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MECHANISMS OF RESISTANCE

Dysfunction of Prohibitin 2 Results in Reduced Susceptibility to Multiple Antifungal Drugs via Activation of the Oxidative Stress-Responsive Transcription Factor Pap1 in Fission Yeast

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ABSTRACT The fight against resistance to antifungal drugs requires a better understanding of the underlying cellular mechanisms. In order to gain insight into the mechanisms leading to antifungal drug resistance, we performed a genetic screen on a model organism, *Schizosaccharomyces pombe*, to identify genes whose overexpression caused resistance to antifungal drugs, including clotrimazole and terbinafine. We identified the *phb2*$^+$ gene, encoding a highly conserved mitochondrial protein, prohibitin (Phb2), as a novel determinant of reduced susceptibility to multiple antifungal drugs. Unexpectedly, deletion of the *phb2*$^+$ gene also exhibited antifungal drug resistance. Overexpression of the *phb2*$^+$ gene failed to cause drug resistance when the *pap1*$^+$ gene, encoding an oxidative stress-responsive transcription factor, was deleted. Furthermore, *pap1*$^+$ mRNA expression was significantly increased when the *phb2*$^+$ gene was overexpressed or deleted. Importantly, either overexpression or deletion of the *phb2*$^+$ gene stimulated the synthesis of NO and reactive oxygen species (ROS), as measured by the cell-permeant fluorescent NO probe DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) and the ROS probe DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate), respectively. Taken together, these results suggest that Phb2 dysfunction results in reduced susceptibility to multiple antifungal drugs by increasing NO and ROS synthesis due to dysfunctional mitochondria, thereby activating the transcription factor Pap1 in fission yeast.

KEYWORDS Phb2, reduced susceptibility to multiple antifungal drugs, Pap1, fission yeast

Invasive fungal infections cause significant morbidity and mortality worldwide and have become a life-threatening problem among immunocompromised patients, such as AIDS patients, those with chemotherapy or organ transplantation, and extremely elderly patients (1–3). Major classes of antifungal drugs for combating fungal diseases, such as azoles, terbinafine, and the polyene drugs (essentially amphotericin B), are known to interfere with the biosynthesis or integrity of ergosterol, an essential component of the fungal plasma membrane (4, 5). However, most of the antifungal drugs suffer from various drawbacks, such as toxicity or drug resistance (3, 6, 7). A better understanding of the mechanisms underlying antifungal drug resistance will facilitate the development of therapeutic strategies to minimize the evolution of antifungal drug resistance or to increase drug effectiveness and reduce toxicity.

We have been studying the mechanisms underlying antifungal drug resistance using the fission yeast *Schizosaccharomyces pombe* as a model organism, since it shares many features with some pathogenic fungi and is amenable to genetic analysis. We

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previously performed several genome-wide screens for altered sensitivity to antifungal drugs in fission yeast and identified a host of genes that are associated with antifungal drug sensitivity (8–10). In this study, in order to gain insight into the mechanisms leading to antifungal drug resistance, we performed a genetic screen to identify genes whose overexpression caused resistance to antifungal drugs in fission yeast, and we isolated the *phb2*$^+$ gene, encoding a homologue of the prohibitin Phb2.

Prohibitin proteins, including prohibitin 1 (Phb1) and prohibitin 2 (Phb2), are ubiquitously expressed and highly conserved from yeasts to humans. It has been reported that Phb2 forms a high-molecular-weight complex with Phb1 in the inner mitochondrial membrane (11, 12) and is implicated in diverse cellular processes, from mitochondrial biogenesis to cell proliferation and embryonic development (13–17). However, no evidence for the involvement of Phb2 or Phb1 in drug resistance has been reported in any species, including pathogenic fungi. In this study, we found that either overexpression of the *phb2*$^+$ gene or deletion of its product led to resistance to multiple antifungal drugs. In addition, deletion of the *phb1*$^+$ gene, but not its overexpression, resulted in resistance to multiple drugs, similar to that caused by dysfunctional Phb2. Notably, deletion of the transcription factor Pap1 abolished the drug resistance caused by Phb2 dysfunction. Furthermore, overexpression or deletion of Phb2 induced the synthesis of intracellular nitric oxide (NO) and reactive oxygen species (ROS), as well as Pap1 activation. Our present data revealed a novel involvement of Phb2 in antifungal drug resistance. Since *S. pombe* Phb1, Phb2, and Pap1 are highly conserved in diverse genera of human-pathogenic fungi, such as *Cryptococcus*, *Aspergillus*, and *Candida*, which are at the forefront of fungal infections, our study may pave the way for the development of novel therapeutic strategies to combat fungal diseases.

RESULTS

Overexpression of the *phb2*$^+$ gene caused antifungal drug resistance. To isolate novel molecules that are associated with antifungal drug resistance, we screened for genes whose overexpression caused resistance to antifungal drugs, including clotrimazole and terbinafine. As described in Materials and Methods, the *phb2*$^+$ gene was cloned by suppression of the lethality caused by clotrimazole and by terbinafine. Nucleotide sequencing of the cloned DNA fragment revealed that the insertion contained bp 1 to 393, representing a truncated *phb2*$^+$ gene, as well as 1,052 bp of its upstream region, corresponding to the promoter region. As shown in Fig. 1A, streak assay showed that wild-type cells grew well in rich YPD medium and failed to grow in the presence of 0.15 μg/ml clotrimazole or 0.09 μg/ml terbinafine. Notably, cells overexpressing the truncated *phb2*$^+$ gene grew well in the presence of 0.15 μg/ml clotrimazole or 0.09 μg/ml terbinafine (Fig. 1A). We then constructed a plasmid containing the full-length *phb2*$^+$ gene and examined the effect of full-length *phb2*$^+$ gene overexpression on antifungal drug resistance by using a spot assay. The results showed that overexpression of the full-length *phb2*$^+$ gene exhibited resistance to both terbinafine and clotrimazole, similar to that of the truncated *phb2*$^+$ gene (Fig. 1B), suggesting that the region encompassing bp 1 to 393 of the *phb2*$^+$ gene was sufficient for the drug resistance phenotypes.

