Differential Roles of a Family of Flavodoxin-Like Proteins That Promote Resistance to Quinone-Mediated Oxidative Stress in *Candida albicans*

Jenna E. Foderaro,^a James B. Konopka^a

^aDepartment of Microbiology and Immunology, Stony Brook University, Stony Brook, New York, USA

ABSTRACT Survival of the fungal pathogen *Candida albicans* within a mammalian host relies on its ability to resist oxidative stress. The four flavodoxin-like proteins (Pst1, Pst2, Pst3, and Ycp4) that reside on the inner surface of the *C. albicans* plasma membrane represent a recently discovered antioxidant mechanism that is essential for virulence. Flavodoxin-like proteins combat oxidative stress by promoting a two-electron reduction of quinone molecules, which prevents the formation of toxic semiquinone radicals. Previous studies indicated that Pst3 played a major role in promoting resistance to the small quinone molecules p-benzoquinone and menadione. Analysis of additional quinones confirmed this role for Pst3. To better define their function, antibodies were raised against each of the four flavodoxin-like proteins and used to quantify protein levels. Interestingly, the basal level of flavodoxin-like proteins differed, with Pst3 and Ycp4 being the most abundant. However, after induction with p-benzoquinone, Pst1 and Pst3 were the most highly induced, resulting in Pst3 becoming the most abundant. Constitutive expression of the flavodoxin-like protein genes from a *TDH3* promoter resulted in similar protein levels and showed that Pst1 and Pst3 were better at protecting *C. albicans* against p-benzoquinone than Pst2 or Ycp4. In contrast, Pst1 and Ycp4 provided better protection against oxidative damage induced by tert-butyl hydroperoxide. Thus, both the functional properties and the relative abundance contribute to the distinct roles of the flavodoxin-like proteins in resisting oxidative stress. These results further define how *C. albicans* combats the host immune response and survives in an environment rich in oxidative stress.

KEYWORDS quinone, p-benzoquinone, flavodoxin-like protein, *Candida albicans*, oxidoreductase, oxidative stress, quinone

Candida albicans is a common commensal organism that can cause lethal systemic infections, especially in an immunocompromised host (1, 2). *C. albicans* is a successful pathogen because it can resist a wide variety of stressors imposed by the host immune system (3, 4). A key line of defense used by mammalian hosts is the attack of *C. albicans* by leukocytes, particularly neutrophils and macrophages, often termed the oxidative burst (5–7). Oxidative stress can damage DNA, lipids, and proteins (5, 7, 8). If left unchecked, the damage disrupts physiological cellular processes, causes mutations, and can result in cell death (8). Many distinct types of reactive oxygen species exist, so cells need several types of detoxifying mechanisms (3, 7, 9–11). The importance of superoxide dismutase activity is highlighted by the fact that *C. albicans* produces cytoplasmic, mitochondrial, and secreted forms of these enzymes to break down superoxide radicals (3, 7, 10). Additional antioxidant mechanisms are found in the cytoplasm, such as catalase, thioredoxin, and glutathione. However, little is known about how cellular membranes are protected (11).

The plasma membrane is highly vulnerable to oxidative attack, as it is exposed at

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Address correspondence to James B. Konopka, James.konopka@stonybrook.edu.

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the cell surface (12). It is essential for *C. albicans* to protect the integrity of this barrier because of the critical roles it plays in virulence. The plasma membrane coordinates a wide range of key functions, including secretion, endocytosis, cell wall synthesis, and polarized morphogenesis, to promote invasive hyphal growth (13). One specific weakness of the *C. albicans* plasma membrane to oxidative attack is that it contains high levels of polyunsaturated fatty acids (PUFAs), which are susceptible to lipid peroxidation (14, 15). Lipid peroxidation of PUFAs is a serious problem, as it starts a chain reaction that propagates the peroxidation to other PUFAs, and the resulting oxidative damage can then spread to proteins and DNA.

Ubiquinol (coenzyme Q), a long-chain quinone molecule embedded in the plasma membrane, can act as an antioxidant to prevent lipid peroxidation and other forms of oxidative damage in the plasma membrane, where other antioxidant mechanisms could not gain access (16–20). However, once ubiquinol has been oxidized to ubiquinone, it must be reduced before it can act again. In *C. albicans*, the reduction of ubiquinone appears to be carried out by a family of four flavodoxin-like proteins (FLPs), Pst1, Pst2, Pst3, and Ycp4 (21). The expression of the FLP genes is induced by oxidative stress, in agreement with the FLPs playing important roles in combatting oxidative stress (22, 23). The FLPs are localized to the inner surface of the plasma membrane and are enriched in MCC/eisosome domains, which are specialized membrane subdomains that promote resistance to several kinds of stress (12, 21, 24).

Studies on the FLP family of proteins that are conserved in bacteria, plants, and fungi have shown that they function as NAD(P)H:quinone oxidoreductases. In particular, they catalyze the two-electron reduction of a quinone to quinol in one step (25, 26). The two-electron reduction promoted by FLPs converts both carbonyl groups on the benzoquinone ring to hydroxyl groups (Fig. 1A). This is highly advantageous, because alternative pathways that act in the absence of FLPs will promote a one-electron reduction of a quinone to a semiquinone, which is a dangerous reactive oxygen species (25). In addition to ubiquinone, cells also encounter a diverse array of quinones. Quinones are six-membered α,β -dienonic rings classified by differences in their carbon skeletons (Fig. 1A) (26). The simplest type of quinone is a benzoquinone; other types contain side groups or additional rings, such as the naphthoquinones, anthraquinones, and phenanthrenequinones (26). A variety of quinones have been found in many organisms, including plants, algae, fungi, lichens, bacteria, insects, viruses, and animals. They are often metabolic by-products, in some cases they are synthesized by cells as defense mechanisms, and they are used as pharmaceutical drugs (26–28). Interestingly, FLPs are an attractive target for the development of new antifungal drugs, as they are required for the virulence of *C. albicans* (21) but are not present in mammalian cells, which use very divergent types of enzymes to reduce quinones (29). Therefore, in this study we examined the function and abundance of the four different FLPs in *C. albicans*.

RESULTS

Small-molecule quinones of various structures inhibit *C. albicans* growth. To better understand the mechanisms used by *C. albicans* to resist quinones, we tested a range of commercially available quinones chosen for their various structures (Fig. 1A). The simplest quinone is p-benzoquinone (BZQ), which represents the basic structural motif for many natural and synthetic molecules (30). 2,3-Dimethoxy-5-methyl-p-benzoquinone, also known as coenzyme Q₀ (CoQ₀), is an early intermediate in the natural synthesis of some quinones, including ubiquinone (coenzyme Q). The structure of CoQ₀ consists of only the benzoquinone head group and lacks the lengthy hydrocarbon tail (Fig. 1A) (31, 32). The final benzoquinone chosen was 2-tert-butyl-1,4-benzoquinone (TBBQ), the oxidation product of tert-butylhydroquinone, a common food additive (27, 33). We also chose two naphthoquinones: menadione (MND) and plumbagin. Naphthoquinones, derived from naphthalenes, are the most commonly occurring quinones, many of which have known antifungal activities (26). Menadione is a synthetic heterocyclic naphthoquinone, also known as vitamin K3, which functions as the

FIG 1 *C. albicans* $\Delta/\Delta/\Delta/\Delta$ mutant shows differential susceptibility to quinones of various structures. (A) Structures and additional information for each of the quinones analyzed in this study. (B to F) The susceptibility of the *C. albicans* wild-type control strain (LLF100A) and $\Delta/\Delta/\Delta/\Delta$ mutant (LLF060A) to p-benzoquinone (B), coenzyme Q₀ (C), menadione (D), plumbagin (E), and TBBQ (F) as determined by halo assays. Cells were spread onto the surface of either synthetic complete or YPD plates (see Materials and Methods), filter disks containing the indicated concentration of compound were placed onto each plate, and then the zone of inhibition (halo) was quantified after 2 days. The absence of a data point at the lowest concentration for TBBQ indicates that *C. albicans* growth inhibition could not be visualized. Results represent the average of at least three independent experiments each done in duplicate. Error bars indicate standard deviations (SD).

water-soluble analog of vitamin K (26, 34). Plumbagin is a plant-derived naphthoquinone that has been studied for broad activity against cancer and pathogenic microbes (28, 35).

