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CHARACTERIZATION OF THE RESPONSE OF *Candida glabrata* (*Nakaseomyces glabratus*) CLINICAL ISOLATES TO NEUTROPHILS

Tesis que presenta

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Constancia de aprobación de la tesis

La tesis "**Characterization of the response of *Candida glabrata* (*Nakaseomyces glabratus*) clinical isolates to neutrophils**" presentada para obtener el Grado de Maestra en Ciencias en Biología Molecular fue elaborada por **Ana Belén Vargas Antillón** y aprobada el **día de mes de año** por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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Acta de Examen

Dedicatorias

A MIS PADRES, ROSALBA Y MARTIN, NO SOLO POR GUIARME POR EL CAMINO DE LA VIDA, SINO TAMBIÉN EN MI TRAYECTORIA PROFESIONAL, POR AYUDARME A SER MEJOR PERSONA CADA DÍA, POR DAR LO MEJOR DE USTEDES A LA DISTANCIA PARA QUE YO LLEGARA HASTA AQUÍ, ES GRACIAS A USTEDES QUE SIEMPRE ENCUENTRO MI CAMINO.

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Abreviaturas

CAA	Casamino Acids
CSP	Caspofungin
CFUs	Colony Forming Units
FLC	Fluconazole
HATs	Histone acetyltransferase
HDACs	Histone desacetylase
H_2O_2	Hydrogen Peroxide
MEN	Menadione
NETs	Neutrophils Extracellular Tramps
NØ	Neutrophils
OD	Optical Density
ON	Overnight
PAMPs	Pathogen-associated Molecular Patters
PRRs	Pattern Recognition Receptors
ROS	Reactive Oxygen Species
RPMI-H-S	RPMI-HEPES-SERUM
YPD	Yeast Extract Peptone Dextrose
YPG	Yeast Extract Peptone Glycerol

Resumen

Las infecciones por patógenos fúngicos están aumentando debido al incremento de la población inmunocomprometida. Algunos de los patógenos fúngicos más comunes del género *Candida* adquieren resistencia a los antifúngicos durante los procesos de infección. *N. glabratus* no solo muestra una resistencia alta a los antifúngicos, sino que también tiene una susceptibilidad relativamente baja a los neutrófilos (NØ). Esto se debe, en parte, a que *N. glabratus* provoca una respuesta inflamatoria de baja intensidad mientras se desplaza silenciosamente dentro del huésped. Como resultado, la activación de los NØ se retrasa, lo que permite al patógeno evadir eficazmente la respuesta inmune.

Dado que *N. glabratus* puede evadir el sistema inmune y resistir parcialmente el ataque de los NØ, evaluamos si existe una microevolución de *N. glabratus* cuando se expone repetidamente de manera secuencial a los NØ.

Utilizamos la cepa estándar del laboratorio de *N. glabratus* (BG14) y dos aislados clínicos diferentes que son resistente (AN378) o susceptible (AN376) a fluconazol (FLC), el antifúngico más comúnmente usado, para exponerlos secuencialmente a NØ y, posteriormente, recuperar las células sobrevivientes. Caracterizamos varios fenotipos de las células que sobrevivieron, incluyendo la disfunción mitocondrial (fenotipo Gly⁻), tiempos de duplicación y supervivencia a una nueva exposición a NØ. Además, para determinar si la acetilación/desacetilación de las histonas participan en la supervivencia a los NØ, construimos mutantes en 2 acetilasas (HATs) y dos desacetilasas de histonas (HDACs) HATs y HDACs y evaluamos su supervivencia a los NØ.

No observamos un aumento en la supervivencia después de tres exposiciones consecutivas a los NØ. Demostramos por primera vez que las HDACs Rpd3 y Hos2 están involucrados en la supervivencia a NØ, mientras que la HAT Gcn5 no participa en esta respuesta.

Palabras clave: *N. glabratus*, neutrófilos, acetilasa de histona, deacetilasa de histona, aislados clínicos, supervivencia**.**

Abstract

Infections by fungal pathogens are increasing due to a rise in the immunocompromised population, some of the most commonly found fungal pathogens of the *Candida* genus acquire resistance to antifungals during the infection of the host. *N. glabratus* is characterized by a relatively low susceptibility to neutrophils (NØ), effectively evading the immune response due to the induction of a low-grade inflammatory response by silently transiting within the host, leading to a delay in neutrophil activation.

Since *N. glabratus* can evade the immune system and partially resist NØ attack, we determined if there is microevolution of *N. glabratus* when serially exposed to NØ that results in mutants or epigenetic variants which confers higher survival to neutrophil.

We used our standard laboratory *N. glabratus* strain (BG14) and two different clinical isolates of which one is resistant (AN378) and the other susceptible (AN376) to fluconazole (FLC), the most commonly used antifungal, to sequentially expose them three consecutive times to NØ. Afterwards, we recovered the surviving *N. glabratus* cells and tested them further for several phenotypes including mitochondrial function (Gly⁻ phenotype), growth rates and survival to a new exposure to fresh NØ. To determine whether histone acetylation/deacetylation participates in survival to NØ, we constructed mutants of histone acetylases (HATs) and deacetylases (HDACs) and their survival to NØ was evaluated.

Three passes through NØ did not increase the resistance to NØ in the *N. glabratus* clinical isolates or thee BG14 strain. We demonstrated for the first time that the HDACs *Rpd3* and *Hos2* are involved in *N. glabratus* survival to NØ, whereas *Gcn5* is not.

Key words: *N. glabratus*, neutrophils, histone acetylase, histone deacetylase, clinical isolates, NØ survival**.**

CHARACTERIZATION OF THE RESPONSE OF *Candida glabrata* (*Nakaseomyces glabratus*) CLINICAL ISOLATES TO NEUTROPHILS

Key words*: N. glabratus*, neutrophils, histone acetylase, histone deacetylase, clinical isolates, survival percentage**.**

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Introduction

1   Infections by fungal pathogens are increasing, in part due to a rise in the
2   immunocompromised population, which turns these infections into a problem of
3   public health. Several species of *Candida*, such as *Candida glabrata (N. glabratus),*
4   have dramatically increased in prevalence as causes of bloodstream infections,
5   partly due to their high intrinsic and acquired resistance to antifungals (Pais et al.,
6   2019). It has been described that the main mechanism of antifungal acquired
7   resistance in *N. glabratus* is due to mutations that confer gain of function (GOF)
8   within the *PDR1* gene, resulting in upregulation of the efflux pumps Cdr1, Cdr2,
9   Snq2, and Qdr2, increasing drug expulsion (De las peñas et al., 2015; Pais et al.,
10   2019; Siscar-Lewin et al., 2021).

11   In addition to its high resistance to fungicides, *N. glabratus* is characterized by a
12   relatively low susceptibility to neutrophils (NØ), effectively evading the immune
13   response (Pais et al., 2019; Urban and Backman, 2020). As the first line of defense,
14   NØ play a crucial role in fighting/preventing infections caused by opportunistic fungal
15   pathogens through active recruitment to infected tissues and organs (Urban and
16   Backman, 2020).

17   According to reports, the recruitment and swarming of NØ to the site of infection is
18   the most common defense against fungi. However, it should be noted that other
19   mechanisms have also been described. NØ are part of the innate immunity, which
20   recognizes pathogen-associated molecular pattern molecules (PAMPs) through
21   pathogen recognition receptors (PRRs) (Ermert et al., 2009; Shantal et al., 2022;
22   Urban and Backman, 2020). Upon recognition of fungal ligands, signaling results in
23   phagocytosis, the release of cytokines and chemokines, and production of reactive
24   oxygen species (ROS), and neutrophil extracellular traps (NETs) (Ermert et al.,
25   2009; Shantal et al., 2022; Urban and Backman, 2020). Furthermore, a study
26   reported that NØ are capable of expelling *N. glabratus* cells after intracellular killing,
27   through a mechanism called "dumping", highlighting this expulsion as an opportunity
28   for other immune cells to ingest fungal cells, present antigens and activate the
29   adaptive immune response (Essig et al., 2015).

However, *N. glabratus* owes its success to the establishment and persistence in the host through the induction of a low-grade inflammatory response by silently transiting within the host, leading to a delay in neutrophil activation (Shantal et al., 2022). Likewise, the development of strategies such as evading phagocytosis and escape to the cytosol, decreasing the production of proinflammatory cytokines, and promoting internalization into non-phagocytic cells achieving its reproduction inside of the mononuclear phagocytes has led to speculation that survival capacity is due to multiple mechanisms such as antioxidant response, combined with the ability to modulate the pH of the phagosome, which in part explain its ability to survive phagocytosis and in fact to take advantage of the immune cells to succeed and persist in the host (Shantal et al., 2022; Siscar-Lewin et al., 2021). Overall, the increase in *N. glabratus* infections and its ability to evade the immune response emphasizes the importance of timely neutrophil activation to trigger immune response to fight potential opportunistic mycosis. This could result in future strategies to treat severe fungal infections.

Previously, our research group found that *N. glabratus* survival to NØ attack depends on cellular mechanisms induced by oxidative stress to maintain the appropriate redox balance *N. glabratus*. We demonstrated that peroxiredoxin Tsa1 and Tsa2, and the double mutant of catalase Cta1 with sulfiredoxin Srx1 are involved in neutrophil survival, since the absence of these proteins leads to a 50% reduction in survival compared to the parental strain BG14. Furthermore, we also showed that the thioredoxin (Trx)/ thioredoxin reductases (Trr*)* system is necessary for NØ survival, as mutants lacking these systems had 10% survival compared to the parental BG14 (Gutiérrez-Escobedo et al., 2023). Finally, null mutants *hst1Δ* and *sum1Δ* showed no difference in survival compared to BG14 (Vázquez-Franco et al., 2022).

Increasing evidence suggests that epigenetic regulation plays a crucial role in drug resistance (Lin et al., 2023; Pfaller et al., 2009; Yu et al., 2022). Histone acetylation and deacetylation are post-translational modifications essential for transcriptional regulation in eukaryotic organisms (Lin et al., 2023; Pfaller et al., 2009; Yu et al., 2022). Histone acetylation levels involve two enzyme families:

2

62 histone acetyltransferases (HATs) and histone deacetylases (HDACs), which add or

63 remove acetyl groups from histone tails, respectively. Gcn5 is a HAT that forms part

64 of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex catalytic subunit (Filler et

65 al., 2021; Lin et al., 2023; Yu et al., 2022). Among HDACs, Rpd3 is known for its role

66 in transcriptional regulation and silencing as a subunit of the Rpd3L complex, while

67 Hos2 is a NAD-dependent histone deacetylase and a subunit of both the Set3 and

68 Rpd3L complexes (Filler et al., 2021; Pfaller et al., 2009). The activity of these

69 modified histones has been implicated in resistance to various antifungals, cell wall

70 disruptors, and virulence in *N. glabratus* (Filler et al., 2021; Lin et al., 2023; Pfaller

71 et al., 2015; Wang et al., 2002; Yu et al., 2022). However, it remains uncharacterized

72 whether *N. glabratus* clinical isolates and reference strains can be induced to evolve

73 *in vitro* by exposure to NØ and whether these histone acetyltransferases and

74 deacetyltransferases are involved in the response and survival to NØ attack.

75   In this study, we demonstrated that consecutive passages through NØ do not

76 exhibit microevolution in terms of increased survival of *N. glabratus* strains against

77 NØ. Moreover, these passages do not confer greater resistance to various stresses

78 such as FLC or hydrogen peroxide (H_2O_2), but rather to temperature. Furthermore,

79 we observed that mutants in genes involved in histone deacetylation (*rpd3Δ* and

80 *hos2Δ*) display lower survival to NØ compared to the parental strain BG14, indicating

81 the involvement of epigenetic mechanisms in the response and survival to NØ

82 exposure, at least for histone deacetylation. Additionally, these mutants reveal

83 different phenotypes with respect to temperature sensitivity and resistance to FLC.

84

Materials and methods

Preparation of _N. glabratus_ strains.

Yeast cells (BG14, AN376 and AN378) were grown to stationary phase (48 h at 30 ° C with agitation) in 5 mL of yeast extract-peptone-dextrose (YPD). The optical density (OD) was determined, and the cells were centrifuged and resuspended in 1 mL of sterile milli-Q water after which the OD_{600} was measured and set at 1 (approximately $2x10^7$ per mL).