Both overexpression and deletion of the *phb2*$^+$ gene caused reduced susceptibility to multiple antifungal drugs. In order to examine the effect of deletion of the *phb2*$^+$ gene on drug sensitivity, we constructed *phb2* deletion cells and performed the spot assay. The results showed that *phb2* deletion cells grew better than wild-type cells on YPD plates containing 0.06 μg/ml clotrimazole or 0.015 μg/ml terbinafine (Fig. 2A). It should be noted that we used lower concentrations of these drugs for Fig. 2A than for Fig. 1 to observe the growth of wild-type cells and Phb2 deletion cells, since fission yeast cells are more sensitive to the drugs than are cells harboring plasmids, probably due to the difference in auxotrophy.

We then investigated whether overexpression or deletion of the *phb2*$^+$ gene altered sensitivity to other antifungal drugs, such as fluconazole, amphotericin B, and caspofungin. The antifungal drug 5-fluorocytosine (5-FC), a prodrug of 5-fluorouracil (5-FU),

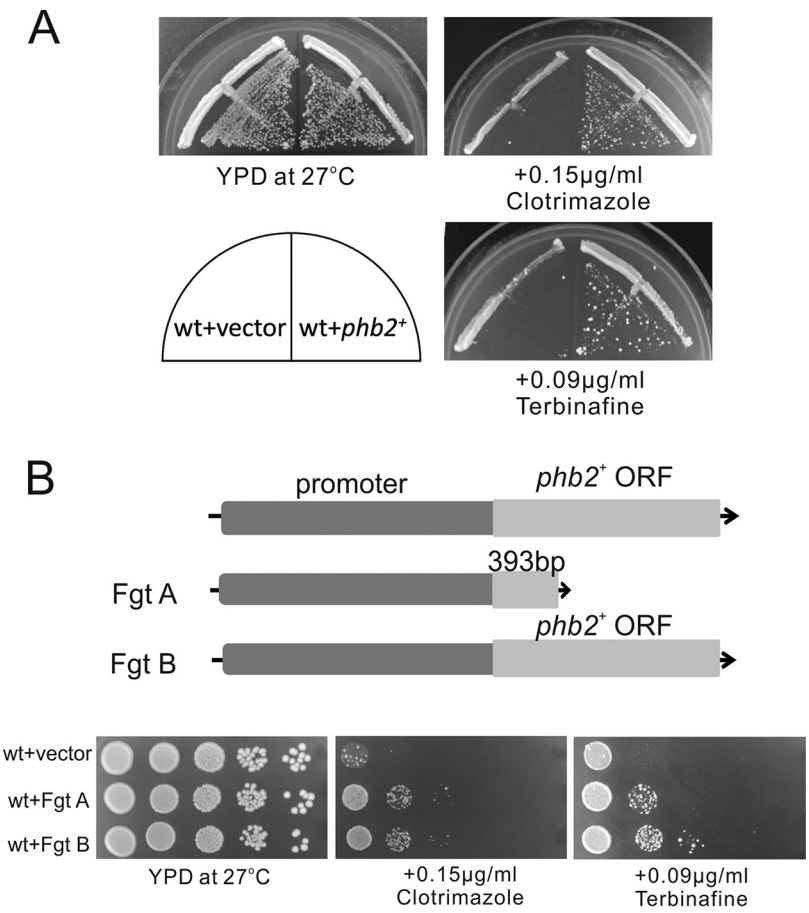

FIG 1 Overexpression of the *phb2*$^+$ gene causes antifungal drug resistance. (A) Wild-type (wt) cells were transformed with either the control vector (+ vector) or the vector containing a 393-bp fragment of the *phb2*$^+$ gene (+ *phb2*$^+$). Cells were then streaked onto plates containing either YPD alone, YPD plus 0.09 μg/ml terbinafine, or YPD plus 0.15 μg/ml clotrimazole and were incubated for 4 days at 27°C. (B) (Top) Structural features of the Phb2 fragments. (Bottom) Wild-type cells were transformed with either the control vector or the vector containing a 393-bp fragment of the *phb2*$^+$ gene (+ Fgt A) or the full-length gene (+ Fgt B) as diagramed above. The cells were then spotted onto plates containing either YPD alone, YPD plus 0.09 μg/ml terbinafine, or YPD plus 0.15 μg/ml clotrimazole and were incubated for 4 days at 27°C.

is changed to 5-FU, which is then transported into the cell by membrane permease and, in turn, inhibits DNA and RNA synthesis (18, 19). Since *S. pombe* is insensitive to 5-FC due to a lack of the enzyme with cytosine permease activity (20), we examined whether overexpression or deletion of Phb2 showed altered sensitivity to 5-FU. Phenylglyoxal, a DNA-damaging agent, was also examined. The results showed that *phb2* deletion cells exhibited significant resistance to all drugs tested here except caspofungin and 5-FU (Fig. 2A). Similarly, overexpression of the *phb2*$^+$ gene also resulted in resistance to all drugs tested except caspofungin and 5-FU (Fig. 2B). These results further suggest that Phb2 is involved in modulating drug resistance and thus is a novel determinant of reduced susceptibility to multiple antifungal drugs.

It has been known that in budding yeast, the Phb1p–Phb2p complex exerts a chaperone-like function and regulates mitochondrial function and biogenesis (21). Therefore, we examined whether Phb1 overexpression caused drug resistance similar to that with Phb2 overexpression. Unexpectedly, our results showed that overexpression of Phb1 did not exhibit resistance to any of the drugs tested here (Fig. 2C). We also investigated whether *phb1* deletion cells exhibited altered sensitivity to these drugs. As shown in Fig. 2A, cells with Phb1 deletion, like cells with Phb2 deletion, displayed significant resistance to all drugs tested here, except caspofungin and 5-FU.

