







Inhibition of Vesicular Transport Influences Fungal Susceptibility to Fluconazole

 Liesbeth Demuyser,^{a,b}  Katrien Van Dyck,^{a,b}  Bea Timmermans,^{a,b}  Patrick Van Dijck^{a,b}

^aVIB-KU Leuven Center for Microbiology, Flanders, Belgium

^bLaboratory of Molecular Cell Biology, Institute of Botany and Microbiology, KU Leuven, Leuven, Belgium

ABSTRACT Fungal infections pose a substantial threat to the human population. They can cause either mild and relatively harmless infections or invasive and often lethal diseases in patients with a weakened immune system. The majority of these human fungal infections are caused by *Candida* species. The limited amount of available therapies, together with the development of resistance against these drugs, strongly emphasizes the need for novel therapeutic strategies. As it is quite time-consuming to introduce completely new drugs to the market, potentiating the efficacy of existing drugs would be a better strategy. Therefore, it is important to identify cellular pathways involved in the development of drug resistance. We found that vesicular transport is involved in fungal susceptibility to the most widely used antifungal drug, fluconazole. We identified specific complexes in the vesicular transport pathway which contribute to fluconazole resistance or tolerance in the model organism *Saccharomyces cerevisiae*. Furthermore, we confirmed our findings in the clinically relevant fungi *Candida albicans* and *Candida glabrata*. Finally, we show that the combination of fluconazole with a specific inhibitor of the vesicular transport pathway increases the susceptibility of *Candida* species, indicating the potential of using vesicular transport as a target in combination therapy.

KEYWORDS *Candida albicans*, *Candida glabrata*, *Saccharomyces cerevisiae*, antifungal drug resistance, antifungal drug tolerance, fluconazole, vesicle transport

There are over 600 different fungal pathogens able to cause either minor or invasive infections in humans (1, 2). The majority of human fungal infections are caused by species of the *Candida* genus, including *Candida albicans* and *Candida glabrata* (3). In most healthy individuals, they are both harmless commensals of the oral cavity, gastrointestinal tract, and/or urogenital tract. However, under conditions involving a reduced immune competence or an imbalance of the microflora, they are able to cause infections (2, 4). Superficial infections are relatively harmless and affect approximately 25% of the worldwide population (5). However, the incidence of severe invasive fungal infections is still rising due to the increase in the number of immunocompromised patients (1). The lack of effective antifungal drugs and the emergence of fungal resistance against these drugs pose a significant threat to human health (6).

The most commonly used antifungal drugs are azoles (7). These fungistatic drugs, with the most studied member being fluconazole, target the ergosterol biosynthesis pathway. Fluconazole binds to the active site of the lanosterol 14 α -demethylase enzyme (Erg11), thereby inhibiting the conversion of lanosterol into ergosterol, which is an essential component of the fungal cell membrane (8, 9). However, long-term exposure of fungal cells to antifungal drugs has different consequences. The cells can develop resistance mechanisms to overcome the inhibitory actions of the drug. Known mechanisms of resistance against azoles include alteration of the target enzyme, overexpression of the gene encoding this target, and increased drug efflux (10, 11).

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Address correspondence to Patrick Van Dijck, Patrick.vandijck@kuleuven.vib.be.

L.D. and K.V.D. contributed equally to this article.

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Resistance mechanisms allow the cells to grow at higher drug concentrations than susceptible cells. Alternatively, cells can develop tolerance to the drug to allow trailing growth at inhibitory concentrations (12). This phenomenon is alternatively referred to as FoG, for fraction of growth (13). The development of new antifungal drugs is important to overcome antifungal resistance. Alternatively, therapeutic strategies can be improved by enhancing the efficacy of existing drugs, such as fluconazole. Therefore, it is important to identify the genes and cellular pathways involved in the development of drug resistance or tolerance. Combination therapy has shown great potential to exert synergism and reduce resistance (14). However, there is still a lack of knowledge concerning the pharmacodynamics, pharmacokinetics, and clinical success of drug combinations (15, 16).