Purification of NØ.

Fifteen mL of venous blood from healthy volunteers was carefully mixed with EDTA (final concentration 1.5-2 mM) and 15 mL of polymorphprep. The cell suspension was centrifuged at 500 g for 35 minutes without using brake or acceleration (to avoid lysis of NØ). The plasma and mononuclear cells were removed and the lower band corresponding to NØ was harvested. The volume of NØ suspension was diluted 1:1 with solution B (5 mM HEPES, 0.425% NaCl) and mixed. The suspension was centrifuged at 400 g for 10 min with slow braking and acceleration (setting 5). The pellet was resuspended in 4 mL of solution C (10 mM HEPES, 0.83% NH_4Cl) and incubated for 10 minutes at 37°C with 5% CO_2 and centrifuged at 400 g for 10 minutes. The pellet was resuspended in 4 mL RPMI-HEPES-SERUM (RPMI-H-S) (1% Serum, 10 mM HEPES in RPMI), and finally, the cells were counted in a Neubauer chamber and adjusted to 2.10^6 cells/mL.

Microevolution of _N. glabratus_ strains.

Neutrophil susceptibility assay was made following the original protocol published by Ermet (2009) with the following modifications: (Gutiérrez-Escobedo et al., 2020).
Three hundred µL of NØ cell suspension were placed in three wells of a 24-well plate. As controls, 300 µL of RPMI-H-S were placed in 3 different wells and incubated at 37°C with 5% CO_2 for 10 min. 30 µL of the yeast suspension was added to the three wells that already contained NØ and to the three wells with only RPMI, and the plate was centrifuged at 400 g for 10 minutes. The suspensions were incubated for

two hours at 37°C with 5% CO_2. Yeast cells were recovered by scraping and reincorporated into a new well with fresh NØ. The exposure to NØ and RPMI-H-S steps was repeated three times (to fresh NØ and RPMI-H-S). The contents of each well were washed with 100 µL of cold, sterile milli-Q water, which caused lysis of the NØ. Then, *N. glabratus* cells were frozen in 15% glycerol and stored at -80°C after the last passage (for the clinical isolates AN376 and AN378, the cells were stored at 4°C after the last passage.

Survival assay.

N. glabratus cells were thawed and recovered in liquid YPD for two overnights (ON) and the survival assay was performed again at 37°C with 5% CO_2 for two hours. The contents of each well were collected in a new well and 100 µl of cold sterile milli-Q water were added to lyse NØ. Each sample was diluted down to 10^{-3} and seeded on YPD plates and incubated for 48 hours at 30°C (Fig. S1). Finally, the Colony Forming Units (CFUs) were counted, and the survival percentage was determined with the following formula (Ermet et al., 2019):

$$\text{\% survival} = \frac{\text{dilution factor } \underline{\textit{(df) x CFU (with neutrophils)}} \text{ X 100}}{\textit{df x CFU (without neutrophils)}}$$

Growth rates.

The doubling times of different *N. glabrata* strains were made following the protocol published by: (Gutiérrez-Escobedo et al., 2023).

To determine the doubling times of BG14, CGM1938 (Gly⁻ generated with ethidium bromide) and cells obtained after exposure to RPMI or NØ, cultures were grown for 48 h in YPD, SC – Ura minimal medium or – amino acids media (CAA) and diluted into their corresponding media. 2×10^5 cells/mL were transferred to multi-wells plates (Oy Growth Curves Ab ltd) containing 300 µL of fresh media. Cells were grown at 30°C and monitored in a Bioscreen C system (Oy Growth CurvesAb ltd) at $OD_{450-580nm}$ every 15 min for 48 h. Doubling times were calculated in the exponential growth phase as $dT = \ln_2/(\ln OD2 - \ln OD1)/(t2-t1)$. dT is doubling time, OD1 is initial optical, OD2 is final optical density, t1 is initial time and t2 is final time. Experiments were done in technical replicates. For statistical analysis, ANOVA two-

144   way test was performed using InStat Graph Pad software (InStat Graph Pad Inc., v.

145   8.0. San Diego, CA, USA). Standard deviations p < 0.05 was considered statistically

146   significant.

Media.

148   Yeast were grown in YPD medium containing 10 g/L yeast extract, 10 g/L

149   peptone, and supplemented with 2% glucose. Synthetic complete medium

150   composition: 1.7 g/L yeast nutrient base. 5 g/L $(NH4)_2SO_4$ and supplemented with

151   0.6% casamino acids and 2% glucose. Yeast extract-peptone-glycerol (YPG)

152   medium contains 10 g/L yeast extract, 10 g/L peptone and supplemented with 3% of

153   glycerol (non-fermentable) carbon source where cells with non-functional

154   mitochondria cannot grow (Gly⁻ phenotype). To prepare YPD plates with selective

155   pressure, media were supplemented with FLC, caspofungin (CSP), H_2O_2, tert-butyl

156   hydroperoxide or menadione.

Spot assay.

158   Dilutions of surviving *N. glabratus* cells were made in a 96-well plate. Each

159   sample was diluted down to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} and spotted onto YPD

160   plates with different concentrations of FLC ([4,8,16, 32,42 and 64 mg/mL]), tert-butyl

161   hydroperoxide ([400, 800, 1200 and 1600 mM]), menadione ([30, 60 and 90 uM]),

162   and H_2O_2 ([5, 10, 15 and 20 mM]) and for thermal stress cells were spotted onto YPD

163   plates which were then incubated at 30°C, 37°C, 42°C, 45°C. Cells were grown for

164   48 hours and then plates were photographed at 24 and 48 hours (Fig. S2).

Reversibility assay.

166   To test the stability of the phenotypes obtained after the microevolution

167   experiment, nine consecutive subcultures in YPD medium were carried out by

168   inoculating 5 µL of an overnight culture of the Gly⁻ strain into 5 mL of fresh YPD

169   (medium without selective pressure). This procedure was repeated after

170   approximately 12 h and repeated consecutively until nine subcultures were

171   completed. After the last subculture, the tubes were incubated at 30°C until the

172   medium reached saturation, at which point the OD_{600} was measured, and cells were

173   adjusted to 1. Serial dilutions were made from 10^{-1} to 10^{-4}. One hundred microliters

174　of the 10^{-2} and 10^{-3} dilutions were plated on YPG agar plates, while 100 microliters

175　of the 10^{-4} dilution were plated on YPD agar plates (Fig. S3).

176　**Yeast transformation.**

177　　　Yeast transformations were carried out with the product obtained from the fusion

178　PCR (full knock-out cassette) using the LiOAc/ssDNA/PEG method (Castaño et al.,

179　2003).

180　**Construction of mutants in the BG14 strain genetic background.**

181　　　Primers were designed with ApE software using the CBS138 reference genome;

182　for the 5' intergenic region primer, we used the complementary sequence of primer

183　#604 (used to amplify the *URA3* selection maker) and for the 3' intergenic region,

184　the complementary sequence of primer #605 (Primers are shown in Table S2).

185　Modules were made by PCR and the full knock-out cassette was obtained by fusion

186　PCR. Two steps were necessary for the fusion, the first step consisted of only 10

187　cycles using the 5' and 3' cassettes and the *URA3* selection marker. The second

188　step was performed in 20 cycles using the corresponding primers (Table S2) to

189　amplify the complete cassette (Fig. S4, Table S1).

190　**Statistical Analysis.**

191　Data distribution was evaluated by D'Agostino-Pearson normality test. Variables with

192　parametric distribution were reported as mean ± standard deviation. Differences

193　between intergroup comparisons were performed using analysis of variance

194　(ANOVA) followed by Turkey or Dunn's multiple comparisons post-hoc test,

195　according to data normality. Analyses were carried out using GraphPad Prism 8.0.1

196　(GraphPad Software Inc., San Diego, CA). For all tests, $p \leq 0.05$ was considered

197　statistically significant.

Results

199　**Microevolution in the standard strain BG14.**

200　　　We induced of microevolution of *N. glabratus* clinical isolates and reference

201　strains by repeated exposure to NØ *in vitro*.

202   Cells were exposed consecutively to NØ and RPMI medium for three 2-h
203   passages (Fig. S1a). Subsequently, cells were recovered for two ON and a survival
204   assay was performed again at 37°C with 5% CO_2 for two hours (Fig. S1b).

205   After the treatments, we obtained cells that formed small colonies compared to
206   the normal size of most of the colonies from strain BG14. This could be due to the
207   appearance of cells called petite, which form small colonies that are unable to use
208   non-fermentable carbon sources such as glycerol or ethanol due to mitochondrial
209   dysfunction.

210   We determined the percentage of petite colonies obtained after three passages
211   through NØ and one survival assay (Table 1).

212   No significant differences were found in the percentage of petites between the
213   strains; however, a higher proportion of petite colonies was observed in BG14 after
214   exposure to NØ

INITIAL STRAIN PHENOTYPE	# PREVIOUS PASSES	% PETITE (Gly⁻)	WITHOUT TREATMENT
BG14 Gly$^+$	0	4.80±2.62 %	2.5%
NØ Gly$^+$	3	2.86±1.03 %	
RPMI Gly$^+$	0	2.91±1.08 %	

Table 3| Percentage of petite colonies obtained after a 2 h exposure to NØ. Abbreviations: BG14 Gly$^+$: BG14 strain under phagocytosis conditions (one survival assay). NØ Gly$^+$: BG14 strain under phagocytosis conditions (three previous passes through NØ plus one survival assay). RPMI Gly$^+$: BG14 strain under RPMI medium conditions plus one survival assay. Data shows no differences between them. Data are shown as mean ± s.d for parametric data. Difference between groups was assessed by Ordinary one-way ANOVA test for parametric data. P values ≤ 0.05 were considered statistically but no differences were found. Data represents the mean of 6 technical replicates, n=2.

We then asked whether *N. glabratus* can be induced to microevolved to NØ resistance by repeated exposure to NØ.

Cells were exposed to NØ and RPMI medium for three passages every two hours and then frozen at -80°C, Subsequently the cells were recovered for two ON and the survival assay was performed again at 37°C with 5% CO_2 for two hours, to determine whether previous repeated exposure to NØ, resulted in higher neutrophil survival rates.

Although there are no significant differences in the survival rates of cells recovered from the different treatments, there is a slight increase in survival trends for Gly$^+$ cells repeatedly exposed to NØ Gly$^+$ (43%) compared to the strain (BG14) without exposure to NØ (39%). However, no difference was observed in the survival rate of NØ Gly$^-$ (40%) compared to the absence of treatment. On the other hand, a lower survival trend was observed in cells treated with RPMI for the Gly$^-$ (20%) and Gly$^+$ (40%) derivatives, compared to BG14, although these differences are not statistically significant.

