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Original Article

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A family of glutathione peroxidases contributes to oxidative stress resistance in *Candida albicans*

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Abstract

Candida albicans is a well-adapted human commensal but is also a facultative pathogen that can cause superficial and systemic infections. Its remarkable capacity to thrive within the human host relies on its ability to adapt and respond to the local environment of different niches. *C. albicans* is able to cope with oxidative stress in a coordinated fashion via upregulation of different protective mechanisms. Here, we unravel the role of a family of glutathione peroxidase (GPx), designated Gpx31, Gpx32, and Gpx33, in oxidative stress resistance. We show that GPx activity in *C. albicans* is induced upon exposure to peroxides and that this enzymatic activity is required for full resistance to oxidative stress. The GPx activity relies on the presence of *GPX31*, with no apparent contribution from *GPX32* and *GPX33* during *in vitro* short-term (3 h) exposure to peroxides. However, a triple *gpx31-33*Δ/Δ mutant exhibited a more pronounced sensitivity than a single *gpx31*Δ/Δ mutant on solid media in the presence of oxidants, suggesting that *GPX32* and *GPX33* may be involved in long-term adaptation to oxidative stress. Interestingly, reintegration of a single allele of *GPX31* was sufficient to restore the wild-type phenotype in both the single and triple mutants. We found that mutants lacking *GPX31-33* were more susceptible to killing by phagocytic cells, suggesting that GPxs are required for full resistance to innate immune effector cells. Despite the sensitivity to oxidative stress and phagocytes, these mutants were not affected in their virulence in the chicken embryo model of candidiasis.

Key words: oxidative stress, glutathione peroxidase, *Candida albicans*, phagocytes.

Introduction

The polymorphic fungus *Candida albicans* is often found as part of the normal microbiota, colonizing niches such as the oral cavity, vagina, and gut [1]. Yet, in susceptible individuals, it can cause clinical diseases that range from superficial mucosal infections to systemic candidiasis [2]. Using oxidative and nonoxidative killing mechanisms, phagocytic cells are one of the first lines of defense during *C. albicans* infections. Upon phagocyte activation, large amounts of superoxide ($O_2^{-}\bullet$) are produced by the nicotinamide adenine dinucleotide phosphate (NADPH) phagocyte oxidase [3]. Superoxide is transformed either spontaneously or enzymatically into other oxygen species, including hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH•). The deleterious effects of H_2O_2 are a consequence of its ability to damage DNA and proteins [4]. Furthermore, unsaturated lipids can undergo peroxidation as a result of the oxidative damage [5,6].

C. albicans senses and robustly responds to oxidative stress via two main signaling pathways: the stress-activated protein kinase Hog1 [7,8] and Cap1-mediated transcriptional regulation of genes involved in oxidative stress response [9–11]. Several gene products are involved in the detoxification of phagocyte-derived oxidants. For example, the surface-associated superoxide dismutases Sod4 and Sod5 contribute to the detoxification of superoxide [12,13], while catalase is responsible for H_2O_2 consumption [14]. However, *C. albicans* possesses additional H_2O_2-responsive components, which are necessary for its detoxification and are also involved in coordination of the response to oxidative stress. The thioredoxin system consists of the H_2O_2 acceptor protein Tsa1 [15] and thioredoxin. Upon oxidation of Tsa1 cysteine residues by H_2O_2, thioredoxin acts as an electron donor to restore the reduced thiol groups of Tsa1. Thioredoxin is reduced via thioredoxin reductase in a NADPH-dependent fashion. Notably, thioredoxin is involved in the activation of Hog1 and in the reversal of the oxidation of Cap1 in response to H_2O_2 [16].

Glutathione peroxidases (GPxs) are enzymes involved in the detoxification of H_2O_2; this is done using glutathione (GSH) as the electron donor. In mammals, these enzymes are selenoproteins in which the cysteine molecules at the active site are replaced by selenocysteine [17], whereas yeasts have conserved cysteine residues in the catalytic site [18]. Upon oxidation of GSH, which is catalyzed by GPx, the resulting disulfide glutathione (GSSG) is reduced by GSH reductases in a process that requires NADPH as the reducing agent. In *Saccharomyces cerevisiae*, three GPxs [18,19] are involved in protection against lipid peroxidation by H_2O_2 (hence designated as phospholipid hydroperoxide GPxs [PhGPxs]) and with additional roles in protection against oxidative stress. A functional divergence exists in these GPxs. For instance, while all three ScGPx enzymes have GPx activity, ScGpx2 exhibits higher activity using the thioredoxin system components (thioredoxin and thioredoxin reductase) compared with GSH and glutathione reductase [20]. ScGpx3 is involved in sensing oxidative stress and in relaying this signal to the transcription activator Yap1 via formation of a disulfide bridge between cysteine residues in Gpx3 and Yap1 that eventually results in a Yap1 intramolecular disulfide bond [21].

Transcriptional profiling of *C. albicans* in response to oxidative stress revealed that GPx-encoding genes are upregulated under this condition [8]. In addition, upregulation of these genes has been reported in response to phagocytes [22,23]. GSH and GPxs may therefore constitute an additional mechanism by which *C. albicans* is able to detoxify oxidative stressors. Here we analyzed the phylogenetic relationships of *C. albicans* GPxs and demonstrated that a family of GPx enzymes related to ScGpx3 is responsible for the GPx enzymatic activity. Mutants lacking *GPX31-33* exhibited increased sensitivity to peroxide and oxidative stress–generating agents. Furthermore, we determined the contribution of GPxs to the interaction of *C. albicans* with macrophages and neutrophils and analyzed the virulence of mutant strains in a chicken embryo model.

Methods

Ethics statement

The blood donation protocol and use of blood for this study were approved by the institutional ethics committee (Ethik-Kommission des Universitätsklinikum Jena, permission no. 2207-01/08). Chicken embryo experiments were performed in compliance with the European and German animal protection laws. According to these laws, no specific approval is needed for work performed in avian embryos before the time of hatching. The experimental protocols were reviewed and approved in regard to ethical and welfare issues by the responsible animal welfare officer. Experiments were terminated at the latest on developmental day 18, 3 days before hatching, by chilling the eggs on ice for 30–60 min.

Sequence analysis and phylogeny

Nucleotide and amino acid sequences were obtained from the *Candida* Genome Database (http://www.candidagenome.org/) and the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). Synteny analysis was performed using the *Candida* Gene Order Browser [24]. Nucleotide and amino acid sequences were aligned in Clustal X [25]. Phylogenetic inferences were performed in Mega 3.1 [26], by the neighbor-joining method, using 1000

bootstraps as the phylogeny test. GPx signature patterns were identified using Prosite (http://prosite.expasy.org/).

Strains and growth conditions

C. albicans strains used in this study are described in Table 1. In all experiments, BWP17 transformed with the integrative plasmid CIp30 was used as the wild-type strain [27]. Functional analysis was performed using uridine prototrophic mutant, and reconstituted strains were transformed with CIp10. Strains were routinely grown in YPD (1% yeast extract, 2% bacto-peptone, 2% D-glucose, and 2% agar, if needed) or SD minimal medium (2% D-glucose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% agar, if needed). Liquid cultures were incubated overnight at 30°C in a shaking incubator at 180 rpm. SD medium supplemented with 20 μg/ml arginine, histidine, and/or uridine, as required, was used for selection of *C. albicans* transformants. TOP10 *Escherichia coli* (Life Technologies, Invitrogen, Darmstadt, Germany) was used for plasmid construction and routinely grown in Luria–Bertani broth (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, and 2% agar, if needed) with 50 μg/ml ampicillin for selection of positive clones. Liquid cultures were incubated overnight at 37°C at 180 rpm. Growth curves were performed in YPD medium without stressors or with 1 mM tert-butyl hydroperoxide (t-BOOH, Sigma-Aldrich, Seelze, Germany); overnight YPD cultures were grown at 30°C and diluted to an optical density at 600 nm of 0.1 in 100 μl of the required medium. The optical density was monitored every 15 min for up to 40 h in a Tecan Infinite M200 microplate reader (Tecan, Crailsheim, Germany). For determination of GPx activity, overnight cultures were diluted to an OD_{600} of 0.2 in fresh YPD and regrown to OD_{600} of 1.0. Cultures were then left untreated or exposed to the required stressor for 3 h at 30°C.

Strain construction

The *gpx31*Δ/Δ (*orf19.86*Δ/Δ) null mutant was generated using a polymerase chain reaction (PCR)–based approach for gene deletion in *C. albicans* [28]. Primers GPX31ko-F and GPX31ko-R were used to amplify deletion cassettes from pFA-HIS1 and pFA-ARG4 templates. *C. albicans* BWP17 was transformed to sequentially disrupt both *GPX31* (*ORF19.86*) alleles. Deletion of each allele was confirmed by colony PCR using a combination of flanking primers and internal primers binding to the disruption cassettes [29]. The homozygous null mutant was transformed with StuI-linearized CIp10 to complement the uridine auxotrophy; correct chromosomal integration in the *RPS10* locus was confirmed by PCR. A similar process

Table 1. *Candida albicans* strains used in this study.

Strain	Genotype	Source
BWP17	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 arg4*Δ::*hisG/arg4*Δ::*hisG his1*Δ::*hisG/his1*Δ::*hisG*	[42]
BWP17 + CIp30	*ura3*Δ:: λ*imm434/ura3*Δ::λ*imm434 arg4*Δ::*hisG/arg4*Δ::*hisG his1*Δ::*hisG/his1*Δ::*hisG RPS10/rps10*Δ::*CIp30-URA3-HIS1-ARG4*	[27]
*gpx31*Δ/Δ Ura$^-$	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 arg4*Δ::*hisG/arg4*Δ::*hisG his1*Δ::*hisG/his1*Δ::*hisG gpx31*Δ::*HIS1/gpx31*Δ::*ARG4*	This study
*gpx31*Δ/Δ + CIp10	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 arg4*Δ::*hisG/arg4*Δ::*hisG his1*Δ::*hisG/his1*Δ::*hisG gpx31*Δ::*HIS1/gpx31*Δ::*ARG4 RPS10/rps10*Δ::*CIp10-URA3*	This study
*gpx31*Δ/Δ *gpx32*Δ/Δ *gpx33*Δ/Δ Ura$^-$	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 arg4*Δ::*hisG/arg4*Δ::*hisG his1*Δ::*hisG/his1*Δ::*hisG [gpx32*Δ *gpx31*Δ *gpx33*Δ*]::HIS1/[gpx32*Δ *gpx31*Δ *gpx33*Δ*]::ARG4*	This study
*gpx31*Δ/Δ *gpx32*Δ/Δ *gpx33*Δ/Δ + CIp10	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 arg4*Δ::*hisG/arg4*Δ::*hisG his1*Δ::*hisG/his1*Δ::*hisG [gpx32*Δ *gpx31*Δ *gpx33*Δ*]::HIS1/[gpx32*Δ *gpx31*Δ *gpx33*Δ*]::ARG4 RPS10/rps10*Δ::*CIp10-URA3*	This study
*gpx31*Δ/Δ + *GPX31*	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 arg4*Δ::*hisG/arg4*Δ::*hisG his1*Δ::*hisG/his1*Δ::*hisG gpx31*Δ::*HIS1/gpx31*Δ::*ARG4 RPS10/rps10*Δ::*CIp10-URA3-GPX31*	This study
*gpx31*Δ/Δ *gpx32*Δ/Δ *gpx33*Δ/Δ + *GPX31*	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 arg4*Δ::*hisG/arg4*Δ::*hisG his1*Δ::*hisG/his1*Δ::*hisG [gpx32*Δ *gpx31*Δ *gpx33*Δ*]::HIS1/[gpx32*Δ *gpx31*Δ *gpx33*Δ*]::ARG4 RPS10/rps10*Δ::*CIp10-URA3-GPX31*	This study

Table 2. Primers used for strain construction.