Previous studies demonstrating that *C. albicans* is susceptible to growth inhibition by p-benzoquinone and menadione were performed by spotting dilutions of cells onto agar medium containing a single concentration of one of these molecules (21). While informative, these assays did not reveal the magnitude of sensitivity for each compound. Therefore, a disk diffusion assay was used to quantify the susceptibility of *C.*

albicans to different quinones. Paper filter disks containing different amounts of each compound were placed onto the surface of plates covered with *C. albicans*. The diameters of the resulting halos formed by the inhibition of cell growth were then measured to provide a quantification of the sensitivity of cells to each compound. *C. albicans* was susceptible to inhibition by all five quinones in this assay (Fig. 1B to F), although there were differences in the level of sensitivity to each of the quinones, as described below.

To determine whether FLPs played a role in resisting all five types of quinones, we compared a wild-type *C. albicans* control strain with a quadruple mutant lacking all FLPs (*pst1Δ/pst2Δ/pst3Δ/ycp4Δ* mutant; referred to as $\Delta/\Delta/\Delta/\Delta$ for simplicity). In agreement with previous studies (21), the $\Delta/\Delta/\Delta/\Delta$ mutant was more susceptible to p-benzoquinone and menadione (Fig. 1B and F). In addition, the $\Delta/\Delta/\Delta/\Delta$ mutant was more susceptible to the other three quinones tested, indicating the FLPs play a broad role in promoting resistance to different types of quinones. Interestingly, the most potent molecules were TBBQ and plumbagin, which are the most nonpolar of the quinones included in this study. Potency was determined as the concentration of a compound required to generate a halo of growth inhibition that was 10 mm in diameter. In contrast, the least potent was CoQ₉, which contains two methoxy side groups, making it more polar than TBBQ and plumbagin. This trend suggests that the potency of the different quinones is related at least in part to their hydrophobicity and, hence, their ability to more easily pass through the plasma membrane.

Pst3 plays a major role in resistance to quinones. Previous studies indicated *PST3* plays an important role in promoting resistance to quinones, because a *pst3Δ* mutant was the only single FLP mutant that displayed increased sensitivity to p-benzoquinone and menadione in spot assays (21). Since those assays were not quantitative, we used the quantitative disk diffusion halo assay to confirm the importance of *PST3* and also to test the other FLP mutants against a broader range of quinone molecules. Homozygous mutants lacking both copies of one FLP gene (*pst1Δ*, *pst2Δ*, *pst3Δ*, or *ycp4Δ*) were tested. Of these mutants, the *pst3Δ* strain always showed the greatest increase in susceptibility (Fig. 2, red versus black lines). In every case its level of sensitivity was between that of the $\Delta/\Delta/\Delta/\Delta$ mutant, which had the greatest loss of resistance, and the wild-type control strain.

The majority of halo assays for the other three FLP mutants (*pst1Δ*, *pst2Δ*, and *ycp4Δ*) clustered around the wild type, indicating that these mutants were not significantly increased in sensitivity to the different quinones. However, we found that the FLP mutants displayed differential susceptibility to p-benzoquinone (Fig. 2A), which was not apparent in the previous spot assays (21). This confirms that halo assays are a more quantitative measurement for susceptibility. Nonetheless, the results support the conclusion that *PST3* plays an important role in resisting quinones.

Specificity of anti-FLP antibodies. To better understand how the different FLPs function, we wanted to examine the production of their proteins. One consideration in trying to quantify these proteins is that three of them are roughly the same size, making it essentially impossible to distinguish from one another via Western blotting using only one antibody or a common epitope tag (*Pst1*, 21.3 kDa; *Pst2*, 21.7 kDa; *Pst3*, 21.3 kDa; and *Ycp4*, 29.8 kDa) (Fig. 3). In addition, C-terminal epitope tagging is not optimal, because we cannot predict the impact such large tags would have on the individual protein functions. This is of particular concern for *Ycp4*, which has a C-terminal extension of 90 amino acids ending in a CAAX box, thought to be a site of lipid modification. Therefore, four polyclonal antibodies were raised to allow probing of all four FLPs in a single culture of cells to avoid disrupting protein function with a tag.

Proteins produced in *Escherichia coli* were used to immunize rabbits, and resulting antibodies were screened by Western blotting against *C. albicans* lysates generated from wild-type and $\Delta/\Delta/\Delta/\Delta$, *pst1Δ*, *pst2Δ*, *pst3Δ*, and *ycp4Δ* mutant strains. Each antibody generated was highly specific for the FLP against which it was raised. Western blots revealed bands that were of the appropriate size in wild-type cell extracts but not in an extract from a mutant lacking the FLP against which the antibody was raised or the $\Delta/\Delta/\Delta/\Delta$ mutant strain (Fig. 3). Interestingly, each FLP could also be detected in

FIG 2 *C. albicans* strains lacking single FLP genes show differential sensitivity to quinones. Quantification of halo assays comparing the susceptibility of each *C. albicans* strain to the quinone indicated on the x axis. (A) p-benzoquinone, (B) coenzyme Q₀, (C) menadione, (D) plumbagin, and (E) TBBQ. Strains used include the wild-type control strain (LLF100A) and $\Delta/\Delta/\Delta/\Delta$ mutant (LLF060A), *pst1* Δ (LLF052), *pst2* Δ (LLF059), *pst3* Δ (LLF036), and *ycp4* Δ (LLF037) strains. The absence of a data point at the lowest concentration for TBBQ indicates that *C. albicans* growth inhibition could not be visualized. Results represent the averages from at least three independent experiments, each done in duplicate. Error bars indicate SD.

strains lacking one of the other FLP genes. For example, the anti-Pst1 antibody detected a protein in the wild-type and *pst2* Δ , *pst3* Δ , and *ycp4* Δ mutant strains but not in the $\Delta/\Delta/\Delta/\Delta$ or the *pst1* Δ mutant strain (Fig. 3). The ability of the other proteins to be detected in strains lacking other FLPs indicates that they are not dependent on each other for their stability. This implies Pst3 is not the most important FLP simply due to effects on the production of other FLPs.

FIG 3 Antibodies raised in rabbits are highly specific for each FLP. Western blotting was performed using log-phase cells grown in YPD. Whole-cell lysates were probed with each newly generated antibody to detect FLPs. Anti-actin was used as a loading control. Strains used include the wild-type control strain (LLF100A) and $\Delta/\Delta/\Delta/\Delta$ mutant (LLF060A), *pst1* Δ (LLF052), *pst2* Δ (LLF059), *pst3* Δ (LLF036), and *ycp4* Δ (LLF037) strains. Images shown are representative of three independent experiments performed on different days.

FIG 4 FLPs are produced at different basal levels in *C. albicans*. (A) Western blots comparing FLP protein production level in *C. albicans* strains to ectopically expressed FLPs of known concentration in *E. coli*. Strains used include the wild-type control strain (LLF100A), Δ/Δ/Δ/Δ mutant (LLF060A), and add-back strains where the Δ/Δ/Δ/Δ mutant was complemented with a single copy of each FLP, +PST1 (LLF064), +PST2 (LLF081), +PST3 (LLF066), and +YCP4 (LLF082), which are referred to as Δ/Δ/Δ/Δ + FLP for simplicity. Asterisks indicate the approximate amount of protein found in the wild-type *C. albicans* sample compared with *E. coli* samples. Images shown are representative of three experiments performed on different days. Western blots were normalized to Coomassie-stained gels. (B) Quantification of Western blots to determine basal amount of each FLP produced. Images were analyzed using ImageStudio software. Results represent averages from three independent experiments performed on different days. AU, arbitrary units. Error bars indicate SD, the line indicates the median, and the box indicates maximum and minimum. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; all by one-way analysis of variance (ANOVA). (C) Standard curves generated from signal intensities of purified *E. coli* proteins used to calculate the amount of FLP present in *C. albicans* samples.

FLPs are produced at different basal levels. To start to address whether the important role for Pst3 is due to its functional properties or its abundance, we quantified the basal level of each FLP in wild-type *C. albicans* grown at 30°C. Log-phase cells from each strain were grown and lysates generated as described in Materials and Methods. In parallel, 6×His-tagged FLPs were purified from *E. coli*. Twenty micrograms of whole-cell lysate from each *C. albicans* sample was run on a gel with adjacent lanes containing a dilution series of purified protein samples of each FLP isolated from bacteria (Fig. 4A). Western blot analysis was carried out and a standard curve generated using the signal intensities of the purified proteins. This curve was then used to determine the amount of protein present in each *C. albicans* sample.