Even though differences are not statistically significant, lower survival trends are observed in cells with Gly$^-$ phenotype previously exposed to RPMI medium (Fig. 1).
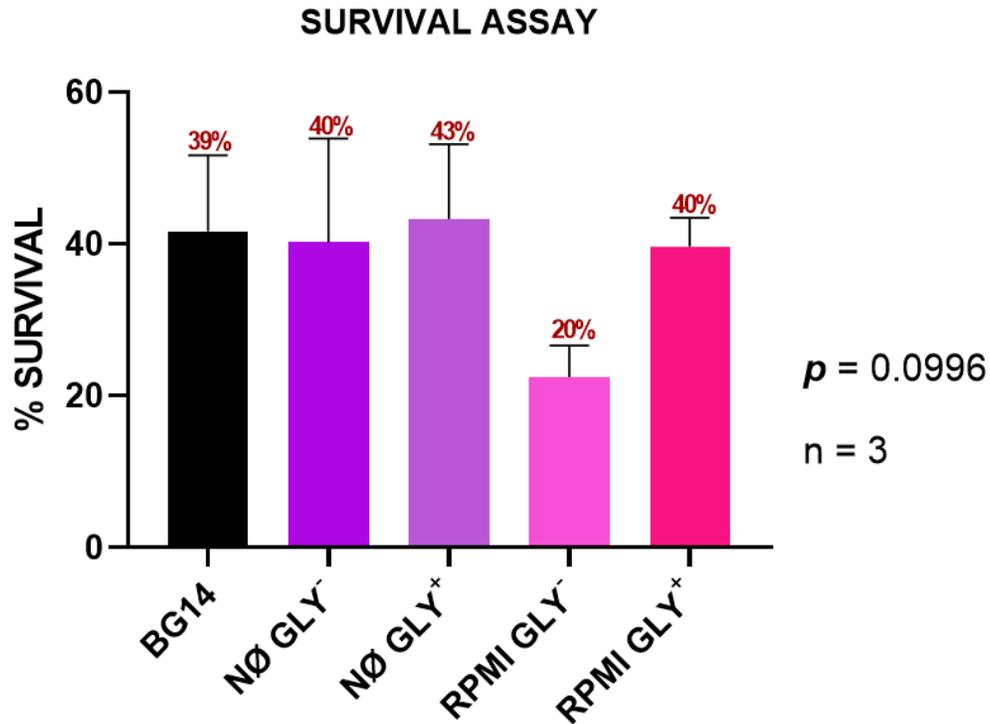
SURVIVAL ASSAY

240

241   **Fig. 1| Survival assay. Reference strain was exposed two hours to NØ (BG14)**; NØGly- and
242   NØGly+ colonies, were exposed to NØ for three passes (six hours) the phenotype was observed, and
243   cells were stored at -80°C, cells were recovered and immediately exposed again to fresh NØ two
244   more hours (eight hours in total) for the survival assay. As controls, Gly+ and Gly- colonies were
245   exposed as above for six hours to RPMI medium (RPMI Gly+ and RPMI Gly- respectively) cells were
246   stored at -80°C, recovered and exposed to NØ for two hours for the survival assay. Difference
247   between groups was assessed by Ordinary one-way ANOVA followed by Tukey's multiple
248   comparisons post-hoc test according to data normality. P-values ≤0.05 were considered statistically
249   significant n=3.

Percentage of petite cells obtained after exposure to NØ (Survival assay).

We then asked whether the percentage of colonies with a Gly⁻ phenotype in strain BG14 increases after the stress condition compared to the no-stress condition.

After repeated exposure to NØ for three consecutive passages, we performed a survival assay by exposing these *N. glabratus* cells to NØ at 37 ° C with 5% CO_2 for two hours. *N. glabratus* cells were diluted down to 10^{-3}, plated on YPD plates, incubated for 24 hours at 30°C and then, we determined CFUs and recorded the colony size phenotype as well as the ability to grow in non-fermentable carbon sources (Gly⁻ phenotype).

No significant differences were found in the percentage of petite colonies between the strains; however, a higher proportion was observed in BG14 after exposure to NØ. A higher percentage of petite colonies was observed in the BG14 strain compared with the no-treatment conditions. These petite colonies were obtained from Gly⁺ colonies after one passage through NØ stress (Table 1).

Gly⁻ phenotype cells are obtained under stress conditions due to neutrophil phagocytosis.

The growth rate of evolved strains in CAA

Since we found Gly⁻ cells that grow more slowly than Gly⁺ cells, we decided to determine the growth rate of cells obtained after treatment with either NØ or RPMI, compared to the BG14 that had undergone no treatment and of the Gly⁺ cells, obtained after the survival assay in CAA (Table 2).

Cell growth was monitored for 2000 minutes at OD_{600} measured every 15 min in a Bioscreen apparatus. The doubling times were determined using the formula **dT = ln₂/(lnOD2-lnOD1)/(t2-t1).**

As shown in Table 2, the doubling time was shorter for the parental strain cells with no treatment (72.88 min) compared to cells that underwent either 3 passes with NØ (84.04 min *p= 0.0088*) or 3 passes in RPMI medium (85.06 min *p= 0.0047*). Gly⁻ strain displays longer duplication time (89.33 min *p= 0.0022* compared to BG14).

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CONDITION	DOUBLING TIME	STANDAR DESVIATION
No treatment	72.88 [a]	0.9673
NØ	84.04 [a-b] **	3.8436
RPMI	85.06 [a-c] **	3.6808
Gly⁻ Control	89.33 [a-d] **	0.2291

279 **Table 4| Growth curve in the synthetic complete medium of BG14 under different treatments**
280 All strains, except for Gly⁻ is CGM1938-petite control strain generated by ethidium bromide-
281 represents a mix of populations. The difference between groups was assessed by ANOVA test for
282 parametric following by Tukey´s multiple comparisons tests. P values ≤ 0.05 were considered
283 statistically significant and are highlighted in bold (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Data represents
284 the mean of 3 technical replicates, n=1. [a]: No treatment, [b]: NØ, [c]: RPMI and [d]: Gly⁻ control.

285 **Three consecutive passes through NØ may decrease chronic H$_2$O$_2$ exposure**

286 **of *N. glabratus*.**

287 To continue phenotypically characterizing the surviving *N. glabratus* cells, we

288 determined the response of the cells that had been exposed to NØ or RPMI for 3

289 consecutive passes to different types of oxidative stress, and antifungals (FLC) (Fig.

290 2).

291   Cells were grown up to stationary phase in 5 mL of YPD medium two ON at 30°C

292 with shaking, the OD was measured (OD$_{600}$) and set at 0.5. Each sample was spotted

293 7 μL on different indicated plates made by spot assay and incubated for 48 hours at

294 30°C.
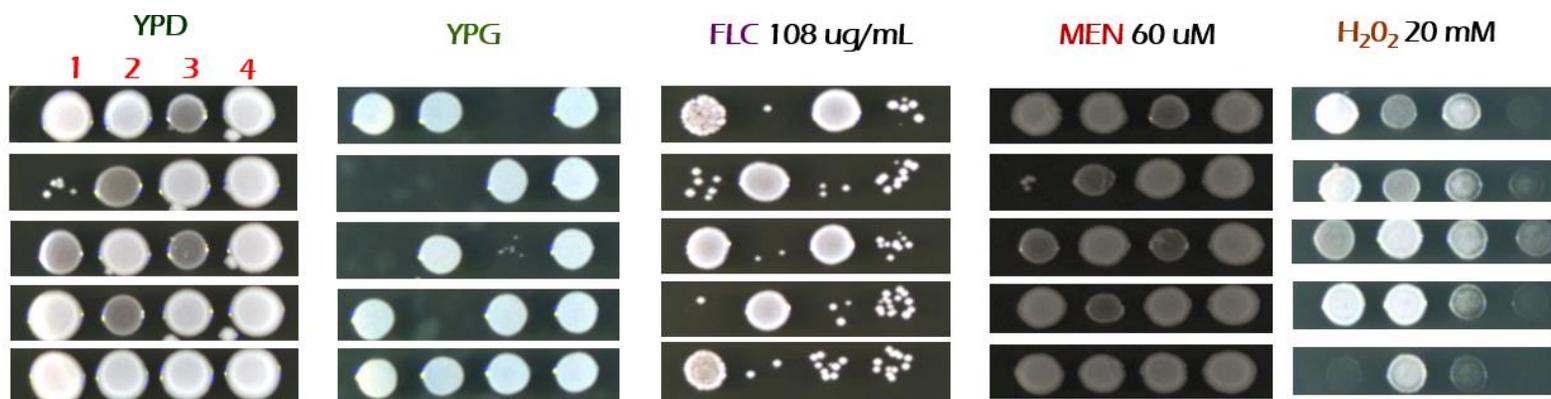
	1	2	3	4
	BG14	Gr NØ	Ch INPUT	NØ R2
	CGM1938	Ch RPMI	Gr INPUT	NØ R3
	Ch NØ	Gr RPMI	Ch INPUT	RPMI R1
	Gr NØ	Ch RPMI	Gr INPUT	RPMI R2
	Ch NØ	Gr RPMI	NØ R1	RPMI R3

Fig. 2| Spot assay under different oxidative stress types and fluconazole. Abbreviations Ch: Gly⁻ phenotype colonies, Gr: Gly⁺ phenotype colonies, FLC: fluconazole, MEN: menadione, H₂O₂: hydrogen peroxide, INPUT: colonies without stress conditions, Rn: technical replicate. Strain CGM1938 is a Gly- strain derived from treatment of BG14 with ethidium bromide. The differences between columns 1 and 2 versus 4 is the number of passes: columns 1 and 2 have 3 passes through NØ plus a survival assay and column 4 has three passes through NØ.

Notably, petite cells (obtained by exposure to NØ and RPMI medium) show greater resistance to FLC compared to Gly⁺ cells exposed to RPMI or NØ; this result was expected from previous reports describing that petite colonies have higher resistance to FLC. On the other hand, no differences were observed in the different oxidative stress conditions compared to the parental strain (BG14) and the CGM1938 (petite control strain created by ethidium bromide). However, greater sensitivity to hydrogen peroxide is observed in the spots from column four (three passes through NØ) compared to columns one and two (three passes through NØ plus a survival assay total of eight passes).

Microevolution in *N. glabratus* clinical isolates.

To test whether there are differences in the response (and adaptation and/or microevolution) to NØ between *N. glabratus* strains, we decided to start the characterization of clinical isolates AN376 and AN378 because these are strains unrelated to our standard laboratory strain BG14 and are isolates obtained from a single patient but differ in their antifungal susceptibility phenotype (Fig. 3).

Cells were exposed to NØ and RPMI medium for three 2-hour passages. Subsequently, cells were stored at 4°C. The cells were inoculated into YPD and incubated for one ON and a survival assay was performed again at 37°C with 5% CO_2 for two hours (Fig. 3).

321

322

323
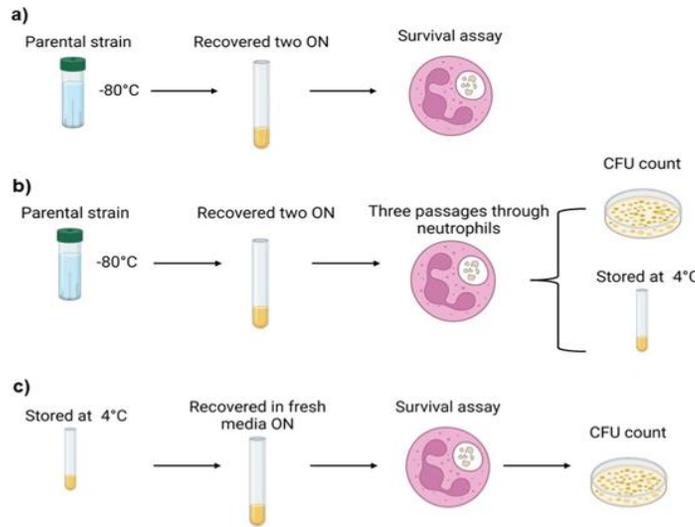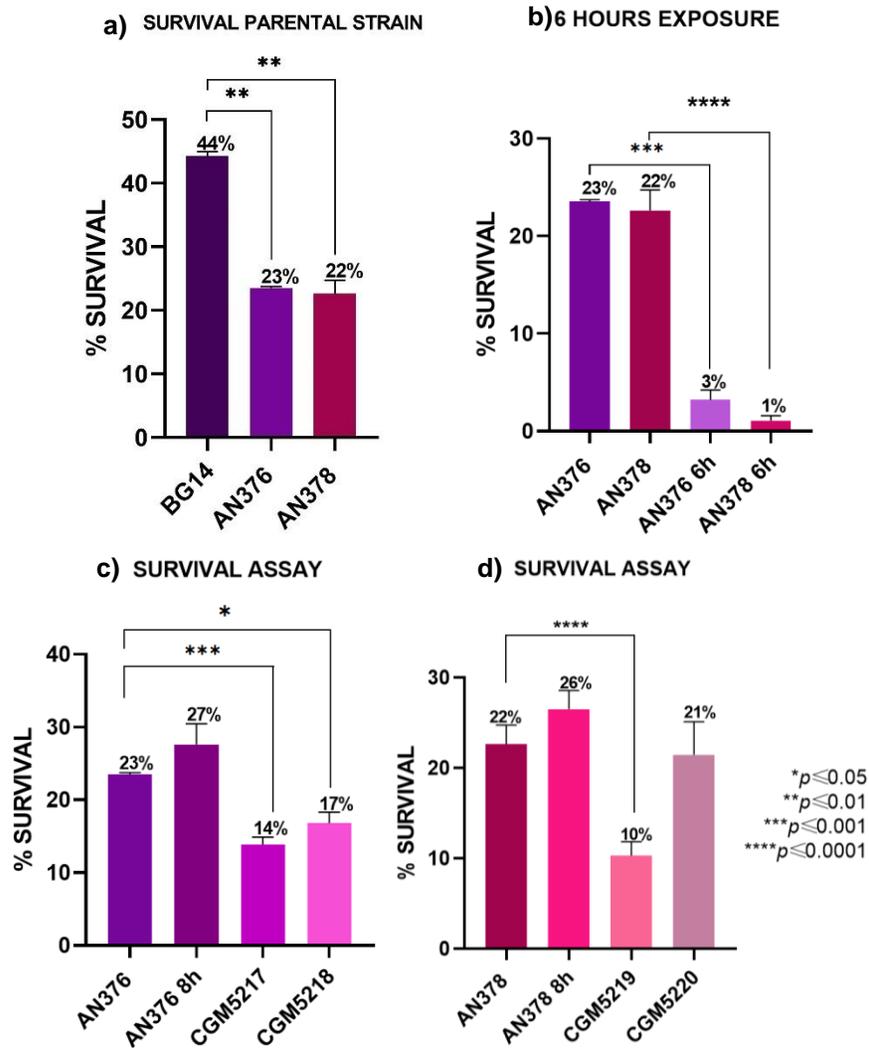
Fig. 3| Survival assay in *N. glabratus* clinical isolates after 3 (2-hour) passages through NØ. a)
Parental strains, exposure to NØ for two h (survival assay) n=3, b) Percent survival after 6 h exposure