Primer	Sequence (5′ – 3′)
GPX31ko-F	tcccatcccttggattgtttttctttttctcttttgcctcccttctctttctttgtattattacttcattacaaagtattcaattatcacatatttacataaa gaagcttcgtacgctgcaggtc
GPX31ko-R	tcaaaataatctcattaagtttattaaagaaaatacatatacaaacaacatgtacatataaaatatggaattaaaatttgtcaatacctatgatgggaaaataaatctgatatcatcgatga attcgag
GPX32ko-F	ccctatcattttcaaatacttcaaacccatcttttctattattagttattttcatttcacttaacctaccgagtttctatttcctccctcaatacttatagctgaagcttcgtacgctgcaggtc
GPX33ko-R	ttaaaaaaaaaaattaaagtacttgtagaaatacgttccagttatagtgaactttgctaatattgatacataattgtacacttcttttgactactcttctatgttctgatatcatcgatgaatt cgag
GPX31-rec F	acgcgtcaatttcagatgataataaaggtgt
GPX31-rec R	gtcgacttccttccctcacttttgt
ARG4-F1	ggatatgttggctactgatttagc
ARG4-R1	aatggatcagtggcaccggtg
HIS1-F1	ggacgaattgaagaaagctggtgcaaccg
HIS1-R1	caacgaaatggcctcccctaccacag
URA3-F2	ggagttggattagatgataaaggtgatgg
RPF-F1	gagcagtgtacacacacacatcttg
GPX31 fwd	aaattggatcattctgttcattg
GPX31 rev	ttcaacaattcttcaatcttggt
GPX32 fwd	agaaattgaaaagtattgtcgtg
GPX32 rev	tgttttaacatttcttcaattcgt
GPX33 fwd	gcactaatgatcaaatagtaacga
GPX33 rev	actgttcaatccttggtgtt
GPX3 fwd	tagtcgaatcttgtagacgc
GPX3 rev	agttgttcaataaacccttcg
ACT1 fwd	tcagaccagctgatttaggtttg
ACT1 rev	gtgaacaatggatggaccag

Underlined nucleotides in GPX31ko-F and -R indicate the annealing regions on pFA-ARG4 and pFA-HIS1 [28]. Underlined nucleotides in GPX31-rec F and R indicate restriction sites for MluI and SalI, respectively.

was followed for the construction of *gpx31-33*Δ/Δ (*orf19.85*Δ/Δ *orf19.86*Δ/Δ *orf19.87*Δ/Δ) triple mutant. Primers GPX32ko-F and GPX33ko-R were used to amplify the deletion cassettes for the sequential disruption of the chromosomal locus containing the three open reading frames (ORFs), and the resulting strain was rendered uridine prototrophic by integration of CIp10. Reconstituted strains were generated by integrating one allele of *GPX31* (*ORF19.86*) in the *RPS10* locus via CIp10. For this purpose, *GPX31* (*ORF19.86*) was amplified from –1567, with respect to start codon, to +336, with respect to stop codon, using the primers GPX31-rec F and GPX31-rec R, and then cloned within the MluI and SalI sites of CIp10 to generate CIp10-GPX31. The resulting plasmid was linearized with StuI and used for transformation of the homozygous uridine-prototrophic *gpx31*Δ/Δ and *gpx31-33*Δ/Δ mutant strains. Primers are listed in Table 2.

GPx activity induction and determination

GPx activity was determined in crude cell extracts of *C. albicans* cultures exposed to 450 μM menadione sodium bisulfite (Sigma-Aldrich, Seelze, Germany), 3 mM H_2O_2, and 1 mM t-BOOH using a GPx assay kit (Cayman Chemicals, MI, USA). Briefly, cultures were harvested by centrifugation at 5000 g for 5 min at 4°C after 3 h of exposure to the appropriate stressor (menadione, H_2O_2, or t-BOOH). Pellets were resuspended in lysis buffer (10 mM potassium phosphate, pH 7.0) with protease inhibitors (20 ng/ml aprotinin, 20 ng/ml leupeptin, 20 ng/ml pepstatin, and 10 μM phenylmethylsulfonyl fluoride (PMSF)) and washed once. Cells were lysed in a cell homogenizer (Precellys, Peqlab, Erlangen, Germany) using 100-μl glass beads, with two bursts at 6000 rpm for 15 s and 5 min on ice in between bursts. Cell extracts were clarified by centrifugation for 10 min at 23000 g at 4°C, and the protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, MA, USA). For determination of GPx activity, 200 μg of protein was assayed using the GPx assay kit according to the manufacturer's instructions. Briefly, 100 μl of assay buffer (50 mM Tris-HCl, pH 7.6; 5 mM ethylenediaminetetraacetic acid [EDTA]) and 50 μl of cosubstrate mixture (containing NADPH, GSH, and glutathione reductase) were mixed with 20 μl of the protein samples in wells in a 96-well plate. The reaction was initiated by addition of 20 μl of cumene hydroperoxide. Absorbance was monitored every minute at 340 nm for 5 min. The slope was calculated for the linear portion of the curve, and the GPx activity was calculated using the NADPH extinction coefficient factor (0.00373 μM^{-1}). One unit was defined as the

amount of enzyme that will cause the oxidation of 1.0 μmol of NADPH to $NADP^+$ per minute at 25°C. GPx activity was determined on at least three occasions, in duplicate.

Quantitative real-time PCR

Expression of *GPX31-33* and *GPX3* was monitored by quantitative real-time PCR (qRT-PCR). RNA was isolated from unstressed cultures and from cultures exposed to 1 mM t-BOOH for 1 h using the RNeasy kit (Quiagen). For qRT-PCR, an EvaGreen-based approach was used. Using this process,100 ng of template cDNA was mixed with 4 μl of 5× EvaGreen QPCR Mix II (Bio & Sell, Feucht, Germany), 0.5 μl forward and 0.5 μl of reverse primer (5 pmol/μl) of the genes of interest (Table 2). Nuclease-free water was added to a final volume of 20 μl. qRT-PCR was performed in a CFX96 real-time PCR detection system (Bio-Rad, München, Germany). The results were analyzed using the Bio-Rad CFX Manager. The expression of *ACT1* (encoding actin) was used as control. Relative changes in gene expression under oxidative stress were quantified in relation to untreated *C. albicans* cells using an efficiency corrected ΔΔ C(t) method. Experiments were performed twice.

Susceptibility to stressors

To determine the effect of different stressors on growth, cells from overnight cultures were washed twice with 1× Dulbecco's phosphate-buffer saline (DPBS); 5 μl of 10-fold serial dilutions (10^6–10^1) were spotted on SD medium containing 450 μM menadione, 3 mM H_2O_2, 2 mM t-BOOH, 1.2 mM sorbitol, 0.6 mM KCl, 400 μg/ml Congo red, 150 μg/ml calcofluor white, 500 μM $CdSO_4$, or 750 μM $AgNO_3$. For ultraviolet (UV)-C radiation exposure, dilutions were spotted in SD medium and exposed to different doses of UV-C light (2–4 mJ/cm^2). Plates were incubated at 37°C, except those containing heavy metals, which were incubated at 30°C. Each experiment was performed at least twice. Representative pictures are shown (Figs. 4 and 5).

For determination of viability after exposure to selected stressors (0.5 mM t-BOOH, 750 μM $AgNO_3$, and 400 μg/ml Congo red), overnight cultures were grown to mid-exponential phase in YPD and exposed to the respective stressor for 3 h at 37°C in a shaking incubator at 180 rpm.

Monocyte-derived macrophage killing assay

Peripheral blood mononuclear cells (PBMCs), obtained from the transfusion medicine unit (Institut für Transfusionsmedizin, Universitätsklinikum Jena), were isolated from buffy coats and separated by gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, Seelze, Germany) according to the manufacturer's instructions. The PBMC fraction was collected and washed with 1× DPBS; the residual erythrocytes were removed by hypotonic lysis. PBMCs were incubated for 2 h at 37°C in 5% CO_2 in a cell-culture Petri dish in Roswell Park Memorial Institute (RPMI) 1640 to allow attachment of monocytes. Nonadherent cells were washed away, and differentiation of macrophages was induced with 10 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF; Immunotools, Friesoythe, Germany) for 2 days in RPMI, followed by 5 days in RPMI + 10% heat-inactivated fetal calf serum (FCS; Life Technologies, Gibco, Darmstadt, Germany) at 37°C in 5% CO_2.

To test the susceptibility of the mutant strains to macrophage killing, adherent monocyte-derived macrophages (MDMs) were detached with 50 mM EDTA and seeded in 96-well plates at a density of 4×10^4 cells in 200 μl RPMI + 10% FCS and incubated overnight at 37°C in 5% CO_2. Overnight cultures of *C. albicans* strains were regrown to exponential phase. Cells from these cultures were washed twice with 1× DPBS and resuspended in RPMI. MDMs were washed with 1× DPBS and infected with 100 μl wild-type mutant and reconstituted strain suspensions in RPMI at a multiplicity of infection (MOI) of 0.0025 to allow approximately 100 microcolonies in the control wells without MDMs. After incubation for 2 days at 37°C in 5% CO_2, plates were centrifuged, medium was carefully removed, and macrophages were lysed by the addition of 100 μl 0.5% Triton X-100. Fungal biomass was determined by means of 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide metabolic assay, adding 100 μl of XTT solution (0.5 mg/ml XTT, 50 μg/ml coenzyme Q_0), and incubating for 1 h at 37°C. Plates were centrifuged, and 100 μl of the supernatant was removed to measure absorbance at 450 nm using a Tecan Infinite M200 microplate reader. Fungal biomass of the wild-type strain was set as 100%. Experiments were repeated with MDMs from at least three donors.