In this study, we used the baker's yeast *Saccharomyces cerevisiae* as a model organism to examine the role of the vesicular transport pathway in fluconazole susceptibility. We screened a library of *S. cerevisiae* deletion strains for decreased growth on fluconazole compared to the wild-type strain and found that impairment of vesicular transport increases susceptibility to this drug. The highly regulated vesicular transport pathway is involved in transporting cargo, such as lipids and proteins, between membrane-bound compartments (17). Proteins are transported from the endoplasmic reticulum (ER) to the Golgi apparatus via vesicles during biosynthesis. Afterwards, proteins are targeted to the plasma membrane, to the vacuole via endosomes, or to the external medium. In the reverse process, proteins can be internalized by endocytosis and transported to the endosomes. Here, they are targeted to the vacuole for degradation or to the Golgi apparatus for recycling (18). Vesicular transport and the correct targeting of vesicles are highly controlled by different proteins at the fusion sites. Soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complexes mediate the fusion of cargo-loaded vesicles to acceptor compartments. The vesicles from donor compartments are coated with tethering complexes, which regulate the specific targeting to the acceptor compartment (19).

Although a link between fluconazole sensitivity and vesicular transport was already suggested previously (20–23), in this project, we identified the involvement of particular complexes in this process. We selected six susceptible *S. cerevisiae* deletion strains which were affected in vesicular trafficking: the *S. cerevisiae pep3Δ* (*Scpep3Δ*), *Scpep5Δ*, *Scpep7Δ*, *Scvps45Δ*, *Scvps51Δ*, and *Scvps54Δ* strains. The *ScPep3* and *ScPep5* proteins are members of the endosomal tethering complex called the class C core vacuole/endosome tethering (CORVET) complex. The CORVET complex is a multimeric protein complex which is required for endocytosis and for the attachment of vesicles to and from the endosome. In addition, they are also members of the homotypic fusion and vacuole protein sorting (HOPS) tethering complex, which is involved in the fusion of vesicles to the vacuole (24). The *ScVps51* and *ScVps54* proteins are both members of the Golgi-associated retrograde protein (GARP) complex, which is involved in retrograde trafficking of vesicles from the endosomes to the Golgi apparatus (25). Finally, the *ScPep7* and *ScVps45* proteins physically interact with each other and are important for vesicle fusion at the endosome (26). For all six deletion strains, we confirmed increased susceptibility by analysis of the fluconazole (FLC) MIC (MIC_{FLC}) and showed a concomitant decrease in retained ergosterol after treatment. In addition, we could confirm the involvement of vesicular transport in fluconazole efficacy against *C. albicans* and *C. glabrata*. Finally, we tested the effect of sorting inhibitors (sortins), which inhibit vesicular transport (27–30), on fluconazole efficacy against several *C. glabrata* and *C. albicans* isolates and showed a synergistic interaction with sortin2.

RESULTS

Interference in vesicular transport increases susceptibility to fluconazole in *S. cerevisiae*. In order to identify the regulatory processes involved in susceptibility to fluconazole, we screened the *S. cerevisiae* haploid *MAT α* deletion collection for reduced growth in the presence of fluconazole (10 or 20 μ g/ml). As described in earlier publications, we added the iron chelator doxycycline to the minimal testing medium (50 or