The drug resistance phenotypes caused by dysfunction of Phb2 or Phb1 were further evaluated by determining the MIC values. As shown in Table 1, cells with Phb2

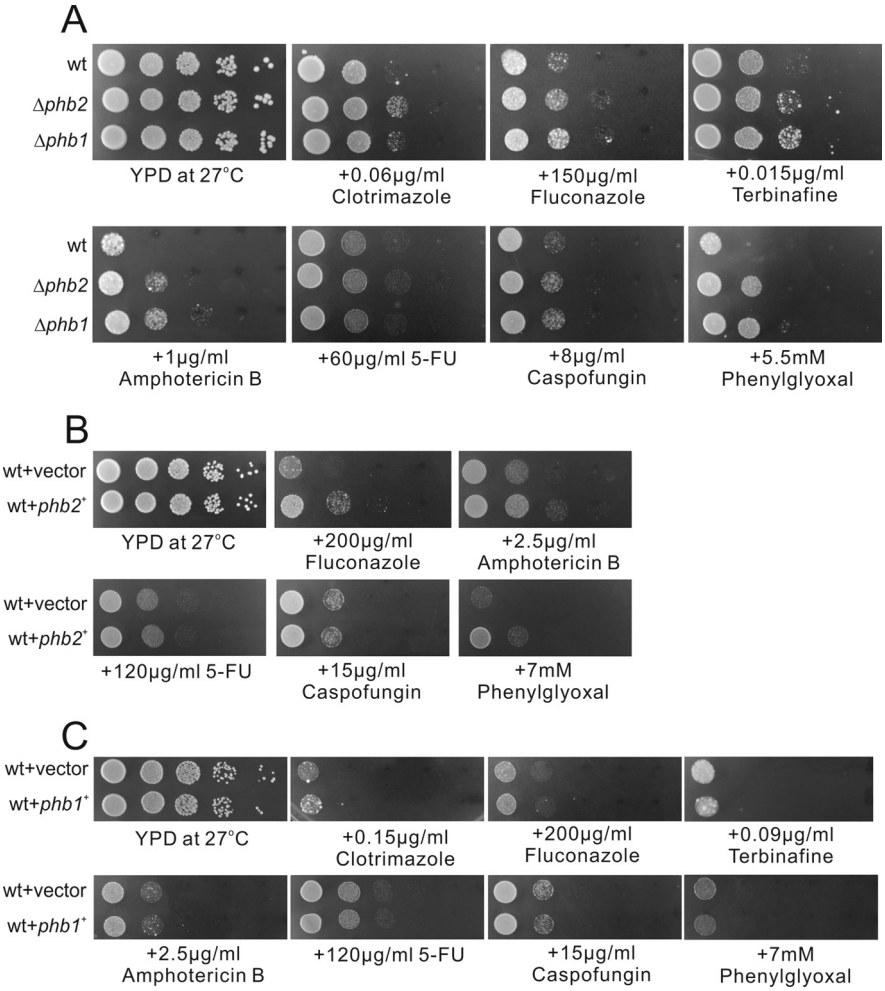

FIG 2 Deletion of the *phb2*$^+$ gene also exhibited drug resistance. (A) Δ*phb2* and Δ*phb1* cells were resistant to clotrimazole, fluconazole, terbinafine, amphotericin B, and phenylglyoxal but not to caspofungin or 5-FU. Wild-type (wt), Δ*phb2*, and Δ*phb1* cells were spotted onto YPD plates without or with 0.06 μg/ml clotrimazole, 150 μg/ml fluconazole, 0.015 μg/ml terbinafine, 1 μg/ml amphotericin B, 60 μg/ml 5-FU, 8 μg/ml caspofungin, or 5.5 mM phenylglyoxal and were then incubated at 27°C for 4 days. (B) Overexpression of Phb2 caused resistance to fluconazole, amphotericin B, and phenylglyoxal but not to caspofungin or 5-FU. Wild-type cells transformed with either the control vector or the vector containing the *phb2*$^+$ gene were spotted onto each plate as indicated and were then incubated at 27°C for 4 days. (C) Overexpression of Phb1 did not exhibit resistance to any drugs tested here. Wild-type cells transformed with either the control vector or the vector containing the *phb1*$^+$ gene were spotted onto each plate as indicated and were then incubated at 27°C for 4 days.

overexpression, but not those with Phb1 overexpression, exhibited higher MIC values of clotrimazole, fluconazole, terbinafine, amphotericin B, and phenylglyoxal than did wild-type cells (Table 1). On the other hand, deletion of either Phb2 or Phb1 resulted in MIC values of clotrimazole, fluconazole, terbinafine, amphotericin B, and phenylg-

TABLE 1 MICs of antifungal agents for *S. pombe* prohibitin overexpression strains

Strain[a]	MIC[b]						
	CLT	FLZ	TRB	AMB	5-FU	CAS	PG
wt + vector	0.060	160.00	0.06	0.50	62.50	4.00	4.00
wt + *phb2*$^+$	0.12	320.00	0.12	1.00	62.50	4.00	8.00
wt + *phb1*$^+$	0.060	160.00	0.06	0.50	62.50	4.00	4.00

[a]wt, wild type.
[b]Expressed in micrograms per milliliter for clotrimazole (CLT), fluconazole (FLZ), terbinafine (TRB), amphotericin B (AMB), 5-fluorouracil (5-FU), and caspofungin (CAS) and in millimolar concentrations for phenylglyoxal (PG).

TABLE 2 MICs of antifungal agents for *S. pombe* prohibitin deletion strains

Strain[a]	MIC[b]						
	CLT	FLZ	TRB	AMB	5-FU	CAS	PG
wt	0.030	80.00	0.015	0.25	31.25	1.00	2.00
Δ*phb2* strain	0.060	160.00	0.030	0.50	31.25	1.00	4.00
Δ*phb1* strain	0.060	160.00	0.030	0.50	31.25	1.00	4.00

[a]wt, wild type.
[b]Expressed in micrograms per milliliter for clotrimazole (CLT), fluconazole (FLZ), terbinafine (TRB), amphotericin B (AMB), 5-fluorouracil (5-FU), and caspofungin (CAS) and in millimolar concentrations for phenylglyoxal (PG).

lyoxal higher than those for wild-type cells (Table 2). Although the MIC values for the Phb mutants were only 2 times higher than those for the wild-type cells, these results are in good agreement with the findings from the spot assay described above, suggesting that the resistance phenotypes are caused by dysfunction of Phb2 or Phb1.