The results indicated there was a 12.9-fold range in the amount of protein produced by the different FLPs (Fig. 4B). Ycp4 is the most abundant protein, accounting for 0.29% of total cell protein in wild-type cells cultured under these standard conditions, followed by Pst3 (0.15%), Pst1 (0.042%), and Pst2 (0.023%) (Fig. 4B). These results vary slightly from the previously published transcriptome sequencing (RNA-seq) data that indicated *PST3* was the most highly expressed FLP transcript, followed by *YCP4*, and with *PST1* and *PST2* tied for the lowest levels (22, 36). This suggests posttranscriptional regulation and/or differential stability influences the levels of these proteins.

FIG 5 FLPs are differentially induced by p-benzoquinone. (A) The panels on the left are Western blots comparing FLP protein production in *C. albicans* strains upon induction with 0.1 mM p-benzoquinone for 1 h. Each blot was probed using the indicated anti-FLP antibody. Images shown are representative of three experiments performed on different days. The image on the right corresponds to a Coomassie blue-stained gel showing that similar amounts of total cell extract were loaded in each lane. (B) Fold change for each FLP quantified after induction with p-benzoquinone. Images were analyzed using Image Studio software, and Western blots were normalized to Coomassie-stained gels. Results represent averages from three independent experiments performed on different days. Error bars indicate SD compared using multiple *t* tests, where, for each FLP, p-benzoquinone-treated sample was compared to the DMSO-treated control (data bar not shown). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

FLPs are induced to different levels after exposure to p-benzoquinone. Since FLPs play an important role in resisting quinones, we quantified the level of each FLP after exposure to p-benzoquinone. Log-phase cells were exposed to 100 μ M p-benzoquinone or dimethyl sulfoxide (DMSO) for 1 h prior to harvesting for Western blotting (Fig. 5A). We note that normalization could no longer be done using actin as a loading control for cells treated with p-benzoquinone, because the presence of this oxidizing agent altered actin levels in cells (data not shown). Therefore, normalization was done using a Coomassie-stained gel to control for loading so that the relative abundance of a broad range of proteins could be examined. Interestingly, the FLPs were differentially induced after treatment with p-benzoquinone, with Pst1 (6.6-fold) and Pst3 (6.3-fold) exhibiting the greatest fold changes over a DMSO control (Fig. 5A and B). Pst2 was also highly induced, with a 4-fold increase in protein level, while Ycp4 was only mildly induced by 2.5-fold (Fig. 5B). When this fold change is used to extrapolate protein abundance after exposure to p-benzoquinone, Pst3 was the most highly abundant FLP in a *C. albicans* cell. If the results are normalized to the least abundant FLP (Pst2), the relative amounts of each FLP are Pst2 (1), Pst1 (3.0), Ycp4 (8.0), and Pst3 (10.7), demonstrating a wide variation in the levels of each FLP. These results confirm the important role that Pst3 plays in resisting quinones.

Pst1 and Pst3 are better able to protect against quinones. Analysis of the FLP levels showed that they varied widely, which limited the ability to conclude whether they have different functions in resisting quinones. To control for protein abundance as a variable and determine whether Pst3 is the most important FLP for promoting resistance to quinones, we next expressed each FLP under the control of the constitutive *TDH3* promoter in the $\Delta/\Delta/\Delta/\Delta$ mutant background. After quantitation of Western blots, we found that the *TDH3* promoter resulted in increased production of all FLPs relative to basal levels of Pst1 (5-fold), Pst2 (10-fold), Pst3 (3-fold), and Ycp4 (5-fold) (Fig. 6A). Interestingly, when relative amounts of each protein were extrapolated from these fold changes, we found relatively equal levels of three of the FLPs when they were normalized to Pst1 (1), Pst2 (1.04), and Pst3 (2.31), but Ycp4 was produced at higher levels (6.84) (Fig. 6B). These strains were then tested for sensitivity to p-benzoquinone. As expected, introduction of *TDH3-PST3* into the $\Delta/\Delta/\Delta/\Delta$ mutant restored resistance to p-benzoquinone to wild-type levels (Fig. 6C). Interestingly, despite having the greatest amount of FLP produced, *TDH3-YCP4* could not restore wild-type levels of resistance in the $\Delta/\Delta/\Delta/\Delta$ mutant (Fig. 6C). *TDH3-PST2* was also unable to restore wild-

FIG 6 Overexpression of Pst1 or Pst3 restores resistance of $\Delta\Delta\Delta\Delta$ mutant to p-benzoquinone. (A) Quantification of Western blots determining relative level of each FLP in wild-type control and *TDH3* promoter-driven strains. Each FLP was normalized to 1 in the wild-type strain (blue bars). Fold increase in FLP level for the *TDH3* promoter-driven strains compared to the wild-type level is shown in red bars. Results represent averages from at least three independent experiments performed on different days. Error bars indicate SD, compared using multiple *t* tests: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. (B) Relative amount of each FLP produced under the control of *TDH3* promoter normalized to *TDH3-PST1* level. Results represent averages from three independent experiments performed on different days. Error bars indicate SD. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; all by one-way ANOVA. (C) Violin plot displaying quantification of halo assays comparing susceptibility of *TDH3* promoter-driven FLPs to 25 mM p-benzoquinone. Inset images show a sample of correlating assay from the bar graph above and are samples of three independent experiments, each done in duplicate. Error bars indicate SD, the dotted line indicates the median, and solid lines indicate 25th and 75th percentiles. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; all by one-way ANOVA.

type levels of resistance, even though the level of Pst2 was now at a level similar to that of Pst3. Interestingly, *TDH3-PST1* was able to restore resistance to p-benzoquinone to essentially the same level as *TDH3-PST3*, indicating that Pst1 can promote strong resistance to quinones when its level of production was increased (Fig. 6C). These results indicate that the function of the different FLPs is due in part to their levels of production and in part to their intrinsic properties.

Pst1 and Ycp4 protect against lipid peroxidation. The new strains with the FLP genes under the control of the constitutive *TDH3* promoter provided an opportunity to compare the ability of each FLP to protect against another type of oxidative stress caused by tert-butyl hydroperoxide (TBHP). To measure this, we exposed cells to TBHP and then assayed the extent of lipid peroxidation by staining *C. albicans* cells with diphenyl-1-pyrenylphosphine (DPPP) (37, 38). Following reaction with lipid peroxides, DPPP can be detected by fluorescence microscopy. All of the *C. albicans* strains we examined exhibited very low levels of staining with DPPP prior to treatment with TBHP (Fig. 7A). However, after treatment with 1.5 mM TBHP, all of the strains showed a significant increase in DPPP staining (Fig. 7B; summarized in Fig. 7C). Analysis of the $\Delta\Delta\Delta\Delta$ strain carrying an empty vector showed that it displayed a significant increase in lipid peroxidation compared to a wild-type control strain (Fig. 7B). Interestingly, the $\Delta\Delta\Delta\Delta$ strain containing *TDH3-PST1* showed even lower levels of DPPP staining than the wild-type control strain, indicating that Pst1 is proficient at protecting against lipid peroxidation. The $\Delta\Delta\Delta\Delta$ strain carrying *TDH3-YCP4* also showed a significantly reduced level of DPPP

FIG 7 Ability of FLPs to counteract lipid peroxidation induced by tert-butyl hydroperoxide (TBHP). The indicated cell types were incubated in the absence of oxidative stress (A) or with 1.5 mM TBHP for 1 h (B) and then stained with DPPP and imaged by fluorescence microscopy (lower) to reveal lipid peroxidation. (Upper) Cells were also imaged by light microscopy. (C) Quantification of DPPP staining. The results represent averages from three independent experiments performed in duplicate on different days. Error bars indicate SD. Significance of results was determined with *t* tests: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

staining, as the level of lipid peroxidation was similar to that of the wild-type control strain. In contrast, $\Delta/\Delta/\Delta/\Delta$ cells expressing either *PST2* or *PST3* under the control of the *TDH3* promoter did not show significantly reduced DPPP staining compared to the $\Delta/\Delta/\Delta/\Delta$ strain carrying an empty vector. Interestingly, the *TDH3-YCP4* strain was better able to resist the lipid peroxidation induced by TBHP (Fig. 7), but it did not show an increased ability to resist quinones (Fig. 6). In contrast, the *TDH3-PST3* strain was better at resisting quinones, but it was not significantly better at resisting the effects of TBHP. These data further indicate that the FLPs have distinct functions.