326 to NØ n=1 (three passes through NØ) AN376: % survival of parental strain; AN378: % survival of
327 parental strain; AN376 6h: three consecutive passages of two h each through NØ, six h in total;
328 AN378 6h: three consecutive passages of two h each through NØ, six h in total. c) Survival assay of
329 strains recovered after the 6 h exposure to NØ (eight hours total exposure): AN376: % survival of
330 parental strain; AN376 8h: three consecutive passages of two h each through NØ, plus one survival
331 assay, eight h in total; CGM5217 and CGM5218: colonies derived from AN376 with eight h exposure
332 in total n=3. d) AN378: % survival of parental strain; AN378 8h: three consecutive passages of two h
333 each through NØ, plus one survival assay, eight h in total; CGM5219 and CGM5220 colonies derived
334 from AN378 with eight h exposure in total n=3. Difference between groups was assessed by Ordinary
335 one-way ANOVA followed by Tukey's multiple comparisons post-hoc test according to data normality.
336 P-values ≤0.05 were considered statistically significant.

338   After three passages through NØ cells, the clinical isolates AN376 and AN378
339 showed significantly lower survival rates (3% and 1% respectively) compared to the
340 reference strain (44%). By comparison, the parental strains display much higher
341 survival rates (AN376: 23%, p = <0.0001, AN378: 22%, p = <0.0001) (Fig. 3a and
342 3b). Interestingly, the population of survivors from the 3 passages through NØ (cells
343 recovered from the survival assay shown in Fig. 3b), have a similar survival rate to
344 that of their respective parental strains. Therefore, we did not see a higher acquired
345 survival rate after the microevolution experiment (AN376: 23% compared to 27%
346 after the microevolution experiment, and AN378: 22% vs 26%, survival after the
347 three consecutive passages through NØ). (AN376: p = 0.2162, AN378: p = 0.2813).
348   We decided to isolate individual colonies of AN376 and AN378 strains after
349 successive passages through NØ. Colony CGM5217 exhibited a small colony
350 phenotype, while CGM5218 displayed a regular colony size originated from the
351 AN376 isolate. Similarly, colony CGM5219 exhibited a regular colony size
352 phenotype, while CGM5220 had a small size; these colonies were derived from
353 AN378. These colonies, once isolated and purified, underwent a survival assay, and
354 the resulting percentages were compared with those of their parental strains and the
355 8-hour population exposed to NØ. In contrast to the survival rate of the AN376
356 parental strain (AN376: 23%), isolated colonies derived from this strain exhibit a
357 reduced survival rate (CGM5217: 14%, p = 0.0004 and CGM5218: 17%, p = 0.0178,
358 respectively), additionally, both CGM5217 and CGM5218 show significantly smaller

359 survival rates (CGM5217: $p = < 0.0001$ and CGM5218: $p = 0.0001$) compared to

360 the AN376 mixed evolved population (AN376 8h: 27% see Fig. 3c).

361   Upon comparing the survival percentages of colonies derived from AN378 (Fig.

362 3d), significant differences were noted between colonies CGM5219 and CGM5220

363 ($p = 0.0002$). Furthermore, it is evident that the isolated colony CGM5219 exhibits

364 lower survival compared to both its parent (CGM5219: 10%, $p = < 0.0001$) and the

365 AN378 population (AN378 8h: 26%, $p = < 0.0001$). Conversely, the survival rate of

366 colony CGM5220 remains similar to that of its parent (CGM5220: 21%, $p = 0.9995$).

367   Consecutive passes through NØ do not confer greater resistance in either of the

368 clinical isolates.

369

Phenotypic characterization of the surviving *N. glabratus* clinical isolates.

We decided to continue phenotypically characterizing the surviving *N. glabratus* cells: we determined the response H_2O_2, antifungals (FLC and CSP), and temperature stress of the cells that had been exposed to NØ for 3 consecutive passes (Fig. 4)

a) [YPD + FLC µg/mL]

b) [YPD + CSP µg/mL]

c) [YPD + H₂O₂ mM]

d)

20
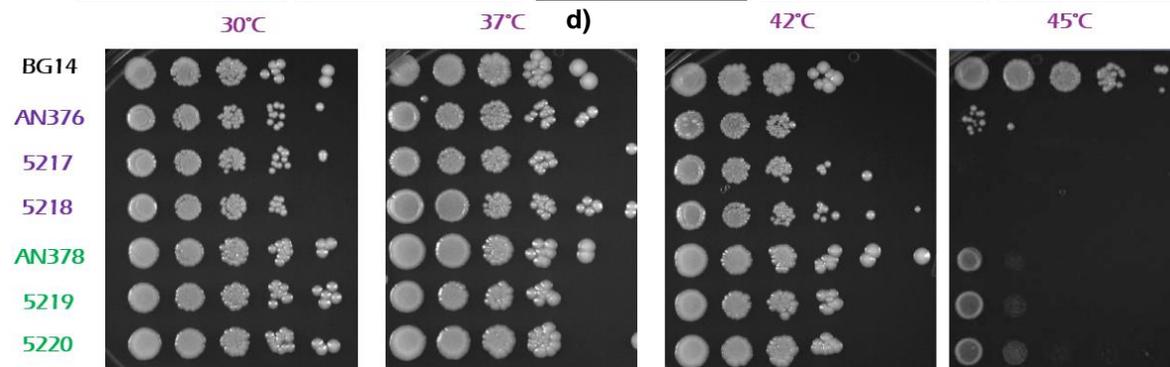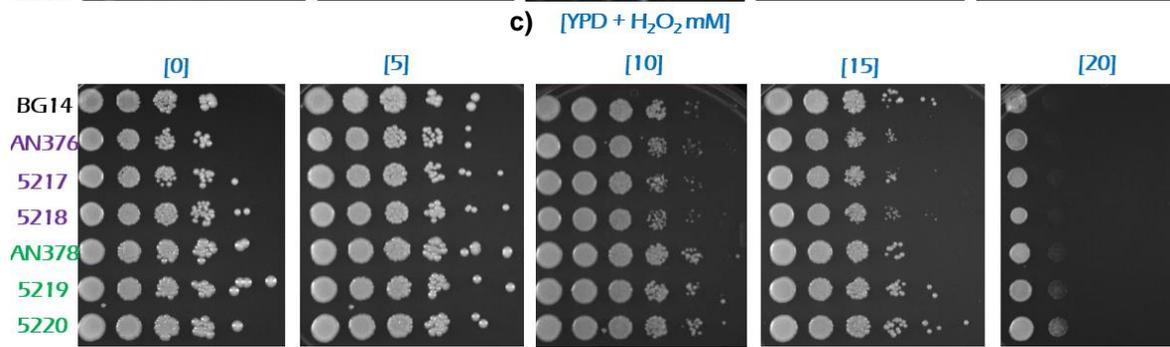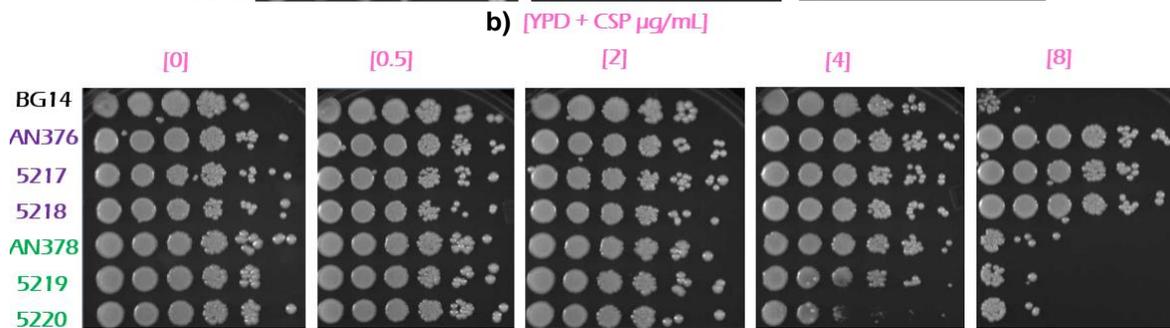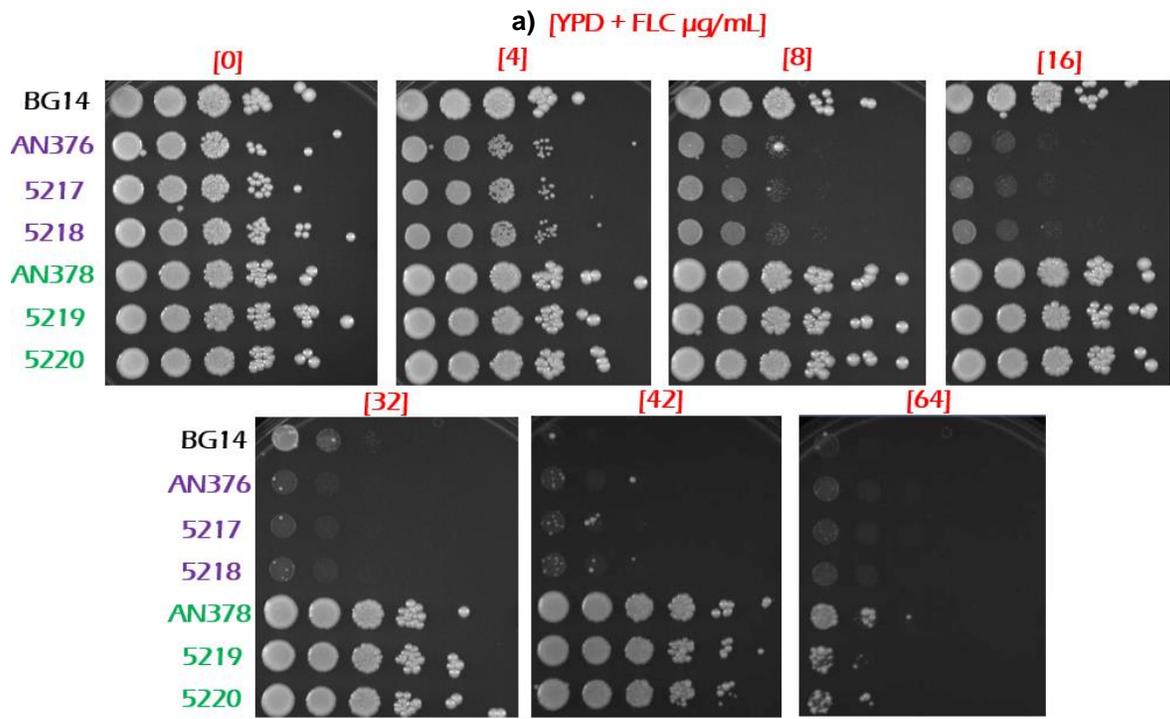
Fig. 4| Spot assay under different oxidative stress types and fluconazole of the *N. glabratus* clinical isolates. Abbreviations FLC: fluconazole, CSP: caspofungin, H_2O_2: hydrogen peroxide, BG14: reference strain. AN376: clinical isolates strain, CGM5217: isolated colony of AN376 strain after six h of exposure to NØ with small colony phenotype, CGM5218: regular colony size originated from the AN376 after six h to NØ, CGM5219: isolated colony of AN378 strain after six h of exposure to NØ with regular colony phenotype, CGM5220: small colony size originated from the AN378 after six h to NØ. n=3

Following neutrophil passages, we isolated and purified colonies from isolate AN376 (CGM5217 and CGM5218) and AN378 (CGM5219 and CGM5220), respectively. These colonies underwent various stress tests, and the results were compared to their parental strains (retrieved from the glycerol stock) and the reference strain BG14.