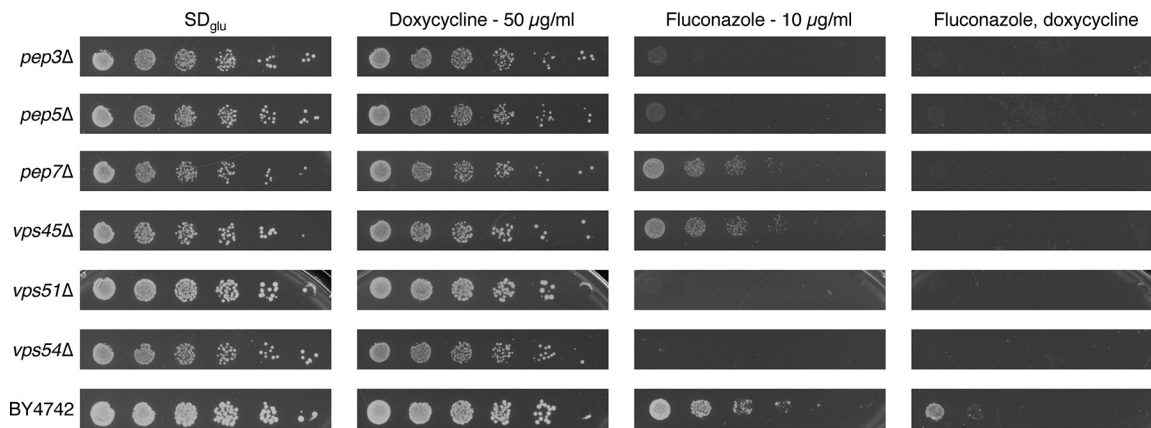


FIG 1 Deletion of genes encoding vesicular transport-regulating proteins increases susceptibility to fluconazole in *S. cerevisiae*. Serial dilutions of six deletion strains and the BY4742 wild-type strain were spotted on SD_{glu} medium containing fluconazole (10 μ g/ml) and/or doxycycline (50 μ g/ml). Pictures were taken after 72 h of incubation at 30°C. At least three biological repeats were tested, and representative results are shown.

100 μ g/ml) to decrease the background growth or tolerance at supra-MICs of the drug and increase the sensitivity of the screening system (31, 32). Strains with enhanced fluconazole susceptibility, being the deletion strains that showed increased susceptibility to the drug, were selected by visually monitoring reduced growth after 3 and 4 days of incubation at 30°C compared to the growth of the BY4742 wild-type laboratory strain (see Table S1 in the supplemental material). The genes deleted in all enhancer strains were clustered, using the online tool FunSpec (33). A list of these genes, classified by gene ontology (GO) annotation, is displayed in Table S2. Using a *P* value cutoff of 0.001, processes mainly related to intracellular vesicle transport were identified as biological processes regulating susceptibility to fluconazole, including Golgi apparatus-to-endosome transport (*ScPEP7*, *ScVPS45*, *ScPEP3*, and *ScPEP5*), vesicle docking involved in exocytosis (*ScPEP7*, *ScVPS45*, *ScPEP3*, and *ScPEP5*), Golgi apparatus-to-vacuole transport (*ScVPS54*, *ScPEP7*, and *ScVPS45*), and regulation of SNARE complex assembly (*ScPEP3* and *ScPEP5*). Many of the involved proteins were localized to the GARP complex (*ScVPS54* and *ScVPS51*), the HOPS complex (*ScPEP3* and *ScPEP5*), or the CORVET complex (*ScPEP3* and *ScPEP5*). Increasing this *P* value ($P < 0.01$) also showed the involvement of other known processes, such as sterol biosynthesis and the general drug response. These processes have been shown to be involved in fluconazole susceptibility by earlier screening setups (22, 34). Parsons et al. performed a similar screening in *S. cerevisiae*, though they used a different experimental setup (22). They found that the *ScPDR16*, *ScPDR5*, and *ScERG* genes, when deleted, confer susceptibility to this drug, as did we. They also identified a number of genes related to vesicular transport to be of importance in drug susceptibility. This partial overlap endorses the accuracy of our screening method, yet it also shows that differences in results occur due to differential experimental setups.

To verify the involvement of vesicular transport in susceptibility to fluconazole, we selected six deletion strains from the mutant collection, the *Scpep3* Δ , *Scpep5* Δ , *Scpep7* Δ , *Scvps45* Δ , *Scvps51* Δ , and *Scvps54* Δ strains, and created at least three independent transformants of each genotype. These strains were used as biological replicates in the following experiments. Initially, spot assays on fluconazole- and doxycycline-containing media were repeated (Fig. 1), confirming increased susceptibility to drugs.