Neutrophil killing assay

Neutrophils were isolated from peripheral blood from healthy donors who provided written informed consent. Briefly, freshly drawn blood was diluted with 1× DPBS and layered on top of 5 ml Polymorphprep (Axis-Shield, Dundee, Scotland), as recommended by the manufacturer. After centrifugation for 45 min at 500 g and 20°C, the plasma and monocyte layer were discarded. The polymorphonuclear cell fraction was collected in a fresh tube that contained 1 volume of 0.5× DPBS and centrifuged for 10 min at 400 g. Residual erythrocytes were removed with ACK (ammonium-chloride-potassium) lysing buffer

(Life Technologies, Invitrogen, Darmstadt, Germany). Neutrophils were washed with 1× DPBS and resuspended in RPMI 1640 + 5% autologous serum.

Neutrophils (10^6 cells/ml) were infected with exponentially growing *C. albicans* cells (10^6 cells/ml) at an MOI of 1 in 200 μl final volume. To test the effect of NADPH oxidase inhibition on mutant survival, neutrophils were pretreated with 1 mM apocynin (Sigma-Aldrich, Seelze, Germany) for 30 min before infection with *C. albicans* strains. Infected neutrophils were incubated for 3 h at 37°C and 5% CO_2. Parallel samples containing fungal cells only were processed under the same conditions. After incubation, 1 ml cold water was added to each sample to lyse the neutrophils. Fungal cells were collected by centrifugation for 5 min at 16000 g at 4°C and 1 ml of the supernatant was removed. Next, 400 μl of XTT solution was added and samples were incubated for 1 h at 37°C. After incubation, samples were centrifuged for 5 min at 16000 g at 4°C. Absorbance at 450 nm was determined in the supernatants. Background absorbance (neutrophils only or medium only) was subtracted from the samples. The residual metabolic activity was used as a measurement of survival and calculated as the ratio of the absorbance of the coincubation (*C. albicans* strains and neutrophils) and the absorbance of the *C. albicans* strains alone, multiplied by 100. Survival of the wild-type strain was set to 100%. Experiments were performed four times using neutrophils from different donors.

Chicken embryo infection model

Virulence of mutant strains was assessed in the chicken embryo infection model as described elsewhere [30,31]. Viable embryos were infected with 10^7 *C. albicans* cells on the chorioallantoic membrane (CAM) after 10 days of initial incubation. Infected embryos were monitored for survival by candling once a day for 7 days after infection. Infection was performed in 20 eggs per strain. Experiments were performed twice. Survival data were plotted as Kaplan-Meyer curves.

Statistics

Unless stated otherwise, all experiments were performed three times; data are presented as mean and standard deviation. For two-group comparisons, the two-tailed Student *t* test was used. Egg infection experiments were analyzed by log-rank (Mantel-Cox) test. Statistical analyses were done in GraphPad Prism 5.03.

Results

Identification of *C. albicans* GPx encoding genes

The first aim of this study was to identify genes that putatively code for GPxs. We identified at least four open reading frames coding for this class of enzymes in the *Candida* Genome Database (http://www.candidagenome.org/): *ORF19.85*, *ORF19.86*, *ORF19.87*, and *ORF19.4436*.

We determined the evolutionary relationships of *C. albicans* GPx-coding genes, with the *S. cerevisiae* homologous genes *GPX1* (*YKL026C*), *GPX2* (*YBR244W*), and *GPX3* (*YIR037W*) and from species of the CUG clade, a group of related yeasts that translate the CUG codon as serine instead of leucine [32]. As shown in Figure 1, two clusters can be distinguished. Cluster 1 contains GPx-coding genes from the CUG clade that are syntenically positioned with respect to *ORF19.86* (Fig. 2A). Orthologues of *ORF19.85* and *ORF19.87* are present only in *C. dubliniensis* and *C. tropicalis*, suggesting that their emergence was posterior to the speciation events that separated the common ancestor of *C. albicans*, *C. dubliniensis*, and *C. tropicalis* from the rest of the CUG clade. Interestingly, *ORF19.85*, *ORF19.86*, and *ORF19.87* are positioned contiguously on chromosome 6. As shown in Figure 2A, this chromosomal arrangement is also conserved in the genomes of two closely related yeasts, *C. dubliniensis* and *C. tropicalis*.

Based on this phylogeny, cluster 1 shares close ancestry with *S. cerevisiae GPX3* and *GPX2*, the former encoding a thiol peroxidase with signaling transduction activities via Yap1 [21], while *GPX2* codes for a PhGPx [19]. Cluster 2 includes homologues of *ScGPX1* encoding another lipid GPx [19]. Notably, members of the CUG clade possess at least one GPx in cluster 1 and one in cluster 2, suggesting a functional specialization for each encoded protein. On grounds of the relatedness to *ScGPX3*, we decided to name *ORF19.86* as *GPX31*; *ORF19.85* and *ORF19.87* were designated as *GPX32* and *GPX33*, respectively, since they may have arisen from the common ancestor shared with *GPX31*.

Alignment of the amino acid sequences of *C. albicans* and *S. cerevisiae* GPxs in Figure 2B shows that the residues constituting the catalytic triad CQW are conserved in all compared sequences. The signature patterns around the triad, which are characteristic of this class of enzyme, are also highly conserved.

GPx activity is induced upon exposure to oxidative stress

To determine if *C. albicans* has GPx activity, we measured this activity in exponential cultures grown in YPD in the presence of different inducers. As shown in Figure 3A, unstimulated cultures exhibited basal GPx activity. After 3 h exposure to menadione, a superoxide-generating naphtoquinone [33], a 2.2-fold increase in the GPx activity was observed. However, GPx activity was increased

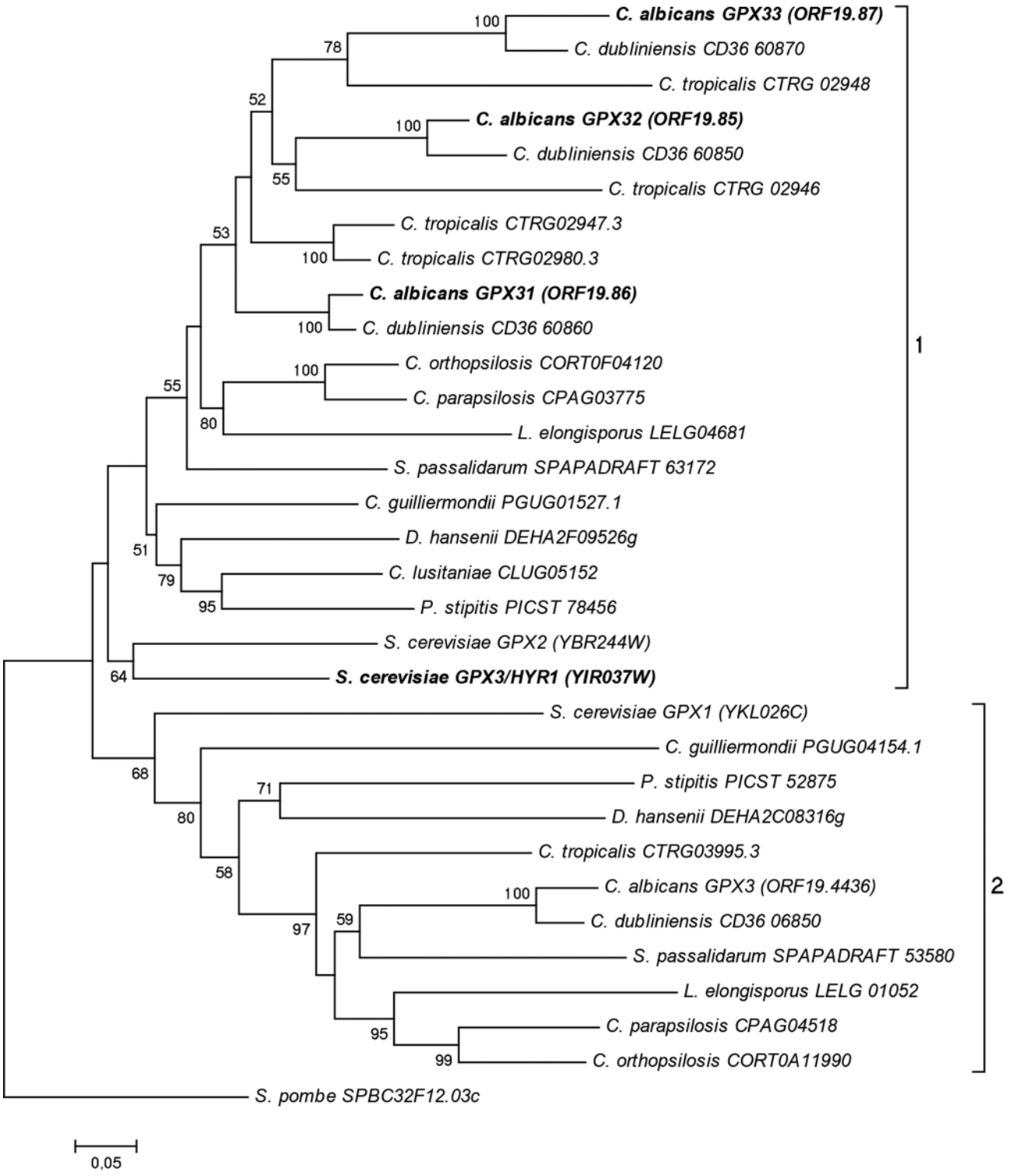
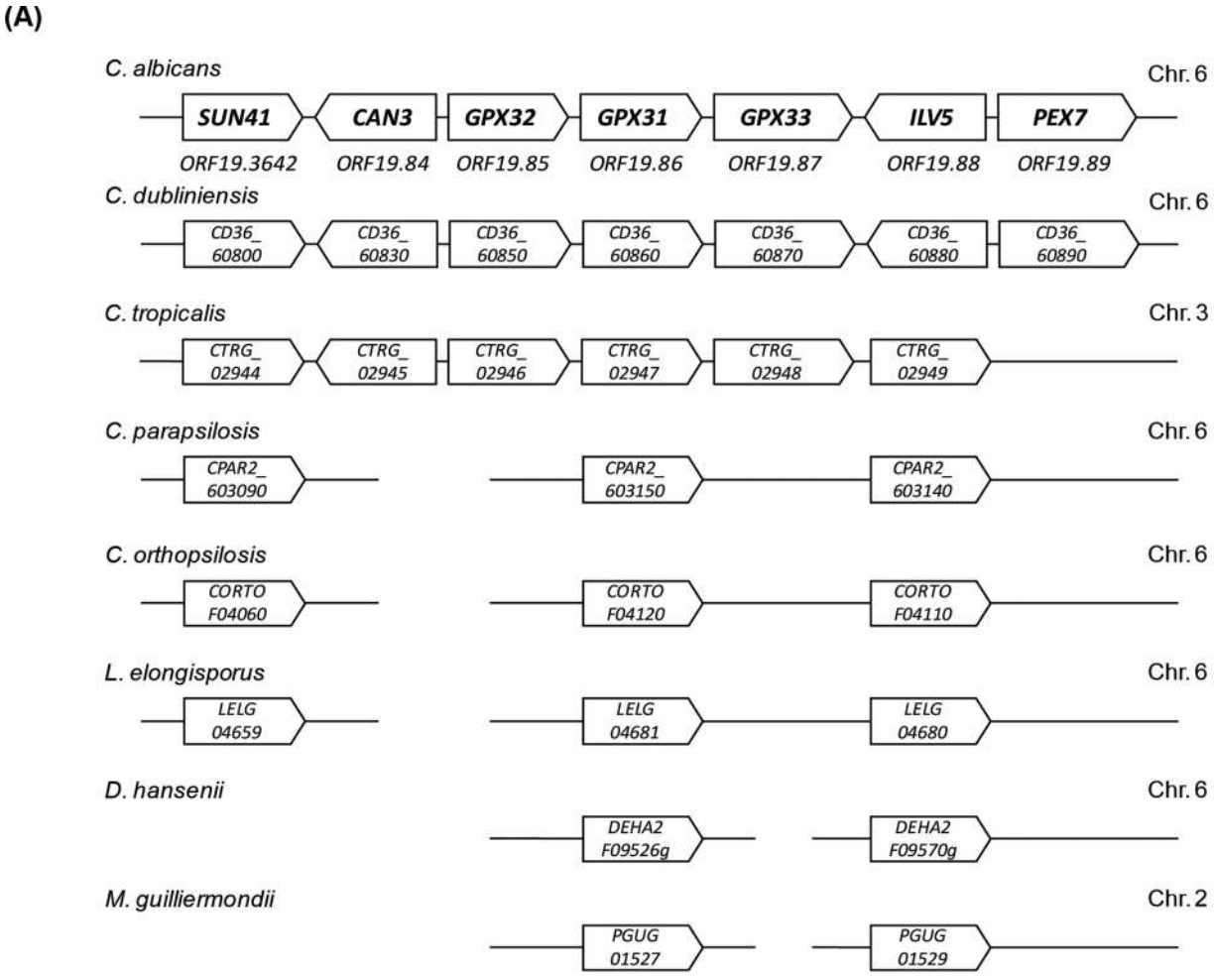