Intracellular localization of Phb2. To study the intracellular localization of the Phb2 protein for further analysis of Phb2 function, we tagged the 3′ end of the *phb2*$^+$ gene with the sequence encoding red fluorescent protein (RFP). Cells overexpressing Phb2-RFP exhibited drug resistance similar to that of cells overexpressing Phb2 without the RFP tag (Fig. 3A). Then we observed the localization of Phb2-RFP when expression was repressed by the addition of 4 μM thiamine to EMM. The results showed that Phb2-RFP localized to largely contiguous tubular structures that were scattered throughout the cytoplasm (Fig. 3B, left). To determine whether these structures indicate a mitochondrion-associated localization of Phb2, Phb2-RFP was examined for colocalization with areas stained with the fluorescent mitochondrial dye MitoTracker Green FM (MTG). As shown in Fig. 3B, Phb2-RFP clearly colocalized with the areas stained by the mitochondrial dye (Fig. 3B, left).

We then wanted to investigate mitochondrial organization and the intracellular localization of the Phb2 protein when Phb2 was overexpressed, since these might be associated with the mechanism of Phb2 drug resistance phenotypes. We determined the effect of Phb2 overexpression on mitochondrial organization by MTG staining. In contrast to cells in control cultures, which exhibited a typical pattern of largely contiguous tubular mitochondrial networks (Fig. 3B, left), we observed that in cultures without thiamine, most Phb2-overexpressing cells exhibited complete or nearly complete fragmentation of mitochondrial organization (Fig. 3B, right). Furthermore, Phb2-

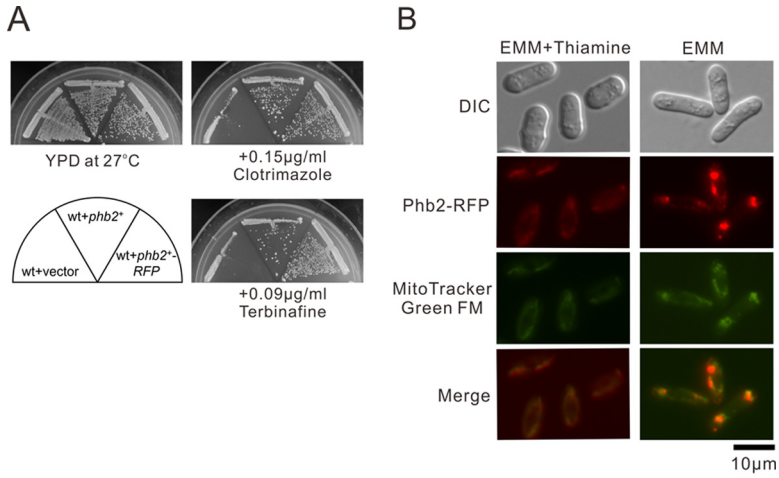

FIG 3 Intracellular localization of Phb2. (A) Wild-type (wt) cells transformed with either the control vector or the vector expressing Phb2, with or without an RFP tag, were streaked onto plates as indicated and were then incubated for 4 days at 27°C. (B) Wild-type cells expressing Phb2-RFP or stained with MTG were grown to log phase and were analyzed by fluorescence microscopy. Bar, 10 μm.

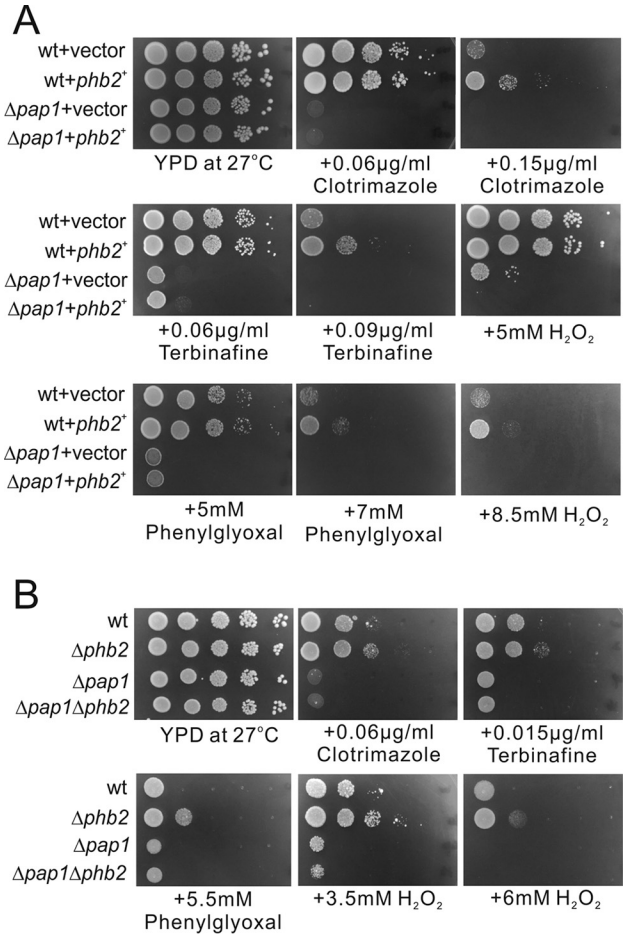

FIG 4 Deletion of the *pap1*$^+$ gene abolished the drug resistance caused by Phb2 dysfunction. (A) Deletion of the *pap1*$^+$ gene abolished the drug resistance caused by overexpression of Phb2. Cells were spotted onto each plate as indicated and were then incubated at 27°C for 4 days. (B) Deletion of the *pap1*$^+$ gene abolished the drug resistance caused by deletion of Phb2. Wild-type (wt), Δ*phb2*, Δ*pap1*, and Δ*pap1* Δ*phb2* cells were spotted onto each plate as indicated and were then incubated at 27°C for 4 days.

RFP fluorescence was found to be aggregated near the cell tips and was not colocalized with MTG staining in Phb2-overexpressing cells (Fig. 3B, right).