To determine whether the increase in susceptibility of the strains impaired in vesicular transport could be linked to a decrease in the MIC of fluconazole (MIC_{FLC}) for these strains, we performed Etest (Fig. S1a) and broth microdilution assays (BDAs) (Fig. S1b). All experiments were performed with at least three independent biological replicates. As can be seen from Table 1, the MIC_{FLC} values for all six deletion strains were lower than those for the BY4742 control strain, indicating lower resistance to the

TABLE 1 MIC_{FLC} values for the analyzed strains determined by Etest and broth microdilution assay analysis^a

Strain	MIC _{FLC} (μg/ml)		
	Etest	Broth microdilution assay	
		50%	90%
<i>S. cerevisiae pep3Δ</i>	2–3	4	4–8
<i>S. cerevisiae pep5Δ</i>	1–2	4	4–8
<i>S. cerevisiae pep7Δ</i>	0.75–1.5	2	2
<i>S. cerevisiae pep45Δ</i>	1.5–3	0.5–1	>128
<i>S. cerevisiae pep51Δ</i>	0.5–0.75	4	4–8
<i>S. cerevisiae pep54Δ</i>	0.75–1	4	4–8
<i>S. cerevisiae</i> BY4742	6–8	16–32	32
<i>C. albicans vps11Δ/Δ</i>	0.5	0.125	0.5
<i>C. albicans</i> YJB6284	0.25	0.125	0.25
<i>C. glabrata vps45Δ/Δ</i>	8	4	8
<i>C. glabrata</i> ATCC 2001	16	8	>128

^aThe MIC_{FLC} was determined by Etest (after 48 h or 72 h) or broth microdilution assay (after 48 h) of 6 deletion strains of *S. cerevisiae* (the *pep3Δ*, *pep5Δ*, *pep7Δ*, *vps45Δ*, *vps51Δ*, and *vps54Δ* strains) and the wild-type strain (BY4742), the *C. albicans vps11Δ/Δ* and *C. glabrata vps45Δ/Δ* deletion strains, and the respective wild-type strains (YJB6284 and ATCC 2001).

drug. Viewing from the Etest assays, the *Scvps51Δ*, *Scvps54Δ*, *Scpep3Δ*, and *Scpep5Δ* strains showed a decrease in trailing growth or tolerance, defined as the supra-MIC growth inside the halo of growth retardation/inhibition. The *Scvps45Δ* and *Scpep7Δ* strains did not show a similar decrease in tolerance. In contrast to the BY4742 and *pep7Δ* strains, the *vps45Δ* strain also showed high trailing growth in the broth microdilution assay; however, this method is less suitable to determine trailing growth due to the insensitivity of the optical density (OD) reading.

To eliminate the possible influence of the auxotrophies in the BY4742 background on our phenotype, we created the deletion genotypes in the prototrophic S288c strain and repeated the MIC_{FLC} analyses. As seen in Fig. S2, the effect of these deletions on growth in the presence of fluconazole remained persistent, indicating that the auxotrophies of the BY4742 reference strain did not interfere in the observed phenotypes.

Interference in vesicular transport lowers the cellular ergosterol content in *S. cerevisiae*. Fluconazole targets the lanosterol 14 α -demethylase enzyme, encoded by *ERG11*, which is involved in the biosynthesis of ergosterol. To determine whether vesicular transport affects the level of this sterol in the cell and could thereby link this process to fluconazole susceptibility, we measured the level of ergosterol using an assay described in the literature (35). In a previous report, we showed that this method yields results very comparable to those obtained by gas chromatography-mass spectrometry-based sterol analysis (32), indicating that it is justifiable to use the technically simpler spectrophotometric technique for the analysis of the cellular ergosterol content. Figure 2 shows the ergosterol level in the BY4742 wild-type strain and mutants in the absence and presence of fluconazole. In the absence of fluconazole, the vesicular transport mutants seemed to accumulate slightly, yet significantly, more ergosterol than the wild-type strain. However, when fluconazole was added, the ergosterol levels of these mutants dropped far below the level for the wild-type strain, linking ergosterol levels to the increased susceptibility to fluconazole. Since the level of ergosterol in the mutants was not lowered in the absence of fluconazole, ergosterol synthesis as such is not a target of vesicular transport. We speculate that upon impairment of vesicular transport, fluconazole somehow accumulates in the cell, thereby lowering the residual amount of ergosterol.