As depicted in Fig. 4a, no differences in susceptibility/resistance to FLC were observed compared to their parental strains. As we had previously determined in the laboratory, isolate AN376 exhibited heightened susceptibility to FLC compared to BG14 and the evolved derivatives, CGM5217 and CGM5218 colonies showed the same susceptibility.

To explore potential alterations in resistance/susceptibility to a different class of antifungals such as CSP (Fig. 4b), which targets the 1-3 β-glucan synthase essential for maintaining *N. glabratus'* cell wall integrity, resistance to CSP was observed in isolate AN376, and its evolved derivatives colonies CGM5217 and CGM5218 [8 µg/mL] compared to BG14 and AN378. Interestingly, the isolated colony CGM5220, which evolved from AN378, displayed increased sensitivity to CSP [4 µg/mL] compared to its parent strain.

In addition, we did not find discernible differences between the strains when compared to each other regarding their response to oxidant stress, H_2O_2 (Fig. 4c), nor to heat shock (Fig. 4d).

Although AN376 and its respective derivates are temperature sensitive at 45°C while AN378 is not as sensitive compared to BG14.

Therefore, serial passages through NØ did not confer greater resistance to FLC or H_2O_2 exposure. Nevertheless, colony CGM5220 exhibited sensitivity to CSP compared to AN378.

413   ***N. glabratus* mutants in histone deacetylases are involved in survival to NØ**

414   **attack.**

415   We next asked whether epigenetic mechanisms, particularly histone acetylation,

416   participate in the response to NØ. To do this, we generated knockout mutants by

417   fusion PCR in the BG14 strain genetic background in *GCN5* (*gnc5Δ*) encoding the

418   histone deacetylase (HAT) Gcn5, and in the genes *RPD3* and *HOS2* (*rpd3Δ* and

419   *hos2Δ*) encoding two histone deacetylases (HDAC). We then measured the survival

420   to NØ of these mutants (Fig. 5).
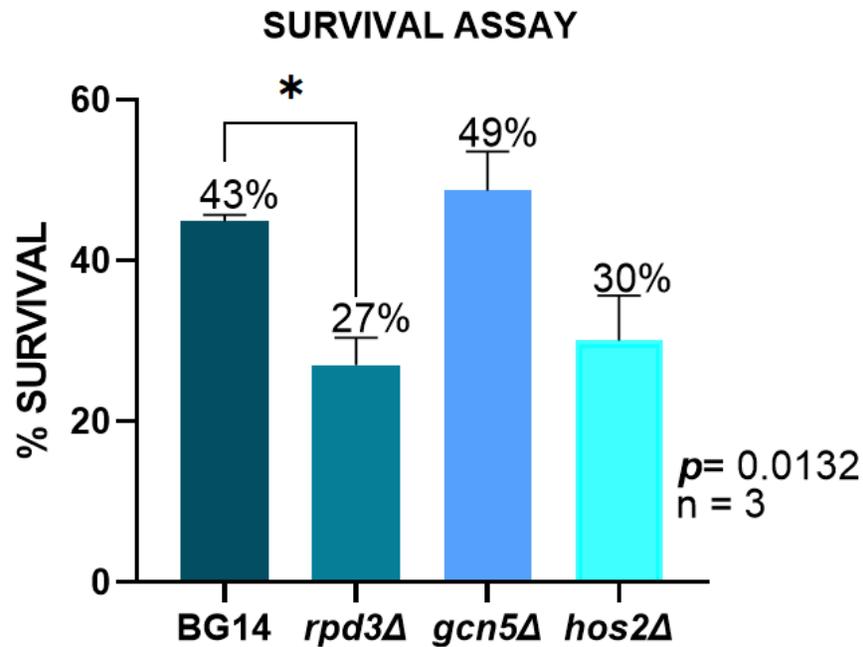
SURVIVAL ASSAY

Fig. 5| Survival assay in *N. glabratus* knockout mutants after 2-hour passages through NØ. The difference between groups was assessed by Ordinary one-way ANOVA followed by Turkey´s multiple comparisons post-hoc test according to data normality (*$p < 0.05$). P-values ≤0.05 were considered statistically significant n=3.

After the survival assay (a single passage through NØ), it is evident that the survival percentage of the *rpd3Δ* strain is lower than that of BG14 (*rpd3Δ*: 43%, $p = 0.0367$ vs BG14: 43%). Although not statistically significant, a trend toward lower survival is also observed in the *hos2Δ* strain (*hos2Δ*: 30%, $p = 0.0801$). Conversely, the *gcn5Δ* mutant exhibited no significant differences in survival percentage compared to the reference strain (*gcn5Δ*: 49%, $p = 0.8470$).

To continue phenotypically characterizing the surviving *N. glabratus* knockout mutants: we determined the response H_2O_2, antifungals (FLC and CSP) and temperature stress of the histone acetylation/deacetylation mutant strains and the derived colonies that had been exposed to NØ.

Phenotypic characterization of the surviving *N. glabratus* knockout mutants.

To continue phenotypically characterizing the surviving *N. glabratus* knockout mutants, we determined the response H_2O_2, antifungals (FLC and CSP) and temperature stress of the histone acetylation/deacetylation mutant strains and the derived colonies that had been exposed to NØ (Fig. 6).

442

a)

[YPD + FLC µg/mL]

[0]　　　　[4]　　　　[8]　　　　[16]　　　　[32]　　　　[64]

BG14
hos2Δ
1
2
gcn5Δ
1
rpd3Δ
2

443

b)

[YPD + CSP µg/mL]

[0]　　　　[0.5]　　　　[2]　　　　[4]　　　　[8]

BG14
hos2Δ
1
2
gcn5Δ
1
rpd3Δ
2

444

c)

[YPD + H$_2$O$_2$ mM]

[0]　　　　[5]　　　　[10]　　　　[15]

BG14
hos2Δ
1
2
gcn5Δ
1
rpd3Δ
2

445

25

d)
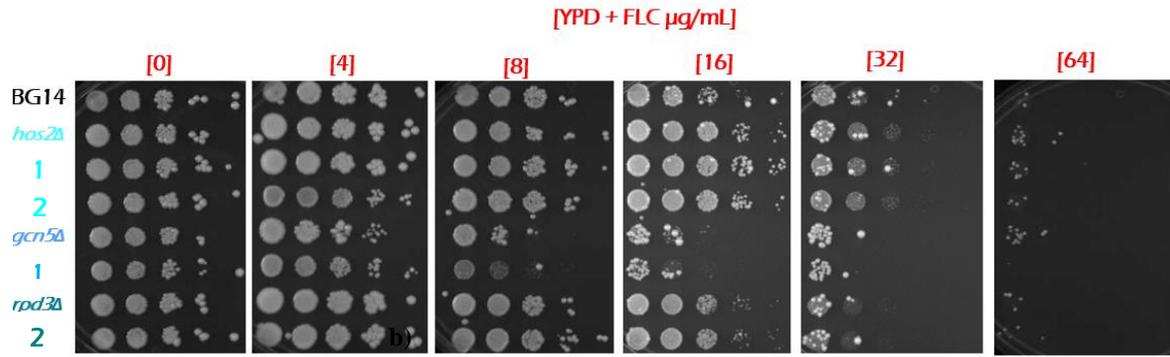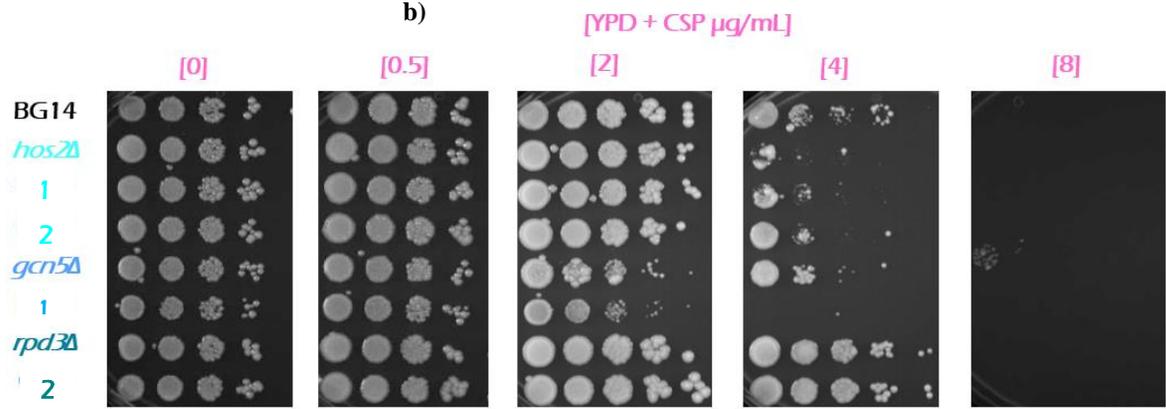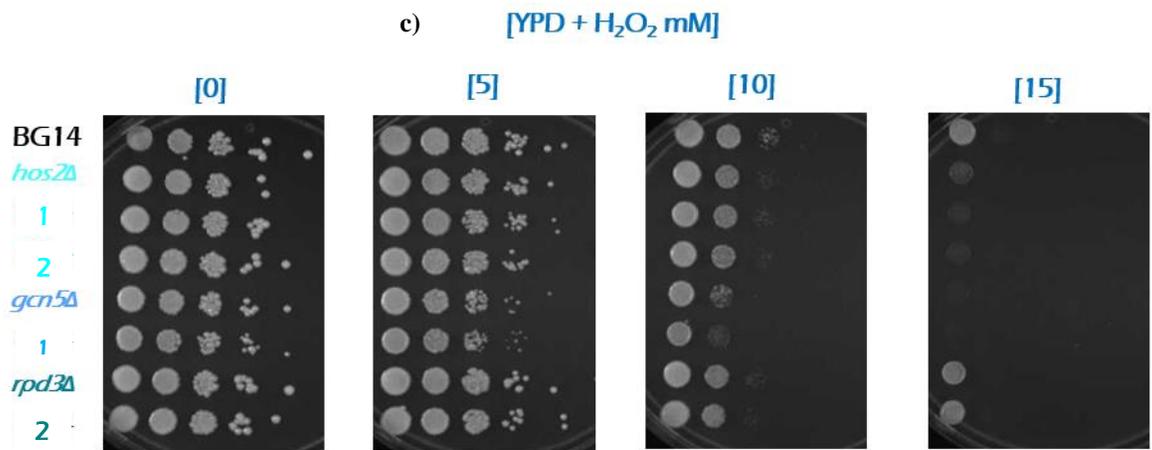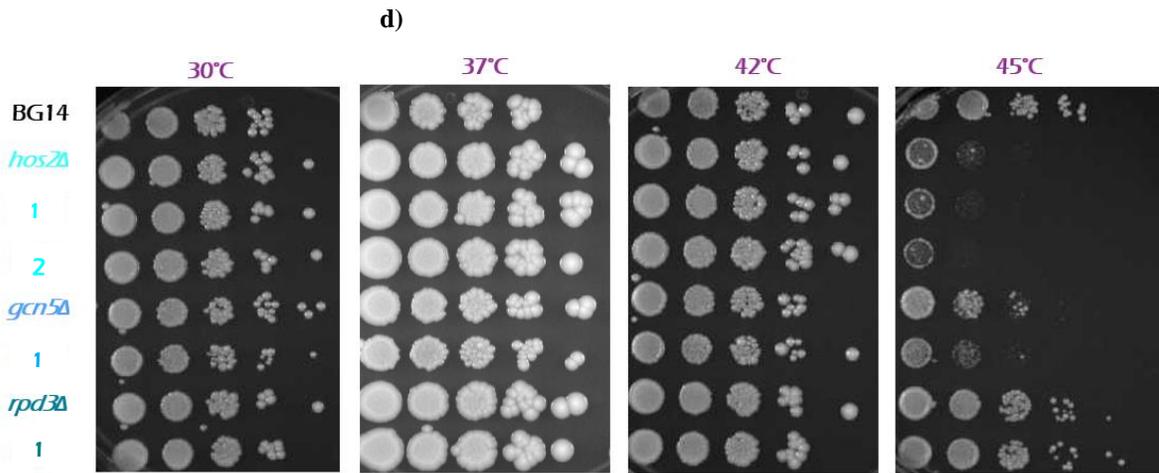
446