Deletion of the *pap1*$^+$ gene abolished the drug resistance caused by overexpression of Phb2. Given that the AP-1-like transcription factor Pap1, one of the major stress-activated transcription factors, plays important roles in multidrug resistance (22–24), we wanted to know the link between Pap1 and the drug resistance caused by Phb2 overexpression. If the Pap1 protein is the true cause of the reduced susceptibility to multiple antifungal drugs observed in Phb2-overexpressing cells, then this resistance phenotype should be lost in strains in which the *pap1*$^+$ gene has been deleted. To this end, we examined the effect of Pap1 deletion on the drug resistance caused by Phb2 overexpression. As shown in Fig. 4A, wild-type cells overexpressing Phb2 showed significantly more resistance to clotrimazole, terbinafine, and phenylglyoxal than control cells (wt + vector); however, Phb2-overexpressing Pap1 deletion cells grew similarly to the control cells (Δ*pap1* + vector) (Fig. 4A) in the presence of clotrimazole, terbinafine, and phenylglyoxal. These results suggest that Phb2 overexpression causes reduced susceptibility to multiple antifungal drugs in a Pap1-dependent manner.

Deletion of the *pap1*$^+$ gene abolished the drug resistance caused by Phb2 deletion. We next investigated whether Pap1 deletion affected the drug resistance caused by deletion of the *phb2*$^+$ gene. The *pap1* deletion was crossed to the *phb2* deletion to construct Δ*pap1* Δ*phb2* double mutants. The results showed that the Δ*pap1*

Δ*phb2* and Δ*pap1* cells were similarly sensitive to clotrimazole, terbinafine, and phenylglyoxal (Fig. 4B). Notably, we found that either overexpression or deletion of the *phb2*$^+$ gene displayed H_2O_2 resistance but that cells with either disruption failed to display H_2O_2 resistance when they lacked Pap1 (Fig. 4A and B). These data further suggest that Pap1 is essential for Phb2-mediated drug resistance and H_2O_2 resistance.

Both overexpression and deletion of the *phb2*$^+$ gene stimulated NO and ROS synthesis. The findings presented above prompted us to explore how Pap1 mediated Phb2-induced drug resistance. Since Pap1 plays an established role in the oxidative stress response in fission yeast (25) and free-radical ROS are signaling molecules that cause oxidative stress (26), we sought to investigate whether ROS production was increased by overexpression or deletion of the *phb2*$^+$ gene. Then ROS levels were measured by labeling with the ROS probe DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate), and the results showed that ROS levels were significantly higher both in cells lacking Phb2 and in cells overexpressing Phb2 than in control cells (Fig. 5A and B).

Since NO is also a free radical and acts as a signaling molecule that modulates various cellular processes, including mitochondrial function (27), we next examined the NO levels in dysfunctional Phb2 cells by labeling with the NO probe DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate). As shown in Fig. 5C and D, both in cells lacking Phb2 and in cells overexpressing Phb2, NO levels were significantly higher than those in control cells.

Both overexpression and deletion of the *phb2*$^+$ gene increased Pap1 mRNA levels. To further test the hypothesis that Phb2 dysfunction activates Pap1 by elevating cellular ROS and NO levels, leading, in turn, to drug resistance, we then examined Pap1 mRNA expression in cells with Phb2 overexpression or deletion. As shown in Fig. 5E, the mRNA level of the *pap1*$^+$ gene was significantly increased when the *pap1*$^+$ gene was overexpressed, while it was decreased when the *pap1*$^+$ gene was deleted. Notably, both overexpression and deletion of Phb2 significantly increased the mRNA level of the *pap1*$^+$ gene (Fig. 5E), suggesting that either Phb2 overexpression or deletion induced Pap1 activation. In addition, we found that Pap1 overexpression exhibited drug resistance phenotypes similar to those with Phb2 dysfunction (Fig. 5F), further suggesting that Phb2 dysfunction causes reduced susceptibility to multiple antifungal drugs via activation of Pap1.

Deletion of the *phb2*$^+$ gene altered the drug sensitivity of *S. cerevisiae*. Given that the *phb2*$^+$ gene was highly conserved, we then investigated whether *phb2*$^+$ deletion was also related to drug resistance in *S. cerevisiae*. As shown in Fig. 6, the *phb2* deletion strain of *S. cerevisiae* was sensitive to clotrimazole, fluconazole, amphotericin B, caspofungin, and phenylglyoxal but resistant to terbinafine and 5-FU. Although the reason for the difference in drug sensitivity between *S. cerevisiae* and *S. pombe* is unknown, the results suggested that dysfunctional Phb2 also plays important roles in the development of drug sensitivity in *S. cerevisiae*.

DISCUSSION

Mitochondrial dysfunction has been identified as an important contributor to drug resistance in human fungal pathogens, but the mechanistic details are far from understood (28). Phb2, as a component of the mitochondrial prohibitin complex along with Phb1, has been reported to be involved in various functions, for example, serving as a receptor for the mitophagic machinery (17). However, evidence for the role of Phb2 in drug resistance has remained lacking. In this study, we found that Phb2 mediated reduced susceptibility to multiple antifungal drugs by activating the transcription factor Pap1 in fission yeast. To our knowledge, this is the first report of the involvement of Phb2 in drug resistance.

Several lines of evidence support the hypothesis that Phb2 dysfunction affects drug resistance by activating Pap1 in fission yeast. First, Phb2 overexpression led to drug resistance but failed to do so when the *pap1*$^+$ gene was deleted. Second, Phb2 deletion also resulted in drug resistance, and deletion of the *pap1*$^+$ gene abolished Phb2-induced drug resistance. Third, either overexpression or deletion of the *phb2*$^+$ gene