DISCUSSION

FLPs have an interesting history in that they were studied well biochemically before their physiological roles in virulence were understood. Originally discovered in 1993, the first FLP was isolated because it copurified from *E. coli* extracts with TrpR, which acts as a repressor of the Trp operon (39). This protein was named WrbA, for tryptophan (*W*) repressor binding protein (39). Analysis of the amino acid sequence of WrbA revealed that it shares the canonical β - α - β -fold of flavodoxins, with a twisted parallel

β -sheet and a flavin mononucleotide binding site (40–42). However, the WrbA β -sheet was found to contain a conserved insertion in strand β 5 that resulted in an additional $\alpha\beta$ -unit (40). This resulted in it being recognized as distinct from flavodoxins; therefore, it became the founding member of the flavodoxin-like proteins. Comparative genome studies led to the realization that the WrbA family of flavoproteins is homologous to several NAD(P)H:quinone oxidoreductases (43–48). WrbA and FLPs from plants, bacteria, and fungi were subsequently shown to catalyze the two-electron reduction of quinones (25, 49, 50). Studies in *C. albicans* were pioneering in showing that the FLPs are important for virulence in a mammalian host (21). Subsequent studies have shown that FLPs are important for the virulence of other organisms, including several species of fungi and bacteria (21, 51, 52).

FLPs promote resistance to diverse set of quinones. The $\Delta/\Delta/\Delta/\Delta$ mutant strain was more susceptible to five different types of quinones, indicating that FLPs can act on a broad range of quinones (Fig. 1). The ability of FLPs to promote resistance to CoQ₀, which consists of the quinone head group of ubiquinone, supports the proposed role of FLPs in reducing ubiquinone so that it can act as an antioxidant in the plasma membrane (21). The results for the other quinones are significant, since they contain side groups with very different structural moieties (Fig. 1A). These results imply that the active site of the FLPs is accessible enough to accommodate bulky side groups, such as methyl, methoxy, and tert-butyl groups, as well as additional ring structures. This conclusion is supported by analysis of the high-resolution structure of *E. coli* WrbA, which has been crystalized with FMN and NADH as well as FMN and p-benzoquinone (49). In these structures, p-benzoquinone was found to stack between the isoalloxazine ring of FMN and Trp-98 of a neighboring subunit of the WrbA tetramer (49). Within this pocket there appears to be sufficient room for the bulkier quinones, such as the naphthoquinones menadione and plumbagin. Note that FLPs are thought to act in a ping-pong mechanism such that the NAD(P)H donates electrons and then leaves before a quinone enters the active site to receive electrons and is reduced, so it is not required that FMN, NAD(P)H, and a quinone all bind at the same time (29, 50). Similar to *C. albicans*, *S. cerevisiae* also contains multiple FLPs (Pst2, Rfs1, and Ycp4), of which ScPst2 and ScYcp4 have been implicated in promoting resistance to oxidative stress (50, 53–58). ScPst2 was recently crystalized and found to share structural similarity and conservation of amino acids around the FMN binding site (50). This suggests that a similar active site exists for *C. albicans* FLPs.

Differential production and function of FLPs. Quantitative analysis of homozygous deletion strains lacking one of the FLP genes showed that they had different levels of susceptibility to quinones (Fig. 2). In particular, the *pst3* Δ mutant was the most sensitive to the different quinones, including p-benzoquinone and menadione. This contrasts with results reported for an *S. cerevisiae* *pst2* Δ mutant, which was more sensitive to p-benzoquinone but was not more susceptible to menadione (50). To determine why the *pst3* Δ mutant had a stronger phenotype than the other FLP mutants, we asked whether the quantity (abundance) or quality (function) of Pst3 was responsible. Interestingly, we found that the FLPs are not produced at the same basal level. Ycp4 and Pst3 are the most abundant under standard culture conditions. Ycp4 was produced at a slightly higher level than Pst3, and both were produced at 6.8- to 12.9-fold higher levels than Pst1 and Pst2 (Fig. 4). The *PST3* and *YCP4* genes are divergently transcribed, which is common in fungal genomes, suggesting some level of coregulation accounts for their similar abundance (59, 60). Consistent with this, RNA-seq indicates that the mRNAs for *PST3* and *YCP4* are more abundant than those for *PST1* and *PST2* (22, 36). However, given that Ycp4 was more abundant than Pst3, the different basal levels of protein production do not account for the different phenotypes of the *pst3* Δ and *ycp4* Δ mutants.

The analysis of protein levels after exposure to p-benzoquinone showed that all of the FLPs were induced, although Pst1 and Pst3 were the most highly induced. Pst3 was induced 8.4-fold (Fig. 5), and since it had a relatively high basal level, Pst3 was the most abundant under these conditions, which likely contributes to its strong role in

promoting quinone resistance. Therefore, to determine if the functional properties (quality) of the protein play a role, we expressed each gene under the control of the constitutive *TDH3* promoter in the $\Delta/\Delta/\Delta/\Delta$ mutant background to determine the relative sensitivity of the strains when the different FLPs were present at similar levels (Fig. 6). Western blot analysis confirmed that the strains produced similar levels of the different FLPs, except that Ycp4 was slightly more abundant. Interestingly, the expression of either *PST1* or *PST3* under the control of the *TDH3* promoter restored resistance to p-benzoquinone to wild-type levels (Fig. 6). This suggests that Pst1 and Pst3 have a similar functional ability to detoxify quinones, although Pst1 typically has less of an effect in cells because it is produced at lower levels than Pst3. In contrast, expressing *PST2* or *YCP4* under the control of the *TDH3* promoter did not restore quinone resistance to the $\Delta/\Delta/\Delta/\Delta$ mutant strain, indicating that these FLPs are functionally distinct from Pst1 and Pst3. However, *YCP4* was functional when expressed from the *TDH3* promoter, as the *TDH3-PST1* and *TDH3-YCP4* strains were better able to promote resistance to oxidative stress caused by TBHP than the *TDH3-PST2* and *TDH3-PST3* strains (Fig. 7). Altogether, these data indicate that the FLPs have distinct functional properties.

Potential of FLPs to act as targets for novel antifungal drugs. New therapeutic approaches are needed to treat systemic candidiasis, as the mortality rate is about 40% (61, 62). The observation that the $\Delta/\Delta/\Delta/\Delta$ mutant was avirulent in a mouse model of systemic infection indicates that FLPs represent potential drug targets (21). One advantage of targeting FLPs is that humans do not have close orthologs. Instead, they use divergent types of quinone reductase, Nqo1 and Nqo2, which lack obvious amino acid similarity with FLPs. Nqo1 and Nqo2 are also distinct from FLPs in that they use FAD as a cofactor instead of FMN, and they form dimers rather than tetramers (29). Our analysis indicates that although Pst3 plays the most important role, it will be important to target all four FLPs to fully block quinone reductase activity. Since this could lead to some challenges, another interesting possibility is to identify quinones that are only toxic after they are reduced by the FLPs. This strategy of using bioactivatable drugs has been explored in cancer chemotherapy to take advantage of the fact that many cancer cells overexpress *NQO1* (63, 64). Quinone compounds have been identified that preferentially kill cancer cells after reduction by Nqo1 into a toxic form (65, 66). Similarly, Nqo1-mediated reduction of benzoquinone-containing ansamycin drugs makes them more potent inhibitors of the Hsp90 chaperone (67). Analogous drugs activated by FLPs could be useful, since Hsp90 inhibitors prevent the emergence of drug resistance in *C. albicans* (68). Another advantage is that drug resistance should not occur through the loss of FLP function, since the cells would then be more susceptible to oxidative stress. Since the FLPs are highly conserved in fungi, the important roles of FLPs in promoting resistance to quinones and oxidative stress provide novel opportunities for new therapeutic strategies aimed at combating infections by *C. albicans* and other fungal pathogens.