Figure 1. Phylogenetic analysis of glutathione peroxidase (GPx) coding genes in members of the CUG clade. Neighbor-joining phylogenetic tree of the GPx family. Bootstrap values >50% are shown on the branches.

4.5-fold upon exposure to H_2O_2 or the organic peroxide t-BOOH for 3 h (Fig. 3A). This indicates that glutathione peroxide activity is induced more strongly in the presence of peroxides than upon superoxide-generated oxidative stress.

We then tested whether GPxs encoded by *GPX31*, *GPX32*, and *GPX33* are responsible for the enzymatic activity detected upon exposure to peroxide. We focused on the GPxs encoded by *ORF19.85–87* on grounds of expression profiles of *C. albicans* under oxidative stress



(B)

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CaGpx31 MSQFYELAPKDAKGEPYPFEQLKGKVVLIVNVASKCGFTPQYKGLEELNKKFADQPVQILGFPCNQFGHQEPGSNEEIGS 80
CaGpx32 MSDFYEFAPNDIKGTPYSFKKLQGKVVLIVNVASKCGFTPQYKGLQDLKQKFADQPVEILGFPCNQFGHQEPGTNEEIEK 80
CaGpx33 RSKFYDLTPLDNSKSPFPFKNLRGKVVLIVNVASRCGFSPQYNGLEQLNKRFANDDFVLLGVPCNQFLWQEPGTNDQIVT 148
ScGpx3  MSEFYKLAPVDKKGQPFPFEDQLKGKVVLIVNVASKCGFTPQYKELEALYKRYKDEGFTIIGFPCNQFGHQEPGSDEEIAQ 80
ScGpx2  TTSFYDLECKDKKGESFKFDQLKGKVVLIVNVASKCGFTPQYKELEELYKKYQDKGFVILGFPCNQFGKQEPGSDEQITE 81
CaGpx3  TARIYTFKIPDAYNNVIDFDQFKNKVTLIVNVASLCGFTPQYKELQLLYEKYHERGLEILGFPCNQFGNQEPLQEEEIVE 87
ScGpx1  MQEFYSFSPIDENGNPFPFENSLRNKVVLIVNVASHCAFTPQYKELEYLYEKYKSHGLVIVAFPCGQFGNQEFEKDKEINK 80

CaGpx31 FQSLNYGVTFPVLDKIEVN---GDNTDPVYKYLKSQKSGVLGLTRIKWNFEKFLIDQNGKVIERFS 143
CaGpx32 YQREYFGVTFPVLSKVETN---GKNAEPVYKFLKSQKPGLLGLHRIMWNFEKFLIDQDGNVVARFS 143
CaGpx33 KQKKKYDVSFQILDKINVN---GEQADPVYKFLKAQKEGLWGTNRVKWNFEKFLIDKNGRVVERYS 211
ScGpx3  FQQLNYGVTFPIMKKIDVN---GGNEDPVYKFLKSQKSGMLGLRGIKWNFEKFLVDKKGKVYERYS 143
ScGpx2  FQQLNYGVTFPIMKKIDVN---GSNADSVYNYLKSQKAGLLGFKGIKWNFEKFLVDSNGKVVQRFS 144
CaGpx3  SQRRNLGVSFPIMKKTKVNIDCDGHESELYKYLKSEKPGEVGFKGVRWNFEKFIVNRKGEVVARFN 153
ScGpx1  FQQDKYGVTFPILHKIRQN---GQKQDPVYKFLKNSVSGKSGIKMIKWNFEKFVVDRNGKVVKRFS 143
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Figure 2. Glutathione peroxidases (GPxs) in *Candida albicans*. (A) Synteny of the GPx-coding genes *GPX31*, *GPX32*, and *GPX33* in selected genomes of the CUG clade. Modified from the *Candida* Gene Order Browser. (B) Amino acid sequence alignment of *C. albicans* and *Saccharomyces cerevisiae* GPxs. Identical residues are shown in white font on black background, while similar residues are shown on gray background. Residues constituting the catalytic triad are indicated by asterisks. Lines indicate signature patterns.

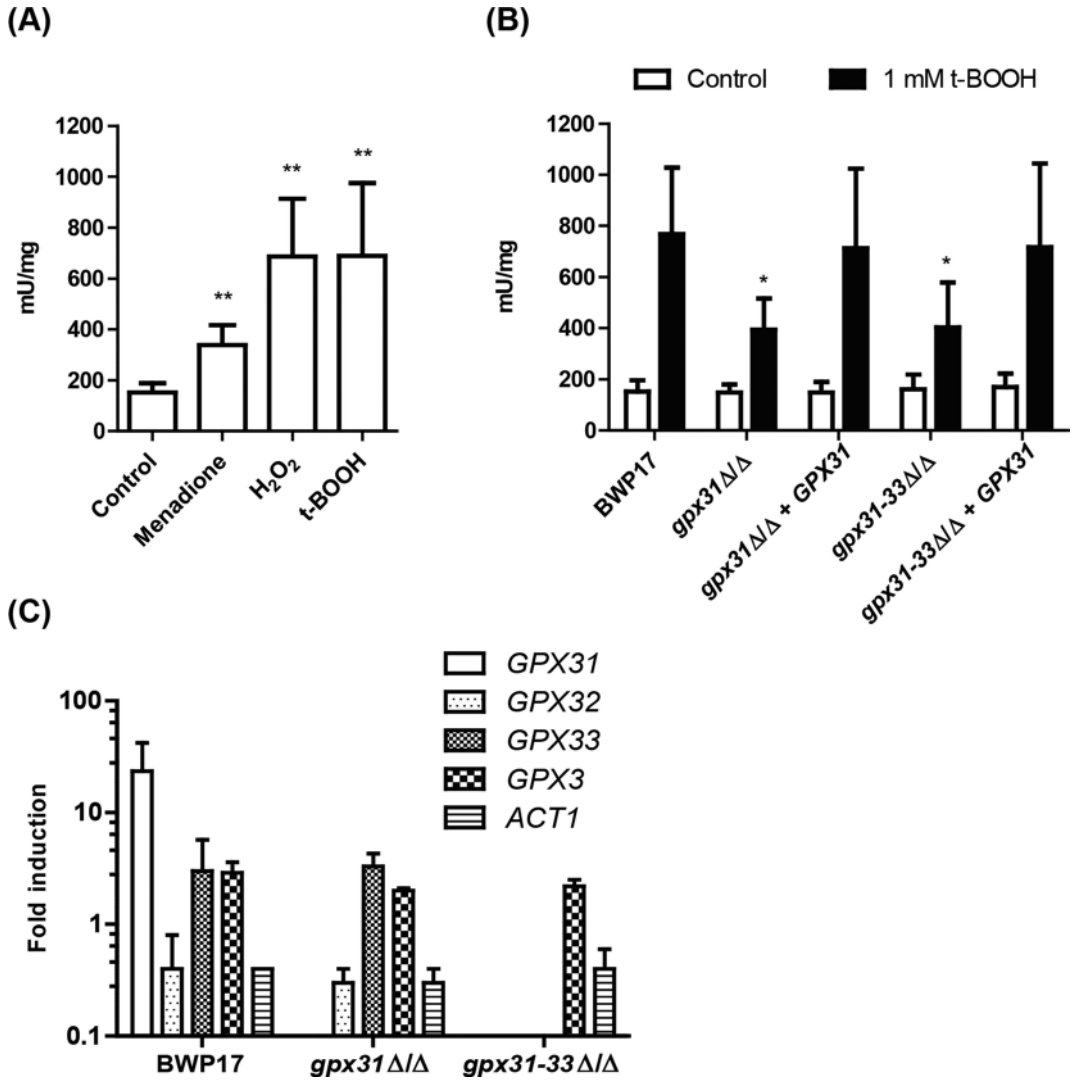

Figure 3. Glutathione peroxidase (GPx) activity is induced by oxidative stress. (A) GPx activity was determined in exponential cultures of *Candida albicans* BWP17 exposed for 3 h to 450 μM menadione, 3 mM hydrogen peroxide, and 1 mM tert-butyl hydroperoxide (t-BOOH). Unstressed cultures (in 1% yeast extract, 2% bacto-peptone, 2% D-glucose) were used as control. (B) GPx activity was determined in *C. albicans* BWP17, *gpx31*Δ/Δ, and *gpx31-33*Δ/Δ and in reconstituted strains *gpx31*Δ/Δ + *GPX31* and *gpx31-33*Δ/Δ + *GPX31* after exposure to 1 mM t-BOOH for 3 h. Unstressed controls were used as controls. Mean from at least three independent experiments. Error bars represent standard deviation (SD). Statistical significance was calculated using *t* test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ compared with control (A) or with wild-type strain BWP17 (B). (C) *GPX31-33* and *GPX3* expression levels were determined by quantitative real-time PCR in the wild-type BWP17 strain and the *gpx31*Δ/Δ and *gpx31-33*Δ/Δ mutant strains exposed to 0.5 mM t-BOOH. Experiments were performed twice. Error bars represent SD.

and coincubation with phagocytes. From all four GPx encoding genes in *C. albicans*, only *ORF19.86* was significantly upregulated upon exposure to oxidative stress (5 mM H_2O_2) [8] and after 1 h and 2 h of exposure to macrophages [22]. During interaction with neutrophils, the three genes were significantly upregulated [23,34]; however, *ORF19.86* was expressed at the highest level. The expression of the GPx encoded by *ORF19.4436* was not regulated at the transcriptional level under any of these conditions.