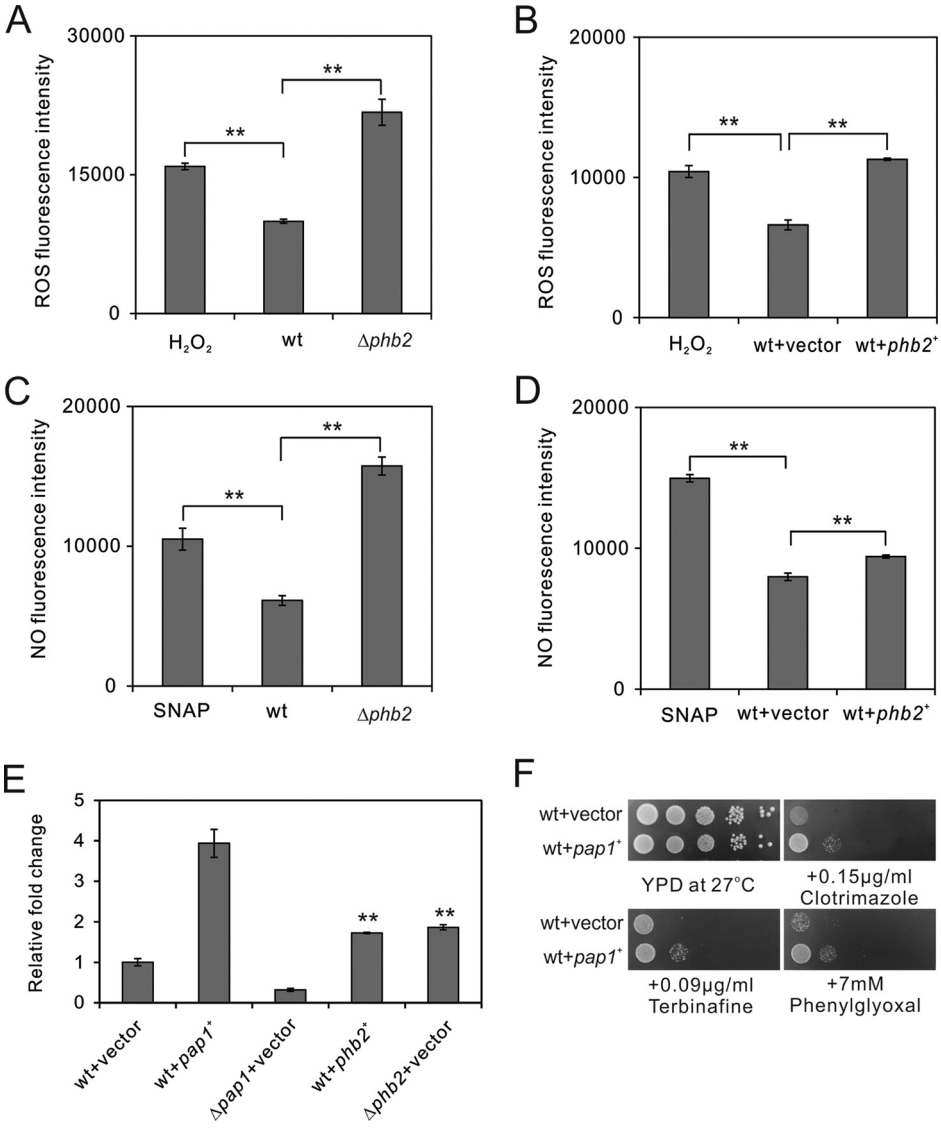

FIG 5 Either overexpression or deletion of the *phb2*$^+$ gene stimulated NO and ROS synthesis. (A through D) ROS levels (A and B) and NO levels (C and D) were increased both in cells lacking Phb2 (A and C) and in cells overexpressing Phb2 (B and D). Cells were grown to log phase, and then ROS production or NO levels were measured by a microplate reader after incubation with a fluorescent probe. Data are expressed as means ± SEM from triplicate assays. Asterisks indicate significant differences (**, $P < 0.01$) from values for wild-type (wt) cells. SNAP, NO-generating *S*-nitroso-*N*-acetylpenicillamine. (E) *pap1*$^+$ mRNA levels were increased both in cells lacking Phb2 and in cells overexpressing Phb2. The cells were grown to log phase; then total RNA was extracted from the harvested cells and was subjected to quantitative RT-PCR for *pap1*$^+$ mRNA. The values were obtained in comparison to those of *act1*$^+$ mRNA and were then normalized to those in wild-type cells. Asterisks indicate significant differences (**, $P < 0.01$) from values for wild-type cells. (F) Overexpression of the *pap1*$^+$ gene exhibited drug resistance similar to that with *phb2*$^+$ gene dysfunction. Cells were spotted onto each plate as indicated and were then incubated at 27°C for 4 days.

increased *pap1*$^+$ mRNA expression. Fourth, overexpression of the *pap1*$^+$ gene caused drug resistance similar to that caused by overexpression or deletion of the *phb2*$^+$ gene. These results suggest that Phb2 dysfunction activates Pap1, which, in turn, leads to drug resistance. It should be noted that overexpression or deletion of the *phb2*$^+$ gene also caused H_2O_2 resistance and that deletion of the *pap1*$^+$ gene suppressed the H_2O_2 resistance, further supporting the above hypothesis. Previous studies have reported that a prohibitin deficiency in plants led to increased sensitivity to H_2O_2 (13), indicating that the role of prohibitin in oxidative stress differs markedly between yeasts and plants. Additionally, our results showed that Phb2 dysfunction causes resistance to all

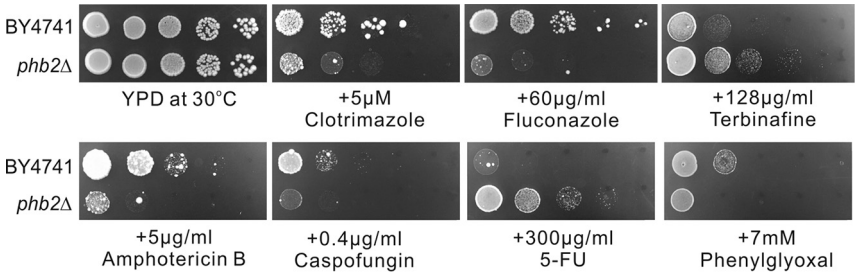

FIG 6 Deletion of the *phb2*$^+$ gene altered the drug sensitivity of *S. cerevisiae*. A *phb2* deletion strain of *S. cerevisiae* was sensitive to clotrimazole, fluconazole, amphotericin B, caspofungin, and phenylglyoxal but resistant to terbinafine and 5-FU. BY4741 and *phb2*Δ cells were spotted onto YPD plates without or with 5 μM clotrimazole, 60 μg/ml fluconazole, 128 μg/ml terbinafine, 5 μg/ml amphotericin B, 0.4 μg/ml caspofungin, 300 μg/ml 5-FU, and 7 mM phenylglyoxal and were then incubated at 30°C for 4 days.

the drugs tested here except 5-FU and the cell wall-damaging agent caspofungin, which inhibits 1,3-β-D-glucan synthase, suggesting that the mechanisms of resistance to 5-FU and caspofungin may be different from those for the other drugs.