MATERIALS AND METHODS

Strains and media. The *C. albicans* strains used in this study appear in Table 1. Cultures were grown in rich YPD medium (2% dextrose, 1% peptone, 2% yeast extract, 80 mg/liter uridine) or synthetic medium (yeast nitrogen base, 2% dextrose) (69). When required, synthetic medium was supplemented with amino acids and uridine. All strains used in this study are fully prototrophic (Table 1). *TDH3* promoter-driven strains were constructed in LLF060A using homologous recombination and ligation cloning as follows. The vector pDDB57 (70) was digested with *Apal* and *SacII*. The *TDH3* promoter was amplified (primer set 1) (Table 2) using a forward primer containing 47 bp of homology upstream of the *SacII* cut site in pDDB57. The open reading frame and 500 bp of accompanying 3'-flanking region were PCR amplified using a forward primer containing 50 bp of homology to the *TDH3* promoter and a reverse primer with 50 bp of homology with pDDB57 (primer sets 2 to 5) (Table 2). Linearized pDDB57, the *TDH3* promoter, and FLP open reading frame/3'-flanking region fragments were then cotransformed into *Saccharomyces cerevisiae* strain BY4741 for homologous recombination to take place. Resulting plasmids were extracted for each FLP and used for subsequent steps. Additional restriction sites were added upstream of the *TDH3* promoter and downstream of the 3'-flanking region of each FLP using PCR. Vectors were digested and newly purified fragments ligated into linearized Clp10-SAT (71). Strains were generated by transforming the resulting *StuI*-digested constructs into LLF060A and selecting with 200 mg/ml nourseothricin (NAT) (71).

TABLE 1 *C. albicans* strains

<i>C. albicans</i> strain	Parent	Genotype
LLF100A	SN152	(Prototrophic wild-type control) <i>arg4Δ/ARG4 leu2Δ/LEU2 his1Δ/HIS1 URA3/ura3Δ::imm IRO1/iro1Δ::imm</i>
LLF052	SN152	(<i>pst1Δ pst1Δ::HIS1/pst1Δ::LEU2 ARG4/arg4Δ</i>
LLF059	SN152	(<i>pst2Δ pst2Δ::HIS1/pst2Δ::LEU2 ARG4/arg4Δ</i>
LLF036	SN152	(<i>pst3Δ pst3Δ::HIS1/pst3Δ::LEU2 ARG4/arg4Δ</i>
LLF037	SN152	(<i>ycp4Δ ycp4Δ::HIS1/ycp4Δ::LEU2 ARG4/arg4Δ</i>
LLF060A	LLF054	($\Delta/\Delta/\Delta/\Delta$) <i>pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt ARG4/arg4Δ</i>
LLF064	LLF054	(+ <i>PST1</i>) <i>pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt PST1::ARG4 arg4Δ/arg4Δ</i>
LLF081	LLF054	(+ <i>PST2</i>) <i>pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt PST2::ARG4 arg4Δ/arg4Δ</i>
LLF066	LLF054	(+ <i>PST3</i>) <i>pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt PST3::ARG4 arg4Δ/arg4Δ</i>
LLF082	LLF054	(+ <i>YCP4</i>) <i>pst3-ycp4Δ::HIS1/pst3-ycp4Δ::LEU2 pst2Δ::frt/pst2Δ::frt pst1Δ::frt/pst1Δ::frt YCP4::ARG4 arg4Δ/arg4Δ</i>
JF365	LLF060A	(Empty vector) <i>rps1::Clp10-SAT1/RPS1</i>
JF379	LLF060A	(+ <i>TDH3p-PST1</i>) <i>rps1::TDH3-PST1-Clp10-SAT1/RPS1</i>
JF399	LLF060A	(+ <i>TDH3p-PST2</i>) <i>rps1::TDH3-PST2-Clp10-SAT1/RPS1</i>
JF355	LLF060A	(+ <i>TDH3p-PST3</i>) <i>rps1::TDH3-PST3-Clp10-SAT1/RPS1</i>
JF422	LLF060A	(+ <i>TDH3p-YCP4</i>) <i>rps1::TDH3-YCP4-Clp10-SAT1/RPS1</i>

Production of polyclonal anti-FLP antibodies. Purified polyclonal antibodies were raised in rabbits against each FLP by GenScript (Piscataway, NJ). Proteins for use as immunogens were produced by expression in bacteria. The coding regions for the FLPs that were expressed included Pst1 amino acids 1 to 198, Pst2 amino acids 1 to 201, Pst3 amino acids 1 to 199, and Ycp4 amino acids 1 to 200. These regions were subcloned into pET28a, resulting in a 6×His-tagged version of each FLP in the plasmids pET28a_PST1, pET28a_PST2, pET28a_PST3, and pET28a_YCP4. Rabbits were immunized (two per FLP), and then affinity-purified antisera were obtained. After screening the antibody samples by Western blotting at 1 mg/ml, the antibody preparation yielding the lowest level of background (i.e., fewest nonspecific bands) was chosen for use in subsequent experiments.

Bacterially expressed FLPs. The open reading frames for the four FLP genes were codon optimized for expression in *E. coli* and cloned into bacterial expression vector pET28a(+) at the NdeI and XhoI sites. Each plasmid was then transformed into *E. coli* strain BL21(DE3) for bacterial expression. A negative-control strain carrying the empty pET28a(+) vector in BL21(DE3) cells was included in parallel in all induction experiments. A single colony of bacteria was used to inoculate 5 ml of LB supplemented with 50 μg/ml kanamycin and incubated overnight at 37°C. The next day, the culture was diluted and grown with shaking at 37°C until an optical density at 600 nm (OD_{600}) of 0.6 was obtained (about 1.5 h). Uninduced samples (1 ml each) were removed and prepared for analysis on an SDS-PAGE gel. The remaining culture was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and then the culture was incubated with shaking at 37°C for 30 min. Samples were removed for analysis of whole-cell lysate and prepared for loading on an SDS-PAGE gel. Larger cell pellets were prepared for protein purification, lysed, passed through a nickel-nitrilotriacetic acid column, and washed, and purified proteins were eluted using imidazole containing elution buffer. The amount of protein obtained was quantified using a Smith/bicinchoninic acid (BCA) assay (Pierce BCA protein assay kit; ThermoFisher Scientific, Waltham, MA).

FLP protein analysis. *C. albicans* strains were grown overnight in YPD at 30°C on a tube roller. Cells were then diluted 1:100 in 20 ml of YPD and grown with shaking until a density of 1×10^7 cells/ml was

TABLE 2 Primers used

Primer name	Primer pair	Sequence
pDDBTDH3pFw	1	GGTAATTATTACTATTACAATCAAAGGTGGTCTCTAGACCGCGGGTGTCTCCTCGTCGACAACGACTGC
TDH3pRev	1	TGTTAATTAATTTGATTGTAAGGTTTG
TDH3pPST1ORFFw	2	GAATTCAAATCAATTAACATCAACAACTTTACAATCAAATTAATTAACAATGGCACAAGGAAAAAGT
pDDBPST1UTRRev	2	AGCTCGGAATTAACCTCACTAAAGGGAACAAAAGCTGGGTACCGGGCCCTCATTATCTCAATGTAT
TDH3pPST2ORFFw	3	GAATTCAAATCAATTAACATCAACAACTTTACAATCAAATTAATTAACAATGTCTAAACCAAGAGT
pDDBPST2UTRRev	3	AGCTCGGAATTAACCTCACTAAAGGGAACAAAAGCTGGGTACCGGGCCCGCATTAATAATTTCTCC
TDH3pPST3ORFFw	4	GAATTCAAATCAATTAACATCAACAACTTTACAATCAAATTAATTAACAATGGCTCCAAAAGTTGC
pDDBPST3UTRRev	4	AGCTCGGAATTAACCTCACTAAAGGGAACAAAAGCTGGGTACCGGGCCCGCACTTGACTACGGTTG
TDH3pYCP44ORFFw	5	GAATTCAAATCAATTAACATCAACAACTTTACAATCAAATTAATTAACAATGAAGATCGCCATTATC
pDDBYCP4UTRRev	5	AGCTCGGAATTAACCTCACTAAAGGGAACAAAAGCTGGGTACCGGGCCCTAAGGGCTTCCCGAATT
MluIPST1UTRRev		GTATCGATACGCGTGATTCATTATCTCAATGTAT
ApaITDH3pFw		CATAATGGGCCCCGATGTTGCTCCTCGTCGACAACG
MluIPST2UTRRev		GTATCGATACGCGTGATGATATTAAATATTCTCC
EcoRVTDH3pFw		GTAGCGATATCGATGTTGCTCCTCGTCGACAACG
MluIPST3UTRRev		GTATCGATACGCGTGATGCACTTGACTACGGTTG
MluIYCP4UTRRev		GTATCACGCGTGATTAAGGGCTTCCCGAATT