We deleted *GPX31* to create the single *gpx31*Δ/Δ mutant in the BWP17 genetic background. Upon exposure to t-BOOH, the single *gpx31*Δ/Δ mutant exhibited only 50% of the GPx activity of BWP17 (Fig. 3B). To check whether the remaining activity was due to the GPxs encoded in *GPX32* and *GPX33*, a *gpx31-33*Δ/Δ triple mutant was constructed by deletion of the three *GPX31*, *GPX32*, and *GPX33* genes. Upon exposure to t-BOOH, GPx activity in the triple mutant was not further reduced. This finding may suggest that of *GPX31, GPX32*, and *GPX33, GPX31* encodes the major GPx. Furthermore, reintroduction of a single allele of *GPX31* in both the single and triple mutants restored the GPx activity to wild-type levels (Fig. 3B). Notably, we observed remaining GPx activity in

both the single and triple mutants exposed to t-BOOH, possibly due to basal expression of the GPx encoded by *GPX3* (*ORF19.4436*).

Next, we investigated the expression levels of the *GPX31-33* and *GPX3* genes by qRT-PCR in the wild-type and mutant strains upon exposure to t-BOOH. As shown in Figure 3C, *GPX31* was upregulated more than 10-fold in the wild type, whereas *GPX32* was downregulated. *GPX33* and *GPX3* were also upregulated, although at lower levels compared with *GPX31*. Absence of *GPX31* did not lead to increased expression of *GPX32/33* or *GPX3* in the *gpx31*Δ/Δ single mutant. Likewise, absence of *GPX31-33* did not induce increased expression of *GPX3* in the *gpx31-33*Δ/Δ triple mutant.

GPXs mediate resistance to peroxide-induced oxidative stress

Having determined that GPx activity is induced upon exposure to oxidative stress, we examined the role of GPxs in the resistance to oxidative stress. As shown in Figure 4A, wild-type, mutant, and reconstituted strains grew equally well in the absence of stress. Interestingly, growth in the presence of menadione was not compromised. H_2O_2 had only a slight inhibitory effect on the growth of *gpx31*Δ/Δ, but the inhibition was more pronounced for *gpx31-33*Δ/Δ. In the presence of t-BOOH, the mutants showed an increased sensitivity as compared with H_2O_2 treatment. This observation suggests that GPxs mediate stress tolerance to peroxides, particularly to organic peroxides. Noticeably, the wild-type phenotype was recovered in both the single and triple mutant upon integration of one *GPX31* allele, suggesting some extent of functional redundancy of *GPX32* and *GPX33* under these conditions.

The inhibitory effect of t-BOOH on the mutants was investigated further in liquid media. As shown in Figure 4B, growth of the mutant strains was comparable to that of the wild-type and reconstituted strains in the absence of stress. In contrast, in the presence of t-BOOH (Fig. 4C), the growth of *gpx31*Δ/Δ was markedly delayed, indicated by an extended lag phase. The lag phase was further prolonged in the *gpx31-33*Δ/Δ mutant. However, both the single and triple mutants were able to reach the logarithmic phase after approximately 20 h and 30 h, respectively. The phenotype was restored nearly to wild-type levels in the reconstituted strains.

Short-term (3 h) exposure to t-BOOH had a detrimental effect on cell viability. As shown in Figure 4D, a 2-log reduction of viable count was observed for the wild-type strain under these conditions. Both the single *gpx31*Δ/Δ and the triple *gpx31-33*Δ/Δ mutants exhibited a trend toward increased susceptibility to t-BOOH; however, the reduction in cell viability was not statistically significant (BWP17 vs. *gpx31*Δ/Δ, *t* test $P = 0.0512$; BWP17 vs. *gpx31-33*Δ/Δ, *t* test $P = 0.0845$).

GPXs necessary for normal resistance to UV light, heavy metals, and cell wall stress

Having established that GPxs are involved in the response to peroxide-induced oxidative stress, we tested whether the *gpx*Δ/Δ mutants exhibited growth sensitivity to other types of stress. We exposed the strains to osmotic stressors (1.2 mM sorbitol; 0.6 mM KCl), UV-C radiation (2–4 mJ/cm^2), heavy metals (500 μM $CdSO_4$, 750 μM $AgNO_3$), and cell wall stress (400 μg/ml Congo red, 150 μg/ml calcofluor white). Growth was unaffected under osmotic stress (not shown). Upon exposure to UV-C light, *gpx31*Δ/Δ exhibited slightly poorer recovery compared with the wild type, and this was further accentuated in the triple *gpx31-33*Δ/Δ mutant (Fig. 5A). Exposure to cadmium had a slight detrimental effect on the growth of these mutant strains, while silver strongly affected the growth of both *gpx31*Δ/Δ and *gpx31-33*Δ/Δ. Complementation with a single copy of *GPX31* restored growth to wild-type levels (Fig. 5B). These observations suggest that GPxs encoded by *GPX31-33* are involved in protection from UV-C light and heavy metal–induced stress. Due to the nature of these stressors, reactive oxygen species may be produced, imposing oxidative stress and rendering the mutants more susceptible to the aforementioned stressors. In the presence of the cell wall stressors, that is, Congo red and calcofluor white, only the growth of the triple *gpx31-33*Δ/Δ mutant was visibly affected (Fig. 5C). However, reintegration of *GPX31* in the *gpx31-33*Δ/Δ mutant was not sufficient to alleviate the growth defect under cell wall stress, suggesting that the GPxs encoded by *GPX32* and/or *GPX33* are needed for normal resistance to cell wall stress.

During short-term (3 h) exposure to $AgNO_3$ and Congo red, no significant differences were observed in the mutant strains compared with the wild-type and reconstituted strains, suggesting that the GPxs encoded by *gpx31-33* are needed for long-term adaptation to these stressors.

GPXs required for normal resistance to phagocytes but dispensable for virulence in the chicken embryo model of candidiasis

Phagocytic cells such as macrophages and neutrophils constitute one of the first lines of defense against *C. albicans* infections and use oxidative mechanisms as part of their candidacidal activities. Given the adaptation defects of the *gpx*Δ/Δ mutants to oxidative stress, we tested the interaction of mutants lacking the *GPX31-33* genes with human MDMs and polymorphonuclear neutrophil granulocytes.

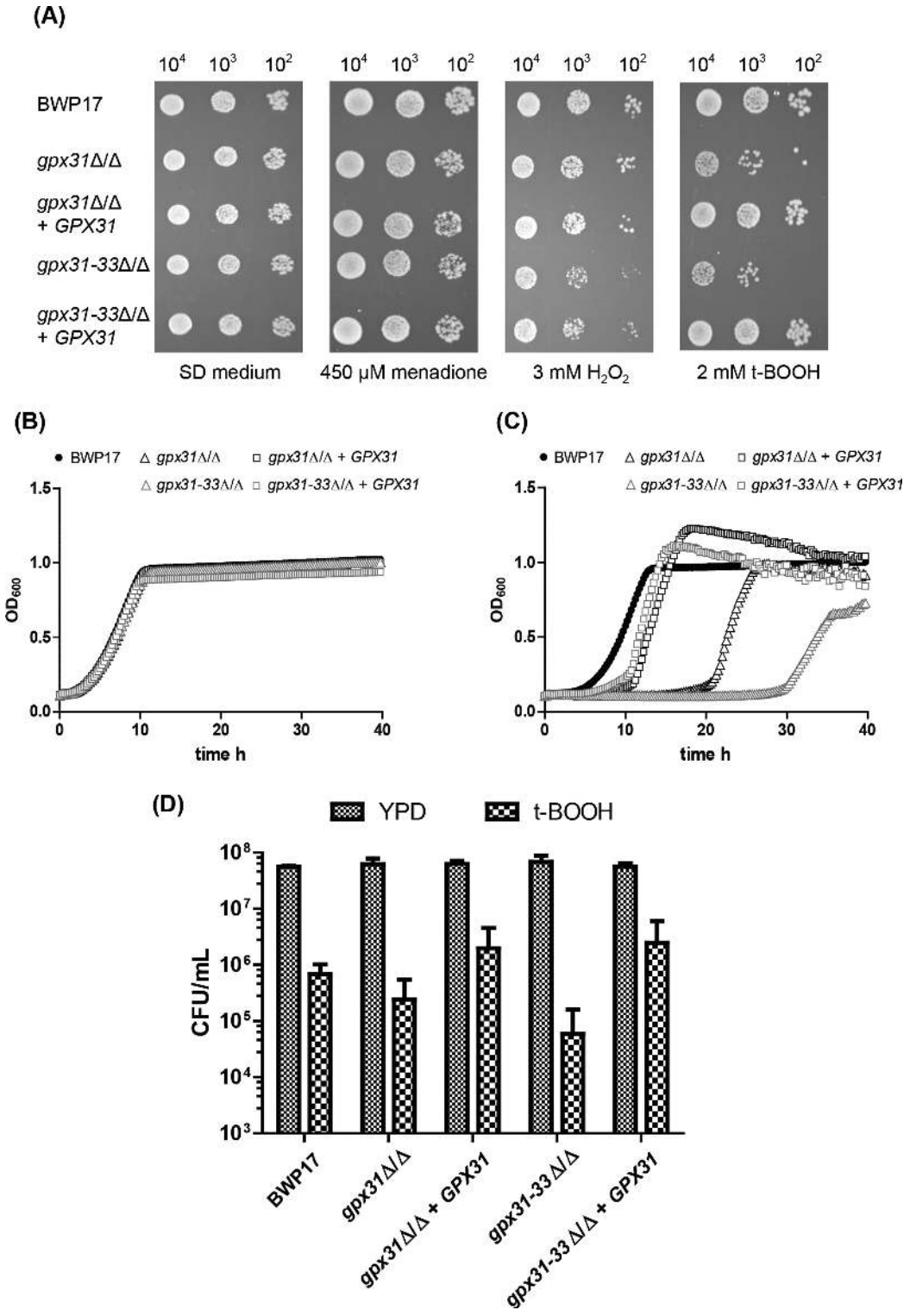

Figure 4. Glutathione peroxidase (GPx) mutants have increased susceptibility to peroxide-induced oxidative stress. (A) Serial dilutions of *Candida albicans* BWP17, *gpx31*Δ/Δ, *gpx31-33*Δ/Δ and reconstituted strains *gpx31*Δ/Δ + *GPX31* and *gpx31-33*Δ/Δ + *GPX31* were spotted on 2% D-glucose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% agar, minimal medium containing 450 μM menadione, 3 mM H_2O_2, and 2 mM tert-butyl hydroperoxide (t-BOOH). Plates were incubated for 2 days at 37°C. Experiments were performed at least twice with similar results. Representative pictures are shown. (B) Growth of wild-type deletion mutant and reconstituted strains in YPD medium (1% yeast extract, 2% bactopeptone, 2% D-glucose) and (C) YPD medium containing 1 mM t-BOOH. OD_{600} was measured every 15 min in a Tecan Infinite M200 microplate reader. Experiments were performed at least twice with similar results. Representative graphs are shown. (D) Viable count of wild-type deletion mutant and reconstituted strains after 3 h exposure to 0.5 mM t-BOOH at 37°C. As control, strains were grown in YPD under the same conditions as in the absence of the stressor. Mean from three independent experiments. Error bars represent SD.