447 **Fig. 6| Spot assay under different oxidative stress conditions and fluconazole of the histone**
448 **acetylation/deacetylation knockout mutants.** Abbreviations FLC: fluconazole, CSP: caspofungin,
449 H_2O_2: hydrogen peroxide, BG14: reference strain. *hos2Δ*: recovered strain, 1: regular size colony
450 isolated of *hos2Δ* strain after two h of exposure to NØ, 2: regular colony size originated from *hos2Δ*
451 after two h to NØ, *gcn5Δ*: recovered glycerol strain, 1: regular size colony isolated of *gcn5Δ* strain
452 after two h of exposure to NØ, *rpd3Δ*: recovered glycerol strain, 1: regular size colony isolated of
453 *rpd3Δ* strain after two h of exposure to NØ. n=2

454

455   After the NØ assay, we isolated and purified colonies obtained from *hos2Δ*
456 (colonies 1 and 2), *gcn5Δ* (1) and *rpd3Δ* (1) respectively and we tested these
457 colonies onto various stress tests compared to their parental strains and BG14
458 strain.

459   The results of chronic stress exposure to FLC are shown in Fig. 6a. FLC
460 sensitivity is evident in the parental strain *gcn5Δ* and its derived colony [16 µg/mL]
461 compared to BG14. We did not find significant differences in resistance to FLC at 32
462 µg/mL observed in the *hos2Δ*.

463   Regarding the resistance/susceptibility phenotypes to CSP (Fig. 6b), no
464 differences were observed between *rpd3Δ* and BG14. In contrast, *hos2Δ* and its
465 derivatives exhibited increased sensitivity at 4 µg/mL compared to the WT.
466 Additionally, the colony derived from *gcn5Δ* after NØ exposure demonstrated
467 significantly greater resistance to CPS [2 µg/mL] compared to its parent and the
468 reference strain. On the other hand, no differences were noted in growth phenotypes
469 when exposed to H_2O_2 (Fig. 6c) versus BG14 strain.

470   Furthermore, when evaluating the response to temperature stress (Fig. 6d), a
471 sensitivity phenotype was observed at 45°C in *hos2Δ* and colonies derived from it

472 and in the *gcn5Δ* two h exposure colony derived from the *gcn5Δ* parental strain,
473 compared to BG14. No differences in the temperature stress response were
474 observed in the *rpd3Δ* mutant compared to the parent strain.

Discussion

Consecutive passages through NØ do not enhance resistance to NØ.

477 Recently some investigations have studied the microevolution of *N. glabratus*
478 during the infection of human hosts and the development of antifungal resistance
479 (Badrane et al., 2023; López-Marmolejo et al., 2024). However, it remains unclear
480 whether *N. glabratus* cells exhibit microevolution *in vitro* when exposed to cells of
481 the innate immune system. Innate immunity plays a crucial role in combating and
482 preventing infections caused by opportunistic fungi. Phagocytic cells, such as NØ,
483 act as the first line of defense by recognizing these pathogenic fungi (Salgado et al.,
484 2021). *N. glabratus* relies on inducing a low-grade inflammatory response and silent
485 transit within the host, delaying neutrophil activation (Shantal et al., 2022). However,
486 the mechanisms by which *N. glabratus* evades neutrophil activity have not been
487 extensively studied.

488 In this study, for the first time, we evaluated *in vitro* microevolution of clinical
489 isolates and a reference strain of *N. glabratus*. Through three passages to induce
490 microevolution, we observed a slight increase in the survival trends of Gly$^+$ cells.
491 Nonetheless, no significant difference was noted in the survival rate of the Gly$^-$
492 phenotype compared to untreated cells (Fig. 1). In clinical isolates AN376 and
493 AN378, three consecutive passages through NØ (evolved cells) resulted in
494 significant loss of viability as measured by a survival assay of the recovered cells
495 (Fig. 2b). However, the isolated evolved survivors of the three passages through NØ
496 (cells recovered from the survival assay shown in Fig. 2b), retained a similar survival
497 rate to their respective parental strain (evolved from AN736: 22%, compared to the
498 parental AN376 27%, and parental strain AN378: 22% compared to evolved AN378:
499 26%; Fig. 2c). Therefore, we do not observe any microevolution that enables it to
500 better survive increased levels to NØ.

501   Although we did not observe enhanced resistance to NØ as a result of
502   microevolution, Badrane et al., 2023 have reported *N. glabratus* clinical isolates
503   resistant to phagocytosis and death by NØ. Overall, passages through NØ do not
504   confer greater resistance in clinical isolates or the laboratory reference strain BG14.
505   Further research is needed to elucidate these mechanisms, which are essential for
506   developing drugs targeting these adaptations.

507   **N. glabratus mutants rpd3Δ and hos2Δ are involved in the survival against**
508   **NØ.**

509   In this study, we present novel findings demonstrating the involvement of HDACs
510   (*Cg*Rpd3 and *Cg*Hos2) in *N. glabratus* survival under NØ stress (Fig. 5), as well as
511   their response to thermal stress (Fig. 6). Rpd3 and Hos2 belong to the same family
512   of class I histone deacetylases in *S. cerevisiae* (Kurdistani and Grunstein, 2003).
513   *Sc*Rpd3 and *Sc*Hos2 show some redundancy in the genes they regulate like, *RNR3*
514   and *HUG1* genes, and elimination of these HDACs leads to hyper-acetylation to
515   different degrees, in several common genes like: *ERG11, GAL7, GAL10*, and *GAL1*.
516   Rpd3 is required for deacetylation of H3, H2B and H4 except for H4K16, and Hos2
517   for deacetylation of H4-K16 (Kurdistani and Grunstein, 2003; Sharma et al., 2007;
518   Wang et al., 2002). Rpd3 activity is associated with the activation of osmotic stress
519   response gene expression in *S. cerevisiae*, as well as in azole resistance in *C.*
520   *albicans*, while Hos2 is necessary for chromatin deacetylation and recruitment to the
521   open reading frames (ORFs) of different genes (De Nadal et al., 2004; Li et al., 2015;
522   Sharma et al., 2007). Our study reveals that the survival percentage of these
523   knockout mutants is lower than that of the parental strain BG14 (*rpd3Δ*: 27%, *hos2Δ*:
524   30% compared to 41% in the parental BG14).

525   Previous studies have reported the involvement of *Sc*Hos2 and *Sc*Rpd3 in
526   regulating the transcriptional activation of DNA damage response genes, directly
527   positively regulating *RNR3* and *HUG1* (Sharma et al., 2007). Fukuda et al., 2013
528   evaluated the transcriptional profile of *N. glabratus* during phagocytosis by NØ and
529   found that 5% of the 339 upregulated genes corresponded to DNA repair processes.
530   Based on this information, we propose that the lower survival percentage observed
531   in our study may be due to the inefficient transcriptional activation of DNA damage

response genes resulting from HDACs elimination. Consequently, the production of ROS from NØ may cause DNA damage that is not adequately repaired. However, further investigation is required to confirm these findings by quantifying ROS production by NØ.

Filler et al., 2021 identified that Rpd3 is necessary for *N. glabratus* for virulence, as the mutant exhibited attenuated virulence in a neutropenic mice model. Additionally, susceptibility assays to host defense peptides revealed that the *Cg*Rpd3 mutant showed susceptibility to human neutrophil peptide-1 (hNP-1), whereas *Cghos2Δ* strain displayed a response similar to its parent strain (CBS138 strain) to hNP-1 but sensitivity to protamine, an antimicrobial compound. We demonstrate the involvement of HDACs in NØ survival mechanisms, possibly induced by defects in DNA repair caused by NØ-derived ROS. However, confirmation of these findings awaits further investigation into ROS production by NØ and DNA repair mechanisms in the mutants.

Gcn5 is not involved in the survival to NØ.

GCN5 is part of the SAGA complex with histone acetyltransferase (HAT) activity. Previous studies have reported the role of CgGcn5 in modulating virulence and regulating replication within macrophages in *N. glabratus* (Yu et al., 2022), although there are no reported data on NØ survival. Our findings indicate that exposing the *gcn5Δ*mutant to NØ did not alter the survival percentage compared to its parent strain, suggesting that Gcn5 may not be involved in NØ resistance, unlike the responses to macrophages where the *gcn5Δ* mutant is more sensitive than the parental strain.

The epigenetic response participates in different types of stress.

Previous studies have evaluated the involvement of the epigenetic response in antifungal resistance mechanisms. In this study, we assessed the response to FLC, CSP, H_2O_2, and temperature, in mutants generated in BG14 background and, colonies derived after NØ confrontation (Fig. 6).

Pfaller et al., 2009 evaluated the synergistic effect of a drug inhibiting *Cg*Hos*2* activity (MGCD290) combined with three different azoles (FLC, posaconazole, and

562 voriconazole). They observed increased sensitivity in clinical isolates of *N. glabratus*
563 previously reported as resistant when treated with this combination. On the other
564 hand, Yu et al., 2022 reported that eliminating *GCN5* increased the sensitivity to FLC
565 in the CBS138 strain. These results align with the findings found in this study, where
566 a slight sensitivity to 4 μg/mL was observed in the *gcn5Δ* and *hos2Δ* compared to
567 the WT. Interestingly, the *rpd3* mutant did not exhibit any phenotypic change. Thus,
568 we confirmed the role of these histone modifiers in resistance to FLC. By observing
569 a phenotype of increased sensitivity in the colony isolated from *gcn5Δ*, we
570 demonstrated that exposure to NØ resulted in greater sensitivity to FLC. However,
571 the potential mechanisms underlying this phenotype remain to be studied.

572 Deleting *rpd3, hos2* and *gcn5* in *N. glabratus* CBS138 background increases
573 susceptibility to CSP and Micafungin (Filler et al., 2021; Schwarzmüller et al., 2014;
574 Yu et al., 2022). Additionally, a synergistic effect has been observed in clinical
575 isolates of *N. glabratus*, showing a 46.7-53.3% increase in sensitivity when treated
576 with MGCD290+CSP, and reversing resistance in isolates with FKS mutations
577 (Pfaller et al., 2015). When we evaluated the response to CSP
578 susceptibility/resistance (Fig. 6b), we observed differences in growth among *gcn5Δ*
579 with BG14 strain. These data are consistent with susceptibility to CSP in the *gcn5Δ*
580 background but not *rpd3Δ*. Interestingly, *hos2Δ* exhibited a slightly increased
581 sensitivity to CPS compared to the WT strain. The results suggest that Hos2 and
582 Gcn5 play an important role in CPS resistance. The resistance to CPS observed in
583 Gcn5 may be attributed to its role in regulating the FKS genes (Yu et al., 2022),
584 which are essential for maintaining the cell wall of *N. glabratus*. However, the
585 mechanisms utilized by Hos2 remain to be studied.

586 When testing H_2O_2 sensitivity, we observed a modest increase in susceptibility in
587 *gcn5* mutant and its derived colony, but not in other strains (Fig. 6c). This confirms
588 previous reports that *Cg*Gcn5 is related to resistance to this type of stress (Filler et
589 al., 2021). Deleting this gene in *N. glabratus* increases sensitivity to H_2O_2 (10mM)
590 compared to CBS138 standard strain (Lin et al., 2023; Yu et al., 2022). However,
591 resistance to H_2O_2 is not affected by mutations in *rpd3Δ* or *hos2Δ* in our study and
592 Filler et al., 2021, suggesting that HAT (*Gcn5*) is responsible for H_2O_2 resistance,
593 while HDACs do not play a significant role.