achieved. Samples exposed to oxidative stress were supplemented with 10 μ l of 100 mM p-benzoquinone for 1 h prior to harvesting. Cells were harvested by spinning at 2,000 rpm for 5 min, washed, and divided into thirds. One sample of each strain was lysed using 1 $\times$ Laemmli buffer (2% SDS, 10% glycerol, 125 mM Tris-HCl, pH 6.8, and 0.002% bromophenol blue) and zirconia beads by 4 rounds of bead beating. 2-Mercaptoethanol was added to a 5% final concentration, and then the samples were boiled at 100°C for 10 min. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose using a semidry transfer apparatus. Blots were probed with the appropriate primary rabbit antiserum (anti-Pst1, anti-Pst2, anti-Pst3, or anti-Ycp4) at 1 μ g/ml in TBS-T buffer (0.1% Tween 20, 2% [wt/vol] bovine serum albumin [BSA], and 0.2% [wt/vol] sodium azide) for 12 h. For blots using actin as a loading control, an anti-actin C4 monoclonal antibody was added to each primary antibody mixture at a 1:500 dilution prior to the 12 h of incubation. The blots were then washed and incubated with an IRDye 680-conjugated anti-rabbit IgG secondary antibody (LI-COR Biosciences, Lincoln, NE) diluted 1:20,000 in TBS containing 0.3% Tween 20 for FLPs alone or an IRDye 680-conjugated anti-rabbit IgG/IRDye 800-conjugated anti-mouse IgG mixture for blots visualizing both FLPs and actin. Blots were washed using TBS-T and visualized by scanning with an Odyssey CLx infrared imaging system (LI-COR Biosciences). The resulting images were analyzed using Image Studio software (LI-COR Biosciences). For Coomassie-stained gels, SDS-PAGE was performed as described above, and then gels were stained in a Coomassie brilliant blue solution (0.1% Coomassie R-250, 40% ethanol, 10% glacial acetic acid) overnight. Gels were destained in water and analyzed using Image Studio software (LI-COR Biosciences). For FLP protein quantification, Western blots were performed using known amounts of bacterially expressed FLPs run in parallel with *C. albicans* lysates. Signal intensities obtained by analysis with Image Studio software (LI-COR Biosciences) were used to generate standard curves against which the signals obtained from *C. albicans* whole-cell lysates were compared. All signals were determined to be within the linear range of each standard curve.

Assays for sensitivity to quinones. *C. albicans* strains were grown overnight in YPD at 30°C with rotation. Cells were diluted to 1.0×10^6 cells/ml in YPD, and 250 μ l was spread onto the surface of a synthetic complete medium agar plate. Dilution series for each compound of interest were prepared, and 10 μ l of each was applied to individual paper filter disks (Becton, Dickinson and Company, Sparks, MD). Disks containing each compound were applied to the surface of plates containing the indicated strains. After incubation at 30°C for 48 h, the diameters of the zones of growth inhibition (halos) were measured. Assays testing susceptibility of menadione were screened similarly, but YPD plates were used in place of synthetic complete medium. Drugs tested included p-benzoquinone (BZQ; Sigma-Aldrich, St. Louis, MO), menadione (MND; Sigma-Aldrich, St. Louis, MO), 2-tert-butyl-1,4-benzoquinone (TBBQ; Cayman Chemical, Ann Arbor, MI), 2,3-dimethoxy-5-methyl-p-benzoquinone (coenzyme Q₉; Cayman Chemical Ann Arbor, MI), and plumbagin (Santa Cruz Biotechnology, Dallas, TX).

Quantification of lipid peroxidation. *C. albicans* cells were grown to log phase in YPD. Cells were treated with or without 1.5 mM tert-butyl hydroperoxide (TBHP; ThermoFisher, Waltham, MA) for 1 h. Suspensions were incubated with 10 μ M DPPP (diphenyl-1-pyrenylphosphine; Cayman Chemical Ann Arbor, MI) for 10 min at 30°C, essentially as described previously (37, 38). Samples were imaged using a Zeiss Axio Imager.Z2 fluorescence microscope and Zeiss AxioCam 702 camera with excitation filter at 330 ± 40 nm and an emission filter at 410 ± 10 nm. Fluorescence intensity was quantified for 100 cells per cell strain per experiment were using Zeiss Zen 3.0 software.

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REFERENCES

- Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. *Sci Transl Med* 4:165rv13. <https://doi.org/10.1126/scitranslmed.3004404>.
- Kullberg BJ, Arendrup MC. 2015. Invasive candidiasis. *N Engl J Med* 373:1445–1456. <https://doi.org/10.1056/NEJMra1315399>.
- Brown AJ, Budge S, Kaloriti D, Tillmann A, Jacobsen MD, Yin Z, Ene IV, Bohovych I, Sandai D, Kastora S, Potrykus J, Ballou ER, Childers DS, Shahana S, Leach MD. 2014. Stress adaptation in a pathogenic fungus. *J Exp Biol* 217:144–155. <https://doi.org/10.1242/jeb.088930>.
- Foderaro JE, Douglas LM, Konopka JB. 2017. MCC/eisosomes regulate cell wall synthesis and stress responses in fungi. *J Fungi* 3:61. <https://doi.org/10.3390/jof3040061>.
- Bogdan C, Rollinghoff M, Diefenbach A. 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol* 12:64–76. [https://doi.org/10.1016/s0952-7915\(99\)00052-7](https://doi.org/10.1016/s0952-7915(99)00052-7).
- Nathan C, Shiloh MU. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A* 97:8841–8848. <https://doi.org/10.1073/pnas.97.16.8841>.
- Brown AJ, Haynes K, Quinn J. 2009. Nitrosative and oxidative stress responses in fungal pathogenicity. *Curr Opin Microbiol* 12:384–391. <https://doi.org/10.1016/j.mib.2009.06.007>.
- Imlay JA. 2013. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat Rev Microbiol* 11:443–454. <https://doi.org/10.1038/nrmicro3032>.
- Pradhan A, Herrero-de-Dios C, Belmonte R, Budge S, Lopez Garcia A, Kolmogorova A, Lee KK, Martin BD, Ribeiro A, Bebes A, Yucel R, Gow