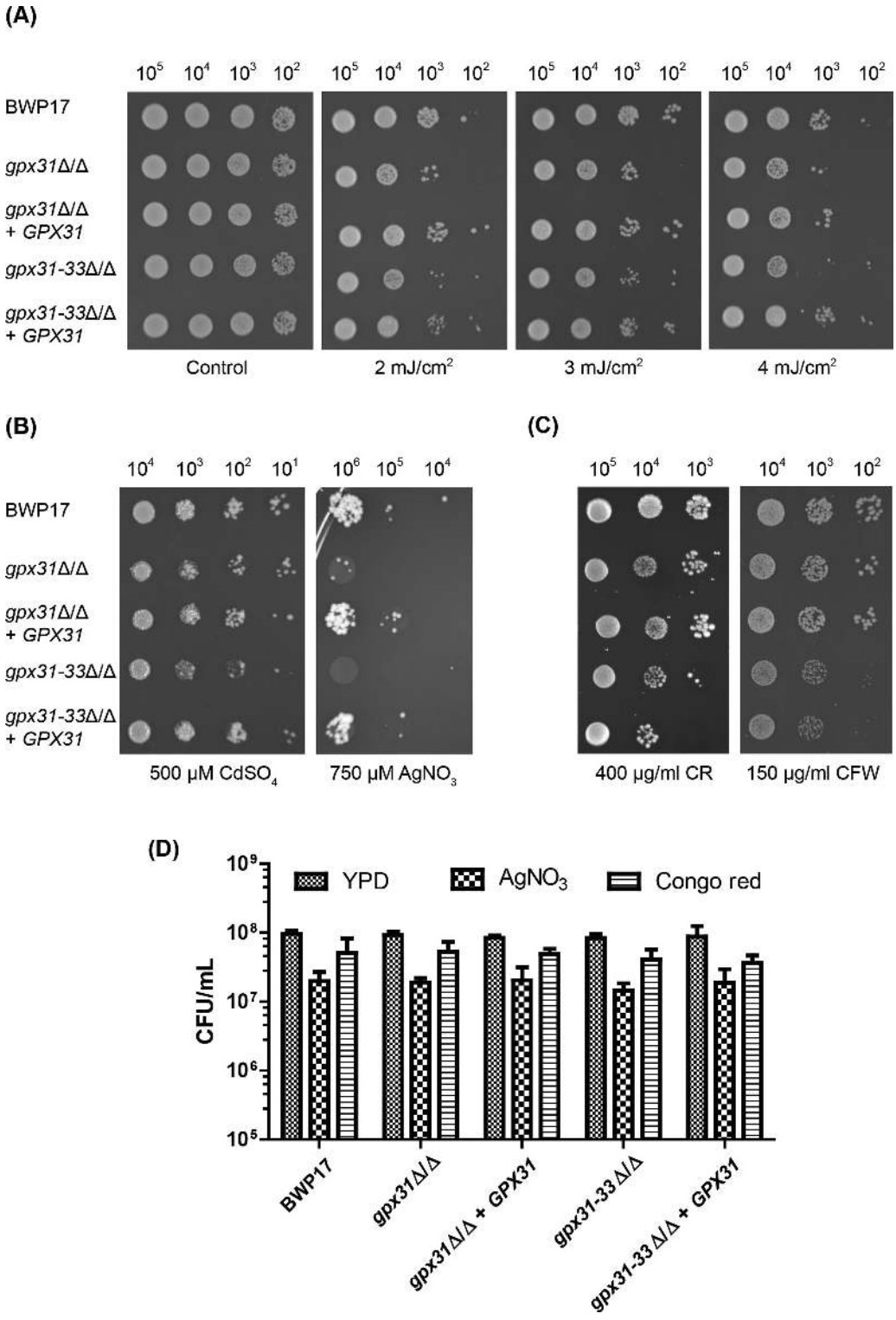

Figure 5. Glutathione peroxidase (GPx) mutants exhibit susceptibility to ultraviolet (UV) light, heavy metal, and cell wall stress. (A) Serial dilutions of *Candida albicans* BWP17, *gpx31*Δ/Δ, *gpx31-33*Δ/Δ, and reconstituted strains *gpx31*Δ/Δ + *GPX31* and *gpx31-33*Δ/Δ + *GPX31* were spotted on 2% D-glucose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% agar, medium and exposed to UV-C light (2 to 4 mJ/cm^2). An unexposed plate was used as control. Plates were incubated for 2 days at 37°C. (B) Drop-test analysis in the presence of 500 μM $CdSO_4$ and 750 μM $AgNO_3$. Plates were incubated for 3 days at 30°C. (C) Drop-test analysis in the presence of 400 μg/ml Congo red (CR) and 150 μg/ml calcofluor white (CFW). Plates were incubated for 3 days at 37°C. All drop-test experiments were performed at least twice with similar results. Representative pictures are shown. (D) Viable count of wild-type deletion mutant and reconstituted strains after 3 h exposure to 750 μM $AgNO_3$ or 400 μg/ml Congo red at 37°C. As control, strains were grown in 1% yeast extract, 2% bacto-peptone, 2% D-glucose under the same conditions as in the absence of the stressor. Mean from three independent experiments. Error bars represent standard deviation.

MDMs were infected with the wild-type, mutant, and reconstituted strains at an MOI of 0.0025 (*C. albicans*:MDMs) and incubated for 2 days until the formation of microcolonies. As shown in Figure 6A, survival of the single *gpx31*Δ/Δ and triple *gpx31-33*Δ/Δ mutants was significantly decreased in the presence of macrophages, indicating that GPxs, especially Gpx31, play a role in survival from attack by macrophages. Reintegration of a single *GPX31* allele in the triple *gpx31-33*Δ/Δ mutant was sufficient to restore the wild-type phenotype.

Survival of the single *gpx31*Δ/Δ mutant during coincubation with neutrophils was not significantly reduced (Fig. 6B), although it exhibited a trend toward reduced survival. In contrast, the survival of the triple *gpx31-33*Δ/Δ mutant was significantly reduced in comparison with the wild-type strain. It is noteworthy that reintegration of a single *GPX31* allele in the *gpx31-33*Δ/Δ background did not restore survival to wild-type levels, suggesting that *GPX32* and/or *GPX33* are needed to fully cope with neutrophil-derived oxidative stress. To test whether the increased sensitivity of the *gpx1–3*Δ/Δ mutant toward neutrophils was due to the exposure to phagocyte-derived oxidative stress, NADPH oxidase was inhibited with apocynin. As shown in Figure 6C, upon apocynin treatment,