594   Finally, we evaluated the response to temperature stress. *Sc*Rpd3 has been
595   proposed to fine-tune the transcriptional response under temperature shock (Kremer
596   and Gross, 2009). There is no other reference in the literature, and further studies
597   are needed to address this question. We evaluated the involvement of the epigenetic
598   response in resistance to temperature shock in *N. glabratus* (Fig. 6d). For the first
599   time, we described that mutants and colonies derived from *hos2Δ* and *gcn5Δ* are
600   more sensitive to growth at 45°C compared to the control. Therefore, we propose
601   that Gcn5 and Hos2 are involved in the heat shock response in *N. glabratus*.
602   In conclusion, our findings show that three passes through NØ do not increase
603   the resistance to NØ in the *N. glabratus* clinical isolates and BG14 strain. We
604   demonstrated for the first time that Rpd3 and Hos2 are involved in the survival of
605   *N. glabratus* to NØ, whereas Gcn5 is not. This discovery could pave the way for
606   identifying new targets for drug development.

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730

Supplementary material

Supplementary tables.

Table S1| Strains generated.

C. glabrata strains	Parental	Genotype	Phenotype		Reference
BG14	BG2	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^+$		Cormack and Falkow 1999
Evolved strains				**Treatment**	
CGM5014	BG14	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^-$	Evolved 3 passes NØ	This work
CGM5015	BG14	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^-$	Survival assay	This work
CGM5016	BG14	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^-$	Evolved 3 passes RPMI	This work
CGM5017	BG14	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^-$	Evolved 3 passes NØ	This work
CGM5018	BG14	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^-$	One pass to RPMI	This work
CGM5019	BG14	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^-$	Evolved 3 passes RPMI	This work
CGM5020	BG14	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^-$	Survival assay	This work
CGM5021	BG14	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^+$	Evolved 3 passes NØ	This work
CGM5022	BG14	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^+$	Survival assay	This work
CGM5023	BG14	*ura3*∆::Tn903 G418^R	Ura$^-$ Gly$^+$	One pass to RPMI	This work

CGM5037	BG14	*ura3Δ*::Tn903 G418^R	Ura$^-$ Gly$^-$	Evolved 3 passes NØ	This work
CGM5038	BG14	*ura3Δ*::Tn903 G418^R	Ura$^-$ Gly$^-$	Evolved 3 passes RPMI	This work
CGM5039	BG14	*ura3Δ*::Tn903 G418^R	Ura$^-$ Gly$^-$	Evolved 3 passes NØ	This work
CGM5040	BG14	*ura3Δ*::Tn903 G418^R	Ura$^-$ Gly$^-$	One pass to RPMI	This work
CGM5041	BG14	*ura3Δ*::Tn903 G418^R	Ura$^-$ Gly$^-$	Evolved 3 passes RPMI	This work
CGMCGM5217	AN376	Clinical isolate	Gly$^+$	Evolved 3 passes NØ	This work
CGMCGM5218	AN376	Clinical isolate	Small, colony, Gly$^{+,}$	Evolved 3 passes NØ	This work
CGMCGM5219	AN378	Clinical isolate	Gly$^+$	Evolved 3 passes NØ	This work
CGMCGM5220	AN378	Clinical isolate	Small colony, Gly$^+$	Evolved 3 passes NØ	This work
CGM5227	AN376	Clinical isolate	Gly$^+$	Evolved 3 passes RPMI	This work
CGM5228	AN378	Clinical isolate	Gly$^+$	Evolved 3 passes RPMI	This work
CGM5229	AN378	Clinical isolate	Gly$^-$	Evolved 3 passes RPMI	This work
CGM5245	AN376	Clinical isolate	Gly$^+$	Evolved 3 passes RPMI	This work
CGM5246	AN378	Clinical isolate	Gly$^+$	Evolved 3 passes RPMI	This work
CGM5247	AN376	Clinical isolate	Small colony, Gly$^+$	Evolved 3 passes RPMI	This work

CGM5248	AN378	Clinical isolate	Small colony, Gly⁺	Evolved 3 passes RPMI	This work
CGM5249	AN376	Clinical isolate	Gly⁻	Evolved 3 passes RPMI	This work

Mutants | | | | **Function** |

CGM5242	BG14	*rpd3Δ::*FRT-*URA3-*FRT	Ura⁺ Gly⁺	Histone deacetylase activity (targets N-terminal H4K5 and H4K12).	This work
CGM5243	BG14	*rpd3Δ::*FRT-*URA3-*FRT	Ura⁺ Gly⁺	Histone deacetylase activity (targets N-terminal H4K5 and H4K12).	This work
CGM5312	BG14	*gcn5Δ::*FRT-*URA3-*FRT	Ura⁺ Gly⁺	Histone acetyltransferase activity (targets N-terminal lysine residues K11/K16 in histone H2B and K9/K14/K18/K23/K27 in histones H3).	This work
CGM5313	BG14	*hos2Δ::*FRT-*URA3-*FRT	Ura⁺ Gly⁺	Histone acetyltransfera	This work

				se activity (targets N-terminal lysine residues K11/K16 in histone H2B and K9/K14/K18/K23/K27 in histones H3).	
CGM5314	BG14	*hos2Δ::*FRT-*URA3*-FRT	Ura+ Gly+	Histone deacetylase activity (N-terminal H4-K16)	This work

734

39

735 **Table S2| Primers**

Strain	Primers	Sequence 5´-> 3´
rpd3Δ	RPD3at-588Fw	GAACGCCATGTATGACAACG
	RPD3at-4Rv	**CGAATTCAGGAACTTGATATTTTT**TCC CTATCGCTGTATTAACC
	RPD3at+24Fw	**GGCTACCACATCGTCTTTG**TA AATAACCCCACCCTCTGC
	RPD3at+522Rv	GACCCAATCCCTTAACTGCC
	RPD3at314_Fw	TGTCGGTGACGATTGTCCCG
	RPD3at816_Rv	ACCACATTGCAACACCACGG
	DiagRPD3at-666Fw	CTTCTCTGCAGACGACACCC
	DiagRPD3at+631Rv	GCATTCTGTCAATGGCACCC
gcn5Δ	GCN5@-968PBFW	GGGAACAGGGTCTACGTATG
	GCN5@-917PBFW	GGAGGTATTGCAGTAAGAGAATG
	GCN5@12PBFW	GACTGTACGCACCGTGTTAG
	GCN5@+4PBFW	**CACGGCTACCACATCGTCTTTG**GTT GGTTAGCTTAAGGTCATGG
	GCN5@-76PBRV	**CGAATTCAGGAACTTGATATTTTT**GCTC CAAAAGGGAAAGCTCTAAC
	GCN5@382PBRV	GACTGTTGATGTAGTACTCGATG
	GCN5@+954PBRV	CGCTGTGAATTCCAAGAGC
	GCN5@+808PBRV	CTAATCCAGCATTACGTGGTG CAAGGTGGTTACAATATTCAATAG
hos2Δ	HOS2@1000PBFW	GTGGACTCATGATCTCAATG
	HOS2@-957PBFW	CCACTTCCACTGTGTCGCAG
	HOS2@60PBFW	GCTCTAACTATGCGCCTCG
	HOS2@+0PBFW	**CACGGCTACCACATCGTCTTTG**CAGCTACAT ACTATTTACGATAATGAGAAG
	HOS2@-39PBRV	**CGAATCGAATTCAGGAACTTGATATTTTTC**GGC TTATAATCACTAGATGAACACTTC
	HOS2@339PBRV	CGGACAATCATCACCAATG
	HOS2@+989PBRV	GTGGCTTTGATAACTTTCCTG
	HOS2@+955PBRV	GTGAATTCCTCAACACAGCC

736

737   **Table S3| Program fusion PCR**

First part		
Buffer 5X	3 µL	
Module 5´	X µL	
Module 3´	X µl	
URA3	X µL	
dNTP	1.5 µL	
iProof	0.3 µL	
Water	3 µL	
	15 µL	

738

98°C	**3:00**	
98°C	**0:30**	
56°C	**0:30**	10x
72°C	**2:30**	
4°C	**1:00**	

Second part		
Buffer 5X	1 µL	
#Prmer Fw	2 µl	
#Primer Rv	2 µL	
	20µL	

98°C	2:00	
98°C	0:20	
62°C	0:20	20x
72°C	2:30	
72°C	3:30	
12°C	1:00	

41

739 **Table S4| Percent reversibility of Gly⁻ to Gly⁺ phenotype**

Strain	Parental	% of reversibility
5014	BG14	0%
5015	BG14	0%
5020	BG14	0%
5037	BG14	0%
5229	AN378	0%

740

741 **Supplementary Figures**

a)
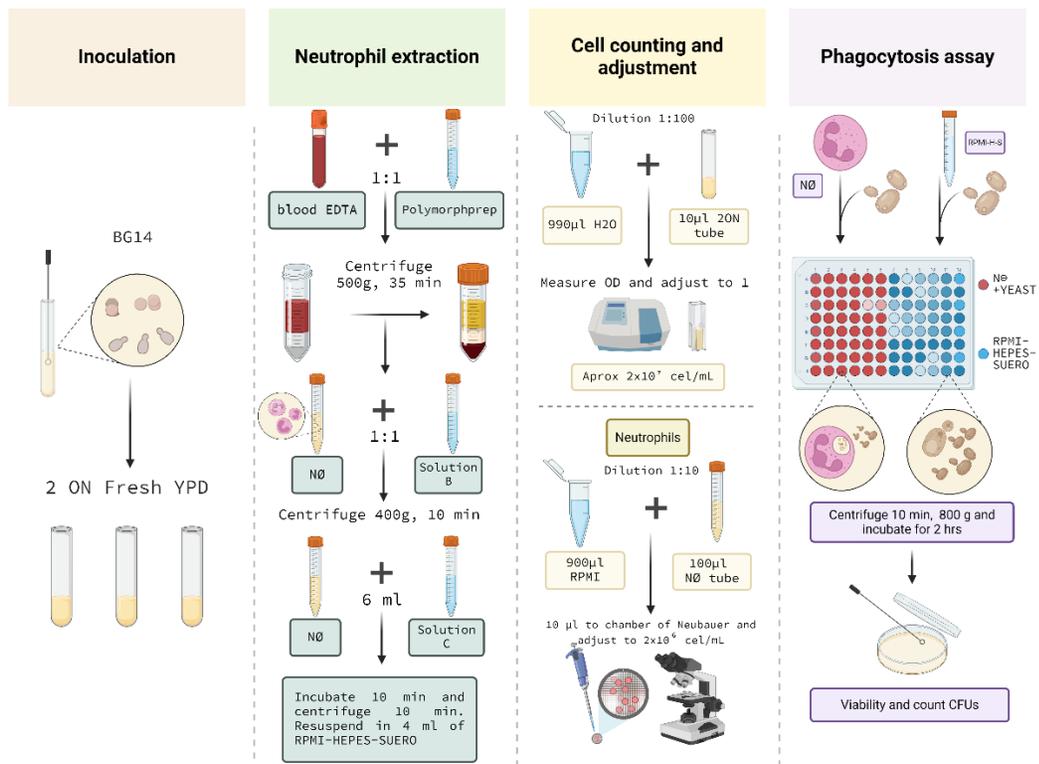
742

b)
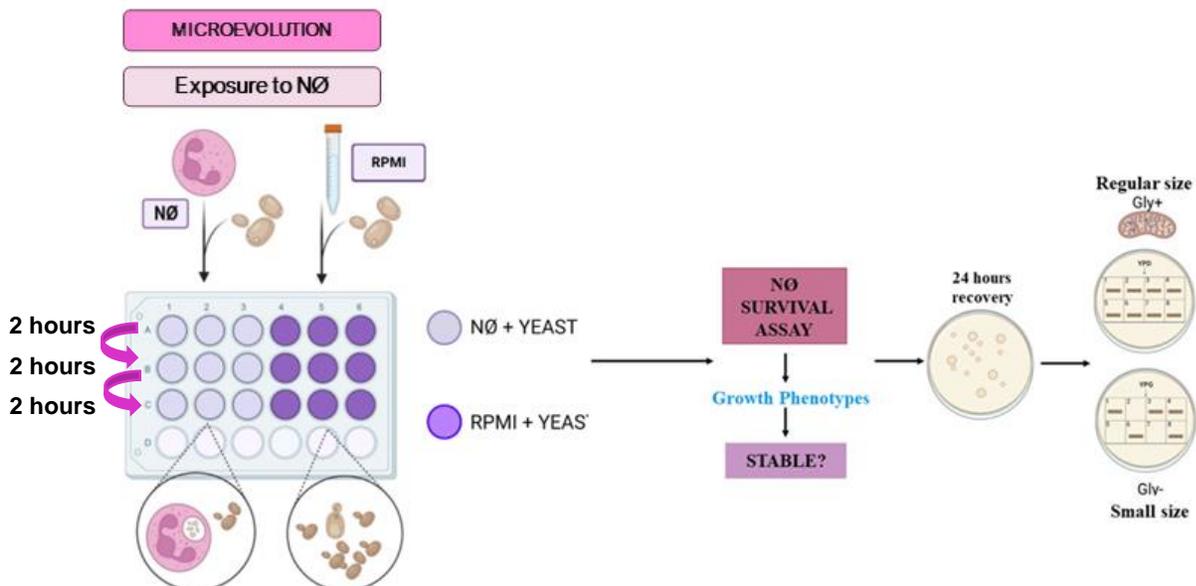
743 **Fig. S1|Schematic representation of experimental approach.** Abbreviature:
744 Optical density (OD), overnight (ON), water (H$_2$O), polymorphonuclear (PMN),
745 colony-forming unit (CFU). a) Schematic representation of survival assay. b)
746 Schematic representation of microevolution of *N. glabratus* cells.