- NAR, Munro CA, MacCallum DM, Quinn J, Brown AJP. 2017. Elevated catalase expression in a fungal pathogen is a double-edged sword of iron. *PLoS Pathog* 13:e1006405. <https://doi.org/10.1371/journal.ppat.1006405>.
10. Chauhan N, Latge JP, Calderone R. 2006. Signalling and oxidant adaptation in *Candida albicans* and *Aspergillus fumigatus*. *Nat Rev Microbiol* 4:435–444. <https://doi.org/10.1038/nrmicro1426>.
 11. Dantas ADS, Day A, Ikeh M, Kos I, Achan B, Quinn J. 2015. Oxidative stress responses in the human fungal pathogen, *Candida albicans*. *Biomolecules* 5:142–165. <https://doi.org/10.3390/biom5010142>.
 12. Lanze CE, Gandra RM, Foderaro JE, Swenson KA, Douglas LM, Konopka JB. 2020. Plasma membrane MCC/eisosome domains promote stress resistance in fungi. *Microbiol Mol Biol Rev* 84:e00063-19. <https://doi.org/10.1128/MMBR.00063-19>.
 13. Douglas LM, Konopka JB. 2016. Plasma membrane organization promotes virulence of the human fungal pathogen *Candida albicans*. *J Microbiol* 54:178–191. <https://doi.org/10.1007/s12275-016-5621-y>.
 14. Catala A. 2010. A synopsis of the process of lipid peroxidation since the discovery of the essential fatty acids. *Biochem Biophys Res Commun* 399:318–323. <https://doi.org/10.1016/j.bbrc.2010.07.087>.
 15. Yin H, Xu L, Porter NA. 2011. Free radical lipid peroxidation: mechanisms and analysis. *Chem Rev* 111:5944–5972. <https://doi.org/10.1021/cr200084z>.
 16. Morre DJ, Morre DM. 2011. Non-mitochondrial coenzyme Q. *Biofactors* 37:355–360. <https://doi.org/10.1002/biof.156>.
 17. Becucci L, Scaletti F, Guidelli R. 2011. Gel-phase microdomains and lipid rafts in monolayers affect the redox properties of ubiquinone-10. *Biophys J* 101:134–143. <https://doi.org/10.1016/j.bpj.2011.05.051>.
 18. Pobezhimova TP, Voinikov VK. 2000. Biochemical and physiological aspects of ubiquinone function. *Membr Cell* 13:595–602.
 19. Do TQ, Schultz JR, Clarke CF. 1996. Enhanced sensitivity of ubiquinone-deficient mutants of *Saccharomyces cerevisiae* to products of autoxidized polyunsaturated fatty acids. *Proc Natl Acad Sci U S A* 93:7534–7539. <https://doi.org/10.1073/pnas.93.15.7534>.
 20. Turunen M, Olsson J, Dallner G. 2004. Metabolism and function of coenzyme Q. *Biochim Biophys Acta* 1660:171–199. <https://doi.org/10.1016/j.bbame.2003.11.012>.
 21. Li L, Naseem S, Sharma S, Konopka JB. 2015. Flavodoxin-like proteins protect *Candida albicans* from oxidative stress and promote virulence. *PLoS Pathog* 11:e1005147. <https://doi.org/10.1371/journal.ppat.1005147>.
 22. Bruno VM, Wang Z, Marjani SL, Euskirchen GM, Martin J, Sherlock G, Snyder M. 2010. Comprehensive annotation of the transcriptome of the human fungal pathogen *Candida albicans* using RNA-seq. *Genome Res* 20:1451–1458. <https://doi.org/10.1101/gr.109553.110>.
 23. Wang Y, Cao YY, Jia XM, Cao YB, Gao PH, Fu XP, Ying K, Chen WS, Jiang YY. 2006. Cap1p is involved in multiple pathways of oxidative stress response in *Candida albicans*. *Free Radic Biol Med* 40:1201–1209. <https://doi.org/10.1016/j.freeradbiomed.2005.11.019>.
 24. Gil-Bona A, Parra-Giraldo CM, Hernaez ML, Reales-Calderon JA, Solis NV, Filler SG, Monteoliva L, Gil C. 2015. *Candida albicans* cell shaving uncovers new proteins involved in cell wall integrity, yeast to hypha transition, stress response and host-pathogen interaction. *J Proteomics* 127:340–351. <https://doi.org/10.1016/j.jprot.2015.06.006>.
 25. Patridge EV, Ferry JG. 2006. WrbA from *Escherichia coli* and *Archaeoglobus fulgidus* is an NAD(P)H:quinone oxidoreductase. *J Bacteriol* 188:3498–3506. <https://doi.org/10.1128/JB.188.10.3498-3506.2006>.
 26. Futuro DO, Ferreira PG, Nicoletti CD, Borba-Santos LP, Silva FCD, Rozental S, Ferreira VF. 2018. The antifungal activity of naphthoquinones: an integrative review. *Ann Acad Bras Cienc* 90:1187–1214. <https://doi.org/10.1590/0001-3765201820170815>.
 27. Ooi N, Chopra I, Eady A, Cove J, Bojar R, O'Neill AJ. 2013. Antibacterial activity and mode of action of tert-butylhydroquinone (TBHQ) and its oxidation product, tert-butylbenzoquinone (TBBQ). *J Antimicrob Chemother* 68:1297–1304. <https://doi.org/10.1093/jac/dkt030>.
 28. Nair SV, Baranwal G, Chatterjee M, Sachu A, Vasudevan AK, Bose C, Banerji A, Biswas R. 2016. Antimicrobial activity of plumbagin, a naturally occurring naphthoquinone from *Plumbago rosea*, against *Staphylococcus aureus* and *Candida albicans*. *Int J Med Microbiol* 306:237–248. <https://doi.org/10.1016/j.ijmm.2016.05.004>.
 29. Carey J, Brynda J, Wolfova J, Grandori R, Gustavsson T, Ettrich R, Smatanova IK. 2007. WrbA bridges bacterial flavodoxins and eukaryotic NAD(P)H:quinone oxidoreductases. *Protein Sci* 16:2301–2305. <https://doi.org/10.1110/ps.073018907>.
 30. Abraham I, Joshi R, Pardasani P, Pardasani RT. 2011. Recent advances in 1,4-benzoquinone chemistry. *J Braz Chem Soc* 22:385–421. <https://doi.org/10.1590/S0103-50532011000300002>.
 31. Zalomaeva OV, Evtushok VY, Maksimov GM, Maksimovskaya RI, Kholdeeva OA. 2017. Synthesis of coenzyme Q0 through divanadium-catalyzed oxidation of 3,4,5-trimethoxytoluene with hydrogen peroxide. *Dalton Trans* 46:5202–5209. <https://doi.org/10.1039/c7dt00552k>.
 32. Adam W, Herrmann WA, Lin J, Saha-Moeller CR. 1994. Catalytic oxidation of phenols to p-quinones with the hydrogen peroxide and methyltrioxorhenium (VII) system. *J Org Chem* 59:8281–8283. <https://doi.org/10.1021/jo00105a058>.
 33. Ooi N, Eady EA, Cove JH, O'Neill AJ. 2016. Tert-butyl benzoquinone: mechanism of biofilm eradication and potential for use as a topical antibiofilm agent. *J Antimicrob Chemother* 71:1841–1844. <https://doi.org/10.1093/jac/dkw100>.
 34. EFSA. 2014. Scientific opinion on the safety and efficacy of vitamin K3 (menadione sodium bisulphite and menadione nicotinamide bisulphite) as a feed additive for all animal species. *EFSA J* 12:3532. <https://doi.org/10.2903/j.efsa.2014.3532>.
 35. Tripathi SK, Panda M, Biswal BK. 2019. Emerging role of plumbagin: cytotoxic potential and pharmaceutical relevance towards cancer therapy. *Food Chem Toxicol* 125:566–582. <https://doi.org/10.1016/j.fct.2019.01.018>.
 36. Naseem S, Min K, Spitzer D, Gardin J, Konopka JB. 2017. Regulation of hyphal growth and N-acetylglucosamine catabolism by two transcription factors in *Candida albicans*. *Genetics* 206:299–314. <https://doi.org/10.1534/genetics.117.201491>.
 37. Takahashi M, Shibata M, Niki E. 2001. Estimation of lipid peroxidation of live cells using a fluorescent probe, diphenyl-1-pyrenylphosphine. *Free Radic Biol Med* 31:164–174. [https://doi.org/10.1016/S0891-5849\(01\)00575-5](https://doi.org/10.1016/S0891-5849(01)00575-5).
 38. Sun L, Liao K, Hang C, Wang D. 2017. Honokiol induces reactive oxygen species-mediated apoptosis in *Candida albicans* through mitochondrial dysfunction. *PLoS One* 12:e0172228. <https://doi.org/10.1371/journal.pone.0172228>.
 39. Yang W, Ni L, Somerville RL. 1993. A stationary-phase protein of *Escherichia coli* that affects the mode of association between the trp repressor protein and operator-bearing DNA. *Proc Natl Acad Sci U S A* 90:5796–5800. <https://doi.org/10.1073/pnas.90.12.5796>.
 40. Grandori R, Carey J. 1994. Six new candidate members of the alpha/beta twisted open-sheet family detected by sequence similarity to flavodoxin. *Protein Sci* 3:2185–2193. <https://doi.org/10.1002/pro.5560031204>.
 41. Grandori R, Khalifah P, Boice JA, Fairman R, Giovanielli K, Carey J. 1998. Biochemical characterization of WrbA, founding member of a new family of multimeric flavodoxin-like proteins. *J Biol Chem* 273:20960–20966. <https://doi.org/10.1074/jbc.273.33.20960>.
 42. Sancho J. 2006. Flavodoxins: sequence, folding, binding, function and beyond. *Cell Mol Life Sci* 63:855–864. <https://doi.org/10.1007/s00018-005-5514-4>.
 43. Laskowski MJ, Dreher KA, Gehring MA, Abel S, Gensler AL, Sussex IM. 2002. FQR1, a novel primary auxin-response gene, encodes a flavin mononucleotide-binding quinone reductase. *Plant Physiol* 128:578–590. <https://doi.org/10.1104/pp.010581>.
 44. Jensen KA, Jr, Ryan ZC, Vanden Wymelenberg A, Cullen D, Hammel KE. 2002. An NADH:quinone oxidoreductase active during biodegradation by the brown-rot basidiomycete *Gloeophyllum trabeum*. *Appl Environ Microbiol* 68:2699–2703. <https://doi.org/10.1128/aem.68.6.2699-2703.2002>.
 45. Cohen R, Suzuki MR, Hammel KE. 2004. Differential stress-induced regulation of two quinone reductases in the brown rot basidiomycete *Gloeophyllum trabeum*. *Appl Environ Microbiol* 70:324–331. <https://doi.org/10.1128/aem.70.1.324-331.2004>.
 46. Brock BJ, Gold MH. 1996. 1,4-Benzoquinone reductase from basidiomycete *Phanerochaete chrysosporium*: spectral and kinetic analysis. *Arch Biochem Biophys* 331:31–40. <https://doi.org/10.1006/abbi.1996.0279>.
 47. Brock BJ, Rieble S, Gold MH. 1995. Purification and characterization of a 1,4-benzoquinone reductase from the basidiomycete *Phanerochaete chrysosporium*. *Appl Environ Microbiol* 61:3076–3081. <https://doi.org/10.1128/AEM.61.8.3076-3081.1995>.
 48. Wrobel RL, Matvienko M, Yoder JL. 2002. Heterologous expression and biochemical characterization of an NAD(P)H:quinone oxidoreductase from the hemiparasitic plant *Triphysaria versicolor*. *Plant Physiol Biochem* 40:265–272. [https://doi.org/10.1016/S0981-9428\(02\)01361-X](https://doi.org/10.1016/S0981-9428(02)01361-X).
 49. Andrade SL, Patridge EV, Ferry JG, Einsle O. 2007. Crystal structure of the NADH:quinone oxidoreductase WrbA from *Escherichia coli*. *J Bacteriol* 189:9101–9107. <https://doi.org/10.1128/JB.01336-07>.
 50. Koch K, Hromic A, Sorokina M, Strandback E, Reisinger M, Gruber K, Macheroux P. 2017. Structure, biochemical and kinetic properties of recombinant Pst2p from *Saccharomyces cerevisiae*, a FMN-dependent