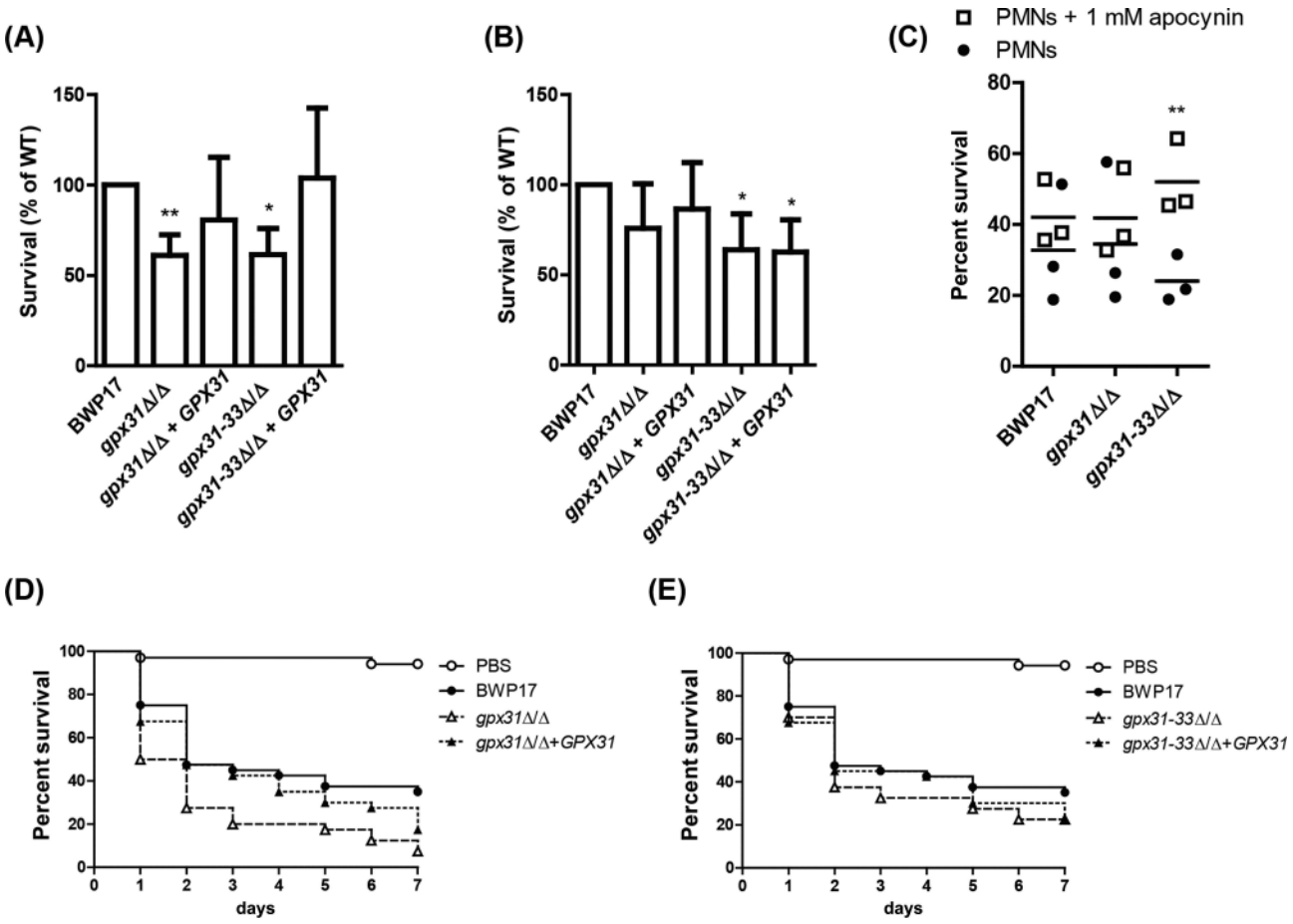

Figure 6. Survival of glutathione peroxidase (GPx) mutants upon exposure to phagocytes and virulence in an *in ovo* infection model. (A) Monocyte-derived macrophages were infected with *Candida albicans* BWP17, *gpx31*Δ/Δ, *gpx31-33*Δ/Δ, *gpx31*Δ/Δ + *GPX31*, and *gpx31-33*Δ/Δ + *GPX31* and incubated for 2 days. Fungal biomass was determined by means of 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay. Wild-type (WT) strain BWP17 was set as 100%. Mean of four independent replicates. Error bars represent standard deviation (SD). Statistical significance was calculated using *t* test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ compared with WT (100%). (B) Freshly isolated neutrophils were infected with *C. albicans* BWP17, *gpx31*Δ/Δ, *gpx31-33*Δ/Δ, *gpx31*Δ/Δ + *GPX31*, and *gpx31-33*Δ/Δ + *GPX31* and incubated for 3 h. Residual metabolic activity was determined by means of XTT assay. Wild-type strain BWP17 was set as 100%. Mean of four independent replicates. Error bars represent SD. Statistical significance was calculated using *t* test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ compared with wild type (100%). (C) Neutrophils were pretreated with 1 mM apocynin prior to infection with *C. albicans* cells. Lines represent mean values of each strain in the presence of neutrophils (filled circles) and apocynin-treated neutrophils (open squares). Results from three replicates are shown. Statistical significance was tested by two-way analysis of variance with Bonferroni post tests. $^{**}P \leq 0.01$. (D and E) Virulence in the embryonated egg infection model. Ten-day-old embryonated hen eggs were infected with *C. albicans* BWP17, *gpx31*Δ/Δ, *gpx31*Δ/Δ+*GPX31*, *gpx31-33*Δ/Δ, and *gpx31-33*Δ/Δ+*GPX31* ($n = 20$ eggs per strain). Eggs were monitored for survival by candling for 7 days. Results are the mean of two independent experiments per strain. Abbreviations: PBS, phosphate-buffer saline; WT, wild type.

survival of the triple *gpx31-33*Δ/Δ mutant from neutrophil attack was significantly increased.

Next, we tested whether GPxs are required for virulence in a chicken embryo model of candidiasis [30,31]. As shown in Figure 6D,E, neither the single *gpx31*Δ/Δ nor the triple *gpx31-33*Δ/Δ mutants showed attenuation of virulence in this infection model. Statistical analyses revealed that the differences between embryo survival infected with mutant and reconstituted strains were not significant (*gpx31*Δ/Δ vs. *gpx31*Δ/Δ + *GPX31*, $P = 0.0572$; *gpx31-33*Δ/Δ vs. *gpx31-33*Δ/Δ + *GPX31*, $P = 0.8333$).

Discussion

C. albicans has evolved as a successful human commensal; however, this fungus also has pathogenic potential under circumstances that favor its overgrowth or compromises the health status of the host. Not surprisingly, *C. albicans* has developed several mechanisms to adapt, respond, and resist host-derived activities [35]. One of the most widely studied responses is that toward phagocyte-generated oxidative stress. *C. albicans* has numerous enzymes and systems to effectively detoxify host-associated oxidative attack [12–14,36,37]. However, the contribution of GPxs in the response of *C. albicans* to oxidative stress and to phagocyte attack has not been determined.

C. albicans belongs to the CUG clade, a group of related yeasts that translate the CUG codon as serine instead of leucine [38]. By comparing the genome sequence of several *Candida* spp., it was shown that gene family expansions are common in members of the CUG clade [32]. We identified a cluster of genes on *C. albicans* chromosome 6 that harbor three open reading frames annotated as putative GPxs: *ORF19.85*, *ORF19.86*, and *ORF19.87*. Orthologous genes are found syntenically positioned in the genomes of the closely related species *C. dubliniensis* and *C. tropicalis* (Fig. 2A). These genes may constitute a novel gene family, expanded previously to the speciation event that led to the emergence of *C. albicans*, *C. dubliniensis*, and *C. tropicalis*. However, this occurred after the common ancestor of these three species separated from the rest of the CUG clade. It is noteworthy that *ORF19.86* orthologues are found in other members of the CUG clade, conserving a high degree of synteny and suggesting that *ORF19.85* and *ORF19.87* arose from duplication of the common ancestor shared with *ORF19.86*. A fourth open reading frame annotated as putative GPx exists in the *C. albicans* genome: *ORF19.4436*. This gene codes for a GPx that is closely related to *S. cerevisiae GPX1*. In fact, all members of the CUG clade have an *ORF19.4436* orthologue (Fig. 1). However, we focused on the GPxs encoded in *ORF19.85–87* since these genes are significantly upregulated in *C. albicans* under oxidative stress [8] and during coincubation with phagocytes [22,23].

Our data show that GPx activity is necessary for *C. albicans* to cope with oxidative stress. However, this enzymatic activity is differentially induced depending on the oxidant species. Menadione induces oxidative stress intracellularly by generating the superoxide radical ($O_2^{-}\bullet$) [33]. Although $O_2^{-}\bullet$ is spontaneously converted into H_2O_2, *C. albicans* possesses three intracellular superoxide dismutases (Sod1, Sod2, and Sod3) that may aid in eliminating this toxic radical [39]. H_2O_2 generated from this reaction can be further detoxified either by catalase or GPxs. As shown here, menadione treatment clearly induced GPx activity. However, the GPx activity was induced even more strongly upon exposure to peroxides. This activity may aid in the detoxification and protection of cellular targets that are susceptible to oxidative damage, such as lipid peroxidation [19]. Deletion of *GPX31* clearly reduced the total GPx activity in *C. albicans* upon exposure to the organic peroxide t-BOOH. However, this reduction, although clearly significant, was only of 50% compared with the wild-type strain. To test whether the remaining GPx activity is due to the expression of the *GPX32* or *GPX33* products, we constructed a triple *gpx31-33*Δ/Δ mutant strain. Surprisingly, deletion of these genes did not cause a further reduction in GPx activity, suggesting that *GPX31* codes for the major GPx in *C. albicans*, while the GPxs coded in *GPX32* and *GPX33* may have some redundancy under the tested conditions. Supporting the hypothesis that *GPX31* is the major GPx induced under oxidative stress, the expression levels of this gene were highly upregulated in the presence of t-BOOH, whereas *GPX32* was downregulated and *GPX33* and *GPX3* were induced at a much lower level (Fig. 3C). A possible explanation for the remaining GPx activity in the absence of *GPX31-GPX33* is that the basal expression of the GPx encoded by *ORF19.4436* is enough to compensate for the deficiency of GPx activity in the mutant strains. Compensatory mechanisms have been observed in *C. albicans*. For example, in a whole blood infection model, expression of *SOD4* is sufficient to render a *sod5*Δ/Δ mutant only partially sensitive to killing in this model [23]. It is also possible that other enzymes involved in GSH metabolism exhibit additional GPx activity. For instance, the GSH S-transferase encoded by *GTT11* displays GSH-dependent peroxidase activity in addition to GSH-conjugating activity [40]. Interestingly, this GSH S-transferase is significantly upregulated under oxidative stress [8] and during coincubation with neutrophils [23].

Although Gpx32 and Gpx33 may have redundant roles during short-term exposure to oxidants, they may be required for long-term adaptation to oxidative stress and related stimuli. This is evidenced by the fact that growth

inhibition of the single *gpx31*Δ/Δ mutant on solid media (Figs. 4A and 5) was modest in comparison to that of the wild-type strain. However, the phenotypes of the triple *gpx31-33*Δ/Δ mutant were indeed more pronounced in the presence of peroxides upon exposure to UV-C light and under heavy metal stress. Of note, growth on solid medium in the presence of menadione caused no visible effect on the growth of either the single or the triple mutant, suggesting that GPxs do not play a role in detoxification of intracellular O_2^-•. Growth assays in liquid media also indicated a role for Gpx31-33 in long-term adaptation to oxidative stress (Fig. 4B,C): the single *gpx31*Δ/Δ mutant was clearly delayed in growth, exhibiting an extended lag phase in the presence of t-BOOH. This extended lag phase was further increased in the triple *gpx31-33*Δ/Δ mutant, suggesting that under this condition, Gpx32/33 can partially complement the function of Gpx31. This is supported by the fact that complementation of either the *gpx31*Δ/Δ or the *gpx31-33*Δ/Δ mutant with a single copy of *GPX31* restored growth to near wild-type levels. Supporting the hypothesis of a role for Gpx31-33 in long-term adaptation to oxidative stress, the cell viability was not significantly affected in the presence of t-BOOH for 3 h in liquid media. Likewise, no differences were observed when the strains were exposed for the short term to heavy metal stress ($AgNO_3$) or cell wall stress (Congo red).

A possible role for Gpx32 and/or Gpx33 in cell wall integrity, suggested by the fact that the *gpx31-33*Δ/Δ triple mutant and the respective *GPX31* reconstituted strain exhibited a growth defect in the presence of cell wall stressors (Fig. 5C), requires further investigation. It should be noted that we could not exclude the possibility that other genetic alterations may have occurred during the construction of the triple mutant and/or the revertant strain, which contribute to the observed phenotypes.

Phagocytes use several killing mechanisms to destroy fungal pathogens [35]. The NADPH oxidase generates copious amounts of O_2^-•, which in turn is transformed into H_2O_2. Different detoxification mechanisms operate in *C. albicans* to overcome phagocyte-derived oxidative attack, such as Sods [12,13] and catalase [14]. Evidence of a role for glutathione-dependent protective mechanisms to overcome phagocyte-generated oxidative stress comes from studies with a mutant defective in the glutathione reductase encoded in *GRX2* [36]. This mutant (*grx2*Δ/Δ) accumulated reactive oxygen species and was hypersensitive to killing by neutrophils [13]. However, the contribution of GPxs for resistance to phagocyte attack was not assessed. Here, we show that mutant strains lacking *GPX31* were clearly more susceptible to macrophage killing (Fig. 6A) and that Gpx32 and Gpx33 do not compensate for the lack of Gpx31. Furthermore, deletion of *GPX32* and *GPX33* did not increase susceptibility to macrophage-mediated killing. This is in accordance with the transcriptional profile of *C. albicans* phagocytosed by macrophages [22], where only *GPX31* was significantly upregulated in response to macrophages.