747

Spott assay
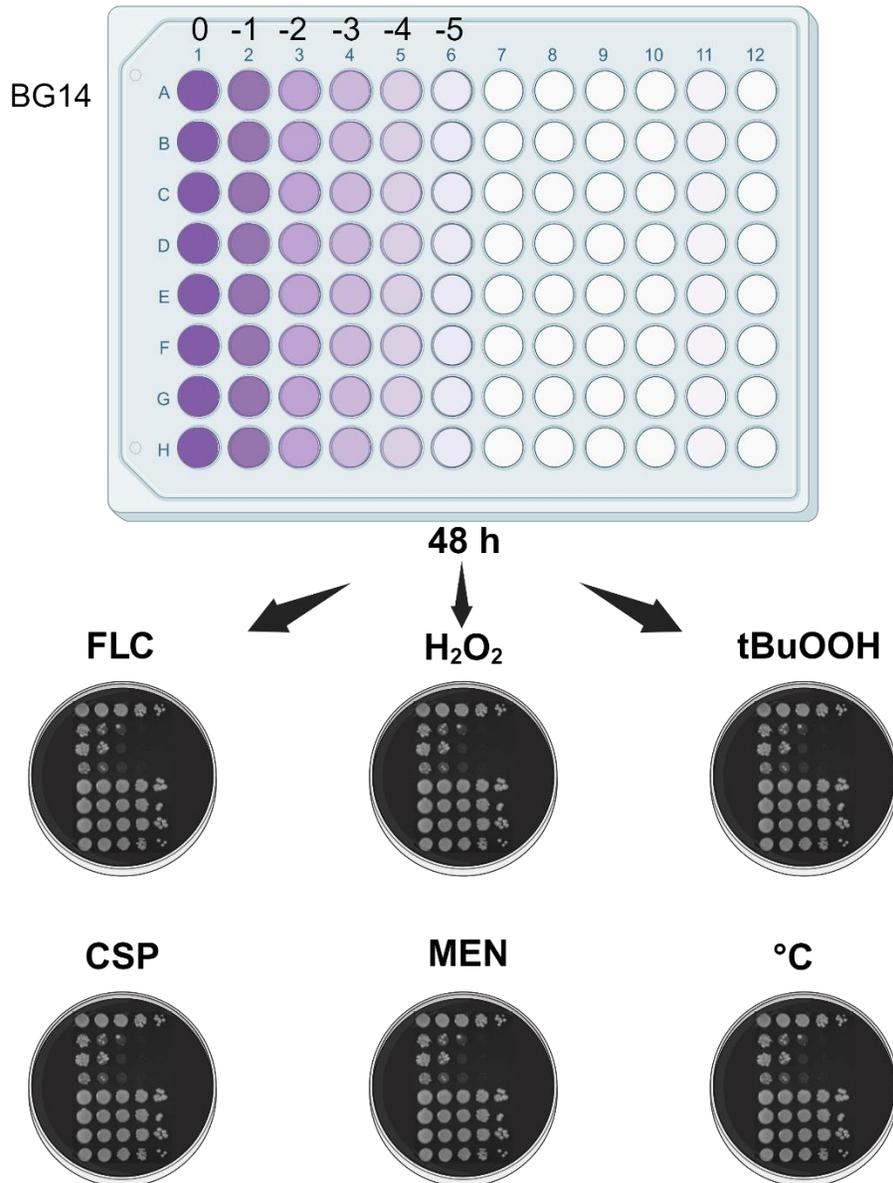
48 h

FLC   H₂O₂   tBuOOH

CSP   MEN   °C

9 consecutive subcultures
YPD medium
5 mL

5 µL   5 µL   5 µL   5 µL   5 µL

-80°C glycerol
stored strain

5 µL

5 µL   5 µL

YPG agar plates

YPD medium
ON 5 mL

YPD agar plates

753
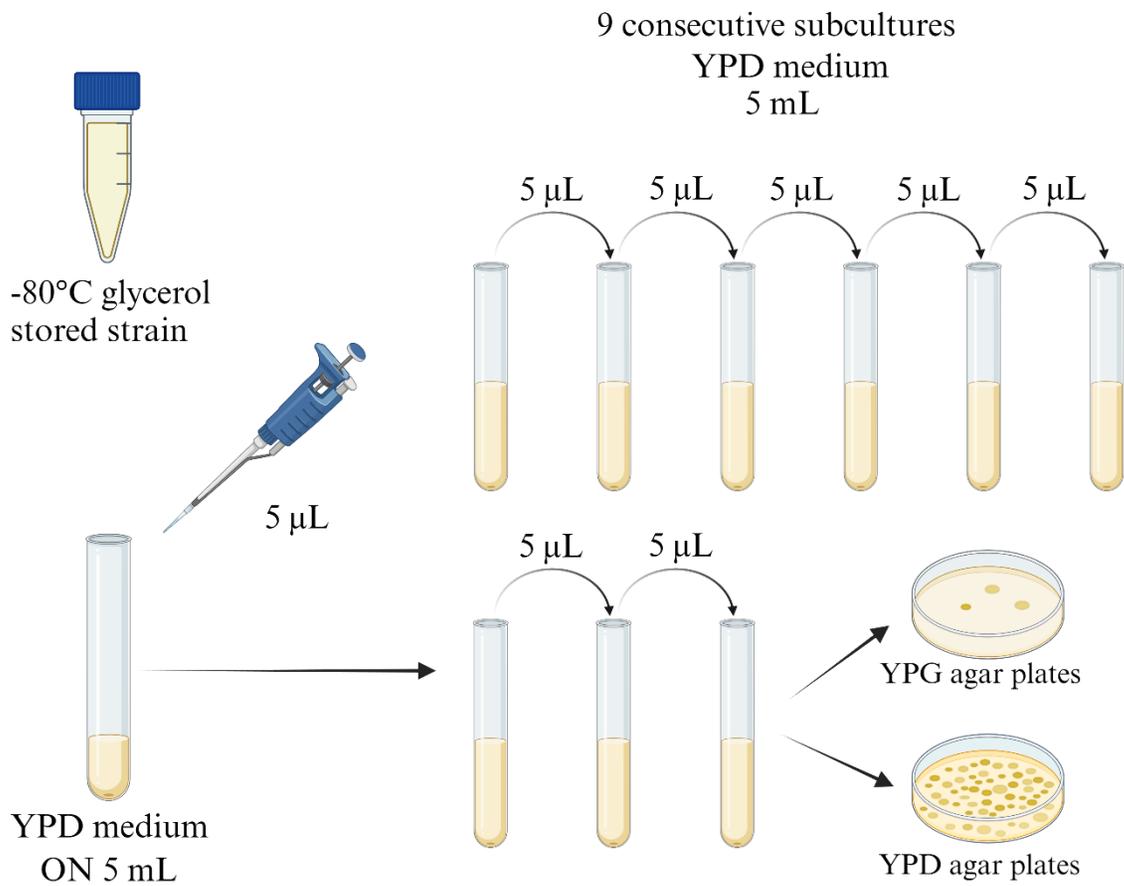
Fig. S6| Schematic representation of reversibility assay.

755
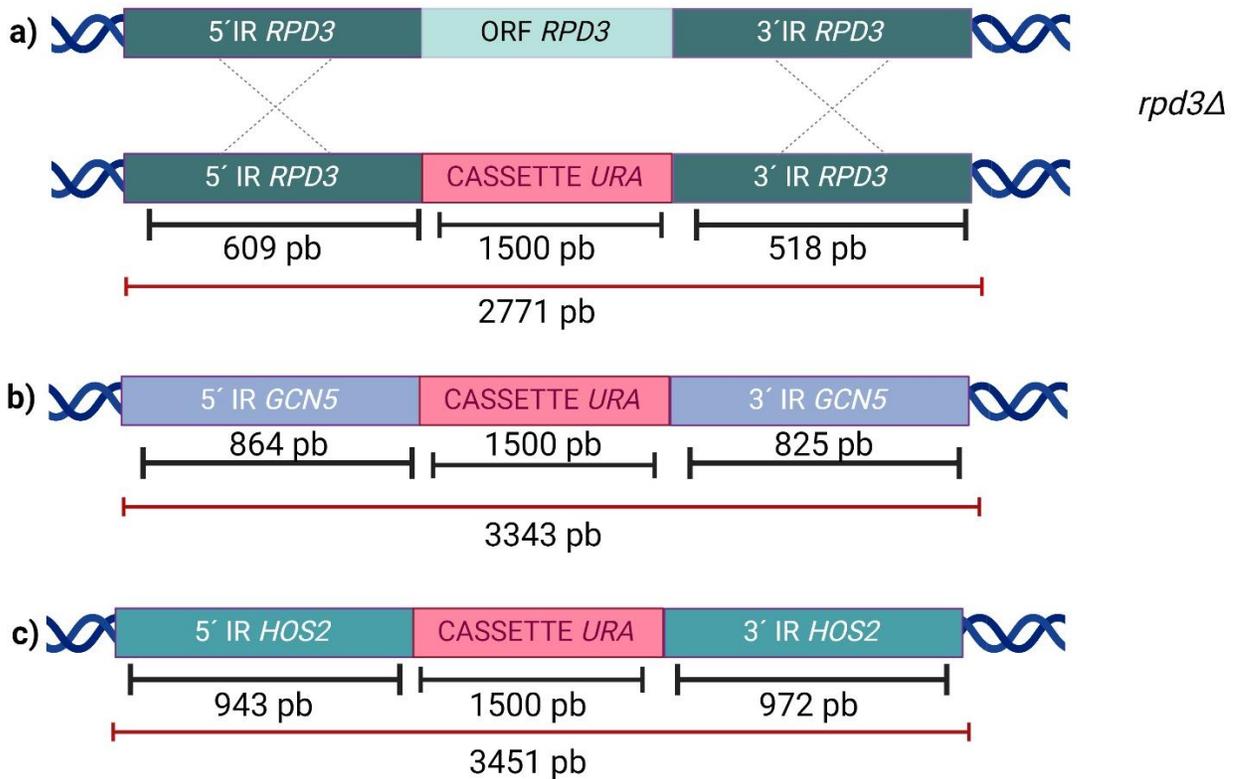
756

Fig. S7| Schematic representation of the experimental approach of fusion PCR. Abbreviation: IR: Intergenic region. a) *RPD3* ORF knockout replaced by *URA3* selection marker. b) *GCN5* ORF knockout replaced by *URA3* selection marker. c) *HOS2* ORF knockout replaced by *URA3* selection marker. All these knockout mutants were performed by a fusion PCR, which is caused by a homologous recombination event.