Furthermore, our results suggest that Phb2 dysfunction causes mitochondrial damage, which results in increasing NO and ROS synthesis. First, Phb2 localized to the mitochondria under normal conditions; however, upon Phb2 overexpression, mitochondria were fragmented, and Phb2-RFP was aggregated abnormally. It has been reported previously that upon prohibitin depletion, mitochondria appeared fragmented and disorganized (29). These findings suggest that overexpression or deletion of Phb2 can cause mitochondrial dysfunction. Second, we found that either overexpression or deletion of the *phb2*$^+$ gene significantly increased NO and ROS levels in fission yeast cells, while mitochondria are the major site of ROS production in the cells. This is quite consistent with the previous report that a lack of prohibitin results in increasing ROS production (12). It has been known that *S. pombe* responds to moderate doses of H_2O_2 by activating the transcription factor Pap1, which accumulates in the nucleus in response to stress (30), and that a Pap1 deletion mutant exhibits H_2O_2 sensitivity (31). Our results showed that overexpression of the *phb2*$^+$ gene caused H_2O_2 resistance in wild-type cells but rendered cells with Pap1 deletion more sensitive to H_2O_2. It is possible that Phb2 overexpression increased ROS levels in cells with Pap1 deletion, which caused an additive inhibitory effect on the growth of these cells with H_2O_2 treatment.

Phb2, as a subunit of the prohibitin complex, exerts many relevant functions through interaction with Phb1. However, our results suggest that overexpression of Phb2, but not Phb1, caused reduced susceptibility to multiple antifungal drugs. It has been shown that the PHB complex is anchored in the mitochondrial inner membrane through N-terminal hydrophobic regions. In budding yeast, the transmembrane domain prediction algorithm TMHMM predicts a transmembrane helix at amino acids 37 to 59 of Phb2, leaving 35 amino acids on the matrix side with the bulk of the protein facing the intermembrane space. However, the homologous helical region at the N terminus of Phb1 is shorter and may not fulfill the requirements for a membrane-spanning domain (12). This may be the reason that Phb2 showed some functions in drug resistance distinct from those of Phb1 in fission yeast. In support of this hypothesis, our results showed that overexpression of the region corresponding to bp 1 to 393 in N-terminally truncated Phb2 caused reduced susceptibility to multiple antifungal drugs.

Collectively, the present data strongly suggest that Phb2 dysfunction causes mitochondrial dysfunction, which induces NO and ROS production and subsequently activates the transcriptional factor Pap1, eventually leading to reduced susceptibility to multiple antifungal drugs. Given that either overexpression or deletion of Phb2 conferred reduced susceptibility to multiple antifungal drugs, *phb2* mutation may cause clinical resistance to these drugs, and Phb2 may serve as a new prognostic and predictive marker for antifungal

TABLE 3 Strains used in this study

Strain	Genotype	Source or reference
S. pombe		
HM123	*h*$^-$ *leu1-32*	Our stock
KP133	*h*$^-$ *leu1-32 ura4-D18 pap1*::*ura4*$^+$	42
CM30	*h*$^-$ *leu1-32 phb2*::*kanMX4*	This study
CM32	*h*$^-$ *leu1-32 phb1*::*kanMX4*	This study
CM54	*h*$^+$ *leu1-32 ura4-D18 pap1*::*ura4*$^+$ *phb2*::*kanMX4*	This study
S. cerevisiae		
CM85	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Beidong Liu
CM86	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 phb2Δ*::*kanMX4*	Beidong Liu

treatment. Mitochondrial dysfunction and antifungal drug resistance are well-documented phenomena in pathogenic yeasts (32, 33). However, *phb2* deletion has never been found in a systematic gene knockout study examining fluconazole sensitivity or resistance. Our findings emphasize the importance of mitochondrial function in maintaining drug sensitivity, which is crucial for the development of future antifungal therapeutic strategies, and our work may provide the possibility of individual treatment optimization for patients with fungal infections.

MATERIALS AND METHODS

Strains, media, and genetic and molecular biology techniques. The *S. pombe* strains and *S. cerevisiae* strains used in this study are listed in Table 3. The budding yeast wild-type strain BY4741 (here designated CM85) and its isogenic derivative mutant CM86 (*phb2*::*kanMX4*) were generously provided by Beidong Liu (University of Gothenburg, Gothenburg, Sweden). The complete and minimal media used were yeast extract-peptone-dextrose (YPD) and Edinburgh minimal medium (EMM), respectively (34). Standard *S. pombe* genetic and recombinant-DNA methods were performed as described previously (35) except where otherwise noted. Gene disruptions are denoted by lowercase letters representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption (for example, *phb2*::*kanMX4*). Gene disruptions are abbreviated by the gene preceded by Δ (for example, Δ*phb2*) in the text and figures. Proteins are denoted by roman letters, of which only the first letter is capitalized (for example, Phb2) (34, 36). All other chemicals and reagents were purchased from commercial sources.

Isolation of the *phb2*$^+$ gene. An *S. pombe* genomic library cloned into the pDB248 vector (37) was transformed into wild-type cells (HM123). The Leu$^+$ transformants were replica plated onto YPD plates at 27°C containing 0.15 μg/ml clotrimazole or 0.09 μg/ml terbinafine. Dozens of primary transformants that showed both clotrimazole resistance and terbinafine resistance were obtained. Plasmid DNA was recovered from transformants that showed plasmid-dependent rescue. The recovered plasmids suppressed the lethality induced by clotrimazole as well as that induced by terbinafine. By DNA sequencing, the suppressing plasmids were found to belong to two classes. One class contained the *phb2*$^+$ gene. The characterization of a gene contained in the second class will be reported elsewhere. Here we focus on the *phb2*$^+$ gene, whose overexpression causes both clotrimazole resistance and terbinafine resistance.