Survival of the *gpx31*Δ/Δ mutant upon exposure to neutrophils was not significantly decreased. However, the triple *gpx31-33*Δ/Δ mutant was clearly reduced in its survival capacity in the presence of neutrophils. As expected from the transcriptional data [23], *GPX32* and *GPX33* are needed to fully cope with neutrophil attack, possibly to counteract oxidative stress. In agreement with the hypothesis that the three Gpx31-33 proteins are needed for full resistance to neutrophils, the triple *gpx31-33*Δ/Δ mutant complemented with only one *GPX31* allele was unable to resist the neutrophil killing at wild-type levels. However, this could also be due to a gene dosage effect. Furthermore, as for the cell wall–sensitive phenotypes discussed above, it cannot be excluded that other genetic alterations were introduced during the construction of the mutant strains and/or the reconstituted strain, which may contribute to the observed phenotypes. Therefore, the precise role of Gpx31-33 proteins remains speculative. However, we provide further evidence that the increased sensitivity of the *gpx31-33*Δ/Δ mutant to neutrophils was due to oxidative killing mechanisms, that is, upon apocynin inhibition of the NADPH oxidase (which is the main source of oxidants in activated neutrophils), survival of this strain was significantly increased. Interestingly, in contrast to this rescue of the *gpx31-33*Δ/Δ mutant, survival of a *grx2*Δ/Δ mutant is not improved upon exposure to apocynin-treated neutrophils [13], suggesting that, although GPxs and reductases are needed to maintain the normal GSH redox balance, glutathione reductase, but not peroxidase, activity may play additional roles in protection against nonoxidative mechanisms. Taken together, we demonstrate that the GPxs, as components of the GSH redox system, are relevant to coping with phagocyte-induced oxidative stress.

Despite their role in resistance to phagocyte attack, the GPxs encoded by *GPX31-33* are dispensable in the chicken embryo model of candidiasis, an infection model that has been successfully used to mimic systemic murine infections [30,31]. We cannot rule out the possibility that GPx activity is necessary for other types of infection (e.g., oral candidiasis in a murine model). However, no major contribution of the GPxs to the overall virulence of *C. albicans* is to be expected because the phenotypes of the deletion mutants in the presence of stressors were only moderate. It must be noted that in the chicken embryo model, *C. albicans* is not immediately exposed to phagocytic cells from the blood, since only few residential phagocytes are present in the CAM. Phagocytic cells are only recruited following

fungal microcolony formation on the CAM and the production of proinflammatory cytokines [30]. This might explain why, despite their involvement in tolerating phagocyte-derived oxidative stress, Gpx31-33 were not required for virulence in this model. Furthermore, although chicken heterophils, which are the avian counterpart of mammalian neutrophils, exhibit the respiratory burst in response to infectious stimuli, the amount of oxidants produced by these cells is smaller than that produced by human neutrophils [41]. Moreover, heterophils lack myeloperoxidase, an enzyme that contributes to the production of oxidants. Thus, in this infection model, the *gpx*Δ/Δ mutants may not be exposed to the full array of oxidants that human neutrophils produce. In addition, *C. albicans* has multiple systems that may operate in parallel as a response to the oxidative stress that the fungus encounters during the course of infection.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References

1. Mavor AL, Thewes S, Hube B. Systemic fungal infections caused by *Candida* species: epidemiology, infection process and virulence attributes. *Curr Drug Targets* 2005; **6**: 863–874.
2. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 2007; **20**: 133–163.
3. Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. *Arch Biochem Biophys* 2002; **397**: 342–344.
4. Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* 2004; **2**: 820–832.
5. Shohet SB, Pitt J, Baehner RL, Poplack DG. Lipid peroxidation in the killing of phagocytized pneumococci. *Infect Immun* 1974; **10**: 1321–1328.
6. Stadler N, Hofer M, Sigler K. Mechanisms of *Saccharomyces cerevisiae* PMA1 H+-ATPase inactivation by Fe2+, H_2O_2 and Fenton reagents. *Free Radic Res* 2001; **35**: 643–653.
7. Smith DA, Nicholls S, Morgan BA, Brown AJ, Quinn J. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. *Mol Biol Cell* 2004; **15**: 4179–4190.
8. Enjalbert B, Smith DA, Cornell MJ et al. Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. *Mol Biol Cell* 2006; **17**: 1018–1032.
9. Alarco AM, Raymond M. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. *J Bacteriol* 1999; **181**: 700–708.
10. Zhang X, De Micheli M, Coleman ST, Sanglard D, Moye-Rowley WS. Analysis of the oxidative stress regulation of the *Candida albicans* transcription factor, Cap1p. *Mol Microbiol* 2000; **36**: 618–629.
11. Wang Y, Cao YY, Jia XM et al. Cap1p is involved in multiple pathways of oxidative stress response in *Candida albicans*. *Free Radic Biol Med* 2006; **40**: 1201–1209.
12. Frohner IE, Bourgeois C, Yatsyk K, Majer O, Kuchler K. *C. albicans* cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. *Mol Microbiol* 2008; **71**: 240–252.
13. Miramón P, Dunker C, Windecker H et al. Cellular responses of *Candida albicans* to phagocytosis and the extracellular activities of neutrophils are critical to counteract carbohydrate starvation, oxidative and nitrosative stress. *PLoS One* 2012; **7**: e52850.
14. Wysong DR, Christin L, Sugar AM, Robbins PW, Diamond RD. Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. *Infect Immun* 1998; **66**: 1953–1961.
15. Urban C, Xiong X, Sohn K et al. The moonlighting protein Tsa1p is implicated in oxidative stress response and in cell wall biogenesis in *Candida albicans*. *Mol Microbiol* 2005; **57**: 1318–1341.
16. da Silva Dantas A, Patterson MJ, Smith DA et al. Thioredoxin regulates multiple hydrogen peroxide-induced signaling pathways in *Candida albicans*. *Mol Cell Biol* 2010; **30**: 4550–4563.
17. Arthur JR. The glutathione peroxidases. *Cell Mol Life Sci* 2000; **57**: 1825–1835.
18. Inoue Y, Matsuda T, Sugiyama K, Izawa S, Kimura A. Genetic analysis of glutathione peroxidase in oxidative stress response of *Saccharomyces cerevisiae*. *J Biol Chem* 1999; **274**: 27002–27009.
19. Avery AM, Avery SV. *Saccharomyces cerevisiae* expresses three phospholipid hydroperoxide glutathione peroxidases. *J Biol Chem* 2001; **276**: 33730–33735.
20. Tanaka T, Izawa S, Inoue Y. *GPX2*, encoding a phospholipid hydroperoxide glutathione peroxidase homologue, codes for an atypical 2-Cys peroxiredoxin in *Saccharomyces cerevisiae*. *J Biol Chem* 2005; **280**: 42078–42087.
21. Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB. A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. *Cell* 2002; **111**: 471–481.
22. Lorenz MC, Bender JA, Fink GR. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* 2004; **3**: 1076–1087.
23. Fradin C, De Groot P, MacCallum D et al. Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol Microbiol* 2005; **56**: 397–415.

24. Fitzpatrick DA, O'Gaora P, Byrne KP, Butler G. Analysis of gene evolution and metabolic pathways using the *Candida* Gene Order Browser. *BMC Genomics* 2010; **11**: 290.
25. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997; **25**: 4876–4882.
26. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 2004; **5**: 150–163.
27. Zakikhany K, Naglik JR, Schmidt-Westhausen A et al. *In vivo* transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. *Cell Microbiol* 2007; **9**: 2938–2954.
28. Gola S, Martin R, Walther A, Dunkler A, Wendland J. New modules for PCR-based gene targeting in *Candida albicans*: rapid and efficient gene targeting using 100 bp of flanking homology region. *Yeast* 2003; **20**: 1339–1347.
29. Martin R, Hellwig D, Schaub Y et al. Functional analysis of *Candida albicans* genes whose *Saccharomyces cerevisiae* homologues are involved in endocytosis. *Yeast* 2007; **24**: 511–522.
30. Jacobsen ID, Grosse K, Berndt A, Hube B. Pathogenesis of *Candida albicans* infections in the alternative chorio-allantoic membrane chicken embryo model resembles systemic murine infections. *PLoS One* 2011; **6**: e19741.
31. Jacobsen ID, Grosse K, Hube B. Embryonated chicken eggs as alternative infection model for pathogenic fungi. *Methods Mol Biol* 2012; **845**: 487–496.
32. Butler G, Rasmussen MD, Lin MF et al. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* 2009; **459**: 657–662.
33. Castro FA, Mariani D, Panek AD, Eleutherio EC, Pereira MD. Cytotoxicity mechanism of two naphthoquinones (menadione and plumbagin) in *Saccharomyces cerevisiae*. *PLoS One* 2008; **3**: e3999.
34. Rubin-Bejerano I, Fraser I, Grisafi P, Fink GR. Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. *Proc Natl Acad Sci U S A* 2003; **100**: 11007–11012.
35. Miramón P, Kasper L, Hube B. Thriving within the host: *Candida* spp. interactions with phagocytic cells. *Med Microbiol Immunol* 2013; **202**: 183–195.
36. Chaves GM, Bates S, MacCallum DM, Odds FC. *Candida albicans GRX2*, encoding a putative glutaredoxin, is required for virulence in a murine model. *Genet Mol Res* 2007; **6**: 1051–1063.
37. Enjalbert B, MacCallum DM, Odds FC, Brown AJ. Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. *Infect Immun* 2007; **75**: 2143–2151.
38. Santos MA, Keith G, Tuite MF. Non-standard translational events in *Candida albicans* mediated by an unusual seryl-tRNA with a 5′-CAG-3′ (leucine) anticodon. *EMBO J* 1993; **12**: 607–616.
39. Hwang CS, Rhie GE, Oh JH et al. Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence. *Microbiology* 2002; **148**: 3705–3713.
40. Garcerá A, Casas C, Herrero E. Expression of *Candida albicans* glutathione transferases is induced inside phagocytes and upon diverse environmental stresses. *FEMS Yeast Res* 2010; **10**: 422–431.
41. Harmon BG. Avian heterophils in inflammation and disease resistance. *Poult Sci* 1998; **77**: 972–977.
42. Wilson RB, Davis D, Mitchell AP. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J Bacteriol* 1999; **181**: 1868–1874.