- NAD(P)H:quinone oxidoreductase. *Biochim Biophys Acta Proteins Proteom* 1865:1046–1056. <https://doi.org/10.1016/j.bbapap.2017.05.005>.
51. Chen Y, Le X, Sun Y, Li M, Zhang H, Tan X, Zhang D, Liu Y, Zhang Z. 2017. MoYcp4 is required for growth, conidiogenesis and pathogenicity in *Magnaporthe oryzae*. *Mol Plant Pathol* 18:1001–1011. <https://doi.org/10.1111/mpp.12455>.
 52. Shimizu T, Hirai S, Yokoyama E, Ichimura K, Noda M. 2015. An evolutionary analysis of nitric oxide reductase gene *norV* in enterohemorrhagic *Escherichia coli* O157. *Infect Genet Evol* 33:176–181. <https://doi.org/10.1016/j.meegid.2015.04.027>.
 53. Cardona F, Orozco H, Friant S, Aranda A, del Olmo M. 2011. The *Saccharomyces cerevisiae* flavodoxin-like proteins Ycp4 and Rfs1 play a role in stress response and in the regulation of genes related to metabolism. *Arch Microbiol* 193:515–525. <https://doi.org/10.1007/s00203-011-0696-7>.
 54. Grossmann G, Malinsky J, Stahlschmidt W, Loibl M, Weig-Meckl I, Frommer WB, Opekarova M, Tanner W. 2008. Plasma membrane microdomains regulate turnover of transport proteins in yeast. *J Cell Biol* 183:1075–1088. <https://doi.org/10.1083/jcb.200806035>.
 55. Kim Y, Chay KO, Kim I, Song YB, Kim TY, Han SJ, Ahn Y, Cho SH, Hoe KL, Ahn BW, Huh WK, Lee SR. 2011. Redox regulation of the tumor suppressor PTEN by glutaredoxin 5 and Ycp4. *Biochem Biophys Res Commun* 407:175–180. <https://doi.org/10.1016/j.bbrc.2011.02.133>.
 56. North M, Tandon VJ, Thomas R, Loguinov A, Gerlovina I, Hubbard AE, Zhang L, Smith MT, Vulpe CD. 2011. Genome-wide functional profiling reveals genes required for tolerance to benzene metabolites in yeast. *PLoS One* 6:e24205. <https://doi.org/10.1371/journal.pone.0024205>.
 57. Delom F, Szponarski W, Sommerer N, Boyer JC, Bruneau JM, Rossignol M, Gibrat R. 2006. The plasma membrane proteome of *Saccharomyces cerevisiae* and its response to the antifungal calcofluor. *Proteomics* 6:3029–3039. <https://doi.org/10.1002/pmic.200500762>.
 58. Pedroso N, Gomes-Alves P, Marinho HS, Brito VB, Boada C, Antunes F, Herrero E, Penque D, Cyrne L. 2012. The plasma membrane-enriched fraction proteome response during adaptation to hydrogen peroxide in *Saccharomyces cerevisiae*. *Free Radic Res* 46:1267–1279. <https://doi.org/10.3109/10715762.2012.704997>.
 59. Seila AC, Core LJ, Lis JT, Sharp PA. 2009. Divergent transcription: a new feature of active promoters. *Cell Cycle* 8:2557–2564. <https://doi.org/10.4161/cc.8.16.9305>.
 60. Xu Z, Wei W, Gagneur J, Perocchi F, Clauder-Munster S, Camblong J, Guffanti E, Stutz F, Huber W, Steinmetz LM. 2009. Bidirectional promoters generate pervasive transcription in yeast. *Nature* 457:1033–1037. <https://doi.org/10.1038/nature07728>.
 61. Brown GD, Denning DW, Levitz SM. 2012. Tackling human fungal infections. *Science* 336:647. <https://doi.org/10.1126/science.1222236>.
 62. Pfaller MA, Diekema DJ. 2010. Epidemiology of invasive mycoses in North America. *Crit Rev Microbiol* 36:1–53. <https://doi.org/10.3109/10408410903241444>.
 63. Starcher CL, Pay SL, Singh N, Yeh IJ, Bhandare SB, Su X, Huang X, Bey EA, Motea EA, Boothman DA. 2020. Targeting base excision repair in cancer: NQO1-bioactivatable drugs improve tumor selectivity and reduce treatment toxicity through radiosensitization of human cancer. *Front Oncol* 10:1575. <https://doi.org/10.3389/fonc.2020.01575>.
 64. Ma X, Moore ZR, Huang G, Huang X, Boothman DA, Gao J. 2015. Nanotechnology-enabled delivery of NQO1 bioactivatable drugs. *J Drug Target* 23:672–680. <https://doi.org/10.3109/1061186X.2015.1073296>.
 65. Dinkova-Kostova AT, Talalay P. 2010. NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. *Arch Biochem Biophys* 501:116–123. <https://doi.org/10.1016/j.abb.2010.03.019>.
 66. Siegel D, Yan C, Ross D. 2012. NAD(P)H:quinone oxidoreductase 1 (NQO1) in the sensitivity and resistance to antitumor quinones. *Biochem Pharmacol* 83:1033–1040. <https://doi.org/10.1016/j.bcp.2011.12.017>.
 67. Guo W, Reigan P, Siegel D, Zirrollo J, Gustafson D, Ross D. 2006. The bioreduction of a series of benzoquinone ansamycins by NAD(P)H:quinone oxidoreductase 1 to more potent heat shock protein 90 inhibitors, the hydroquinone ansamycins. *Mol Pharmacol* 70:1194–1203. <https://doi.org/10.1124/mol.106.025643>.
 68. Hill JA, Ammar R, Torti D, Nislow C, Cowen LE. 2013. Genetic and genomic architecture of the evolution of resistance to antifungal drug combinations. *PLoS Genet* 9:e1003390. <https://doi.org/10.1371/journal.pgen.1003390>.
 69. Sherman F. 2002. Getting started with yeast. *Methods Enzymol* 350:3–41. [https://doi.org/10.1016/S0076-6879\(02\)50954-X](https://doi.org/10.1016/S0076-6879(02)50954-X).
 70. Wilson RB, Davis D, Enloe BM, Mitchell AP. 2000. A recyclable *Candida albicans* URA3 cassette for PCR product-directed gene disruptions. *Yeast* 16:65–70. [https://doi.org/10.1002/\(SICI\)1097-0061\(20000115\)16:1<65::AID-YEA508>3.0.CO;2-M](https://doi.org/10.1002/(SICI)1097-0061(20000115)16:1<65::AID-YEA508>3.0.CO;2-M).
 71. Dennison PM, Ramsdale M, Manson CL, Brown AJ. 2005. Gene disruption in *Candida albicans* using a synthetic, codon-optimised Cre-loxP system. *Fungal Genet Biol* 42:737–748. <https://doi.org/10.1016/j.fgb.2005.05.006>.