomycetous yeasts: 3. Methods and strategies used for sequence analysis and annotation. FEBS Lett 487:17–30. https://doi.org/10.1016/S0014-5793(00)02274-2

Thomé PE (2004) Isolation of a *GPD* gene from *Debaryomyces hansenii* encoding a glycerol 3-phosphate dehydrogenase (NAD^+). Yeast 21:119–126. https://doi.org/10.1002/yea.1070

Thomé PE (2005) Heterologous expression of glycerol 3-phosphate dehydrogenase gene [*DhGPD1*] from the osmotolerant yeast *Debaryomyces hansenii* in *Saccharomyces cerevisiae*. Curr Microbiol 51:87–90. https://doi.org/10.1007/s00284-005-4446-4

Thomé PE, Trench RK (1999) Osmoregulation and the genetic induction of glycerol-3-phosphate dehydrogenase by NaCl in the euryhaline yeast *Debaryomyces hansenii*. Mar Biotechnol 1:230–238. https://doi.org/10.1007/PL00011772

Torres-Quiroz F, García-Marqués S, Coria R, Randez-Gil F, Prieto JA (2010) The activity of yeast Hog1 MAPK is required during endoplasmic reticulum stress induced by tunicamycin exposure. J Biol Chem 285:20088–20096. https://doi.org/10.1074/jbc.M109.063578

Turk M, Montiel V, Žigon D, Plemenitas A, Ramos J (2007) Plasma membrane composition of *Debaryomyces hansenii* adapts to changes in pH and external salinity. Microbiology 153:3586–3592. https://doi.org/10.1099/mic.0.2007/009563-0

Van Wuytswinkel O, Reiser V, Siderius M, Kelders MC, Ammerer G, Ruis H, Mager WH (2000) Response of *Saccharomyces cerevisiae* to severe osmotic stress: evidence for a novel activation mechanism of the HOG MAP kinase pathway. Mol Microbiol 37:382–397. https://doi.org/10.1046/j.1365-2958.2000.02002.x

Vázquez-Ibarra A, Subirana L, Ongay-Larios L, Kawasaki L, Rojas-Ortega E, Rodríguez-González M, de Nadal E, Posas F, Coria R (2018) Activation of the Hog1 MAPK by the Ssk2/Ssk22 MAP3Ks, in the absence of the osmosensors, is not sufficient to trigger osmostress adaptation in *Saccharomyces cerevisiae*. FEBS J 285:1079–1096. https://doi.org/10.1111/febs.14385

Velázquez-Zavala N, Rodríguez-González M, Navarro-Olmos R, Ongay-Larios L, Kawasaki L, Torres-Quiroz F, Coria R (2015) Ineffective phosphorylation of mitogen-activated protein kinase Hog1p in response to high osmotic stress in the yeast *Kluyveromyces lactis*. Eukaryot Cell 14:922–930. https://doi.org/10.1128/EC.00048-15

Velkova K, Sychrova H (2006) The *Debaryomyces hansenii NHA1* gene encodes a plasma membrane alkali-metal-cation antiporter with broad substrate specificity. Gene 369:27–34. https://doi.org/10.1016/j.gene.2005.10.007

Voronovsky AA, Abbas CA, Fayura LR et al (2002) Development of a transformation system for the flavinogenic yeast Candida famata. FEMS Yeast Res 2:381–388. https://doi.org/10.1016/S1567-1356(02)00112-5

Westfall PJ, Patterson JC, Chen RE, Thorner J (2008) Stress resistance and signal fidelity independent of nuclear MAPK function. Proc Natl Acad Sci USA 105:12212–12217. https://doi.org/10.1073/pnas.0805797105

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.