Drug resistance phenotype analysis. The drug resistance of strains was tested by using a streak assay or spot assay as described previously (8). The streak assay is the simplest assay for the assessment of strain growth. Briefly, each strain was streaked out to form single colonies on solid agar plates containing YPD alone or YPD plus drugs, and then the plates were incubated at 27°C for 4 days. The number and size of the colonies were assessed for the estimation of drug resistance after incubation. The semiquantitative spot assay is a classical experimental approach for the study of yeast cell growth due to its accuracy. Briefly, the yeast cells were first grown to log phase and then resuspended in fresh YPD medium to give an optical density at 660 nm (OD_{660}) of 0.3 (approximately 10^7 cells/ml). Five microliters of 10-fold serial dilutions of each yeast culture was spotted onto solid agar plates, and *S. pombe* and *S. cerevisiae* were incubated at 27°C and 30°C for 4 days, respectively. The number and size of the colonies formed on each plate were analyzed for the estimation of drug resistance after incubation.

Determination of MICs. Prior to the fission yeast experiments, the MICs for the strains were tested by microtiter assays according to CLSI guidelines with some modifications as described previously (8). Briefly, the strains were inoculated into 96-well microtiter plates containing 100 μl of YPD medium and different concentrations of drugs to yield 10^5 cells/well. The plates were examined by using a microplate reader (iMark; Bio-Rad) after 48 h of incubation at 27°C. MICs were determined as the lowest concentrations of the drugs causing at least 50% inhibition of growth relative to that with the control. All MIC assays were performed in duplicate.

Gene deletion. The strains carrying deletions of the *phb2*$^+$ or *phb1*$^+$ gene, with a genetic background of *h*$^+$ *leu1-32 ura4-D18 ade6-M210* or *h*$^+$ *leu1-32 ura4-D18 ade6-M216* and the *kanMX* cassette, were purchased from Bioneer (South Korea) (38). We constructed *phb2* or *phb1* deletion cells that were not auxotrophic for uracil or adenine by a genetic cross between wild-type HM123 cells and the strains described above to make CM30 and CM32, respectively (Table 3).

Plasmid construction. The *phb2*$^+$ open reading frame (ORF) was amplified by PCR (forward primer, 5′-GAAGATCTCATGAATCGTCAAAGACCGTTCCAGCAG-3′; reverse primer, 5′-AAGGAAAAAAGCGGCCGCCT

TTAATATCATCTAAAAG-3′) from genomic DNA and was subcloned into BglII/NotI sites of pBluescript SK(+) (Stratagene, USA). To assess subcellular localization, the Phb2 protein was tagged with RFP at its C terminus as described previously (39). The cells expressing Phb2-RFP exhibited the same drug resistance as cells expressing Phb2 without the RFP tag.

MitoTracker Green FM staining of *S. pombe* cells. Mitochondrial localization was detected by staining *S. pombe* cells with MitoTracker Green FM (MTG) (Invitrogen, USA). Briefly, *S. pombe* cells were cultured overnight at 27°C in liquid EMM containing 4 μM thiamine to mid-log phase. Staining was carried out by adding 1 μl MTG solution (0.1 mM MTG in dimethyl sulfoxide [DMSO]) to 100 μl of cell culture and incubating at 27°C with shaking for 20 min. Stained cells were washed three times with EMM and were resuspended in approximately 10 μl of EMM prior to the preparation of samples for microscopy. Stained cells were visualized by fluorescence microscopy using a Nikon Eclipse Ni-U microscope equipped with a DS-Qi2 camera (Nikon Instruments Inc., Japan).

RNA extraction and RT-PCR. Total RNA was extracted from fission yeast cells lysed by TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA purity was assessed with a spectrophotometer. The resultant total RNA was used to synthesize cDNA using the GoScript reverse transcription system (Promega, USA). The resultant cDNA was subjected to semiquantitative PCR with the Thunderbird SYBR qPCR mix (Toyobo, Japan). The *pap1*$^+$ primers for real-time reverse transcription-PCR (RT-PCR) were 5′-TCACCCTGATGAAGTTCCGC-3′ and 5′-AGGTATGCTCGTTCCTTGGC-3′. Act1 was used as an internal control, and the *act1*$^+$ primers for RT-PCR were 5′-TTCAAGCCCTTGCTCCTAGC-3′ and 5′-TACC AGGTCCGCTCTCATCA-3′. The fluorescent signals were detected and analyzed with the Applied Biosystems 7500 real-time PCR system (ABI).

Detection of intracellular NO and ROS. Intracellular NO and ROS were detected as described previously (40), with some modifications. Briefly, fission yeast cells were grown in EMM until the OD_{660} reached 0.3 and were then washed three times with 1× phosphate-buffered saline (PBS). To determine intracellular NO levels, DAF-FM DA (Beyotime Institute of Biotechnology, Shanghai, China) was added to the cells at a final concentration of 5 μM, and the cells were then incubated for 1 h in the dark at 30°C. For the quantification of intracellular ROS production, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Beyotime Institute of Biotechnology, Shanghai, China) was added to the cells at a final concentration of 10 μM, and the cells were then incubated for 30 min in the dark at 30°C. After treatment with DAF-FM DA or DCFH-DA, cells were washed to remove excess probe and were transferred to 96-well plates. The readout was performed in a microplate reader (Tecan Infinite M200 PRO) with fluorescence excitation at 488 nm and emission at 525 nm, and the data were expressed as fluorescence units.

Miscellaneous methods. Light microscopy methods, such as fluorescence microscopy and differential interference contrast (DIC) microscopy, were performed as described previously (39, 41). Database searches were performed using the National Center for Biotechnology Information BLAST network service and the PomBase database search service for the fission yeast *S. pombe* (http://www.pombase.org/).

Statistical analyses. Quantitative data were expressed as means ± standard errors of the means (SEM). Multiple comparisons were statistically analyzed by Student's *t* test. The difference was considered to be significant if the *P* value was <0.05. All statistical analyses were performed using the SPSS software package (version 16.0; SPSS, Inc., Chicago, IL, USA).

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