Vesicular transport mutants with increased fluconazole susceptibility show lowered expression of genes encoding drug efflux pumps. We showed that, specifically in the presence of fluconazole, the ergosterol content of the six vesicular transport mutants was lower than that in the wild-type strain. We hypothesized that a relative increase in the intracellular drug concentration compared to that in the wild

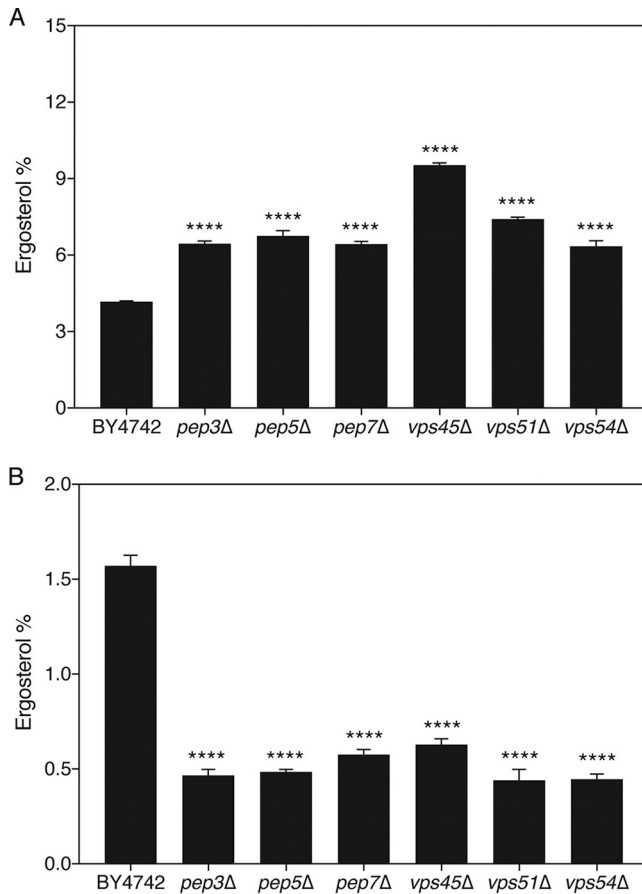


FIG 2 Proper vesicular transport is essential to retain large amounts of ergosterol upon fluconazole treatment in *S. cerevisiae*. The deletion strains and wild-type strain were grown in SD_{glu} medium for 48 h at 30°C in the absence (A) or the presence (B) of 20 μ g/ml fluconazole. Sterols were extracted, and the level of ergosterol was monitored spectrophotometrically. Average levels together with the standard error of the mean (SEM) are displayed. Statistical analysis was conducted by one-way analysis of variance with the Bonferroni correction. ****, $P < 0.0001$.

type would be caused by a lower level of drug efflux. It was reported earlier that deletion of *ScVPS3*, which encodes a protein involved in transporting proteins to the vacuole as a member of the CORVET complex, lowers drug efflux in *S. cerevisiae* (36). We postulated that the same would be true for other mutants with mutations in the vesicular transport pathway. We aimed to determine whether vesicular transport affects the expression of genes encoding known drug efflux pumps in *S. cerevisiae*. We tested expression of the main genes encoding drug efflux pumps, *ScPDR5*, *ScPDR10*, *ScPDR11*, *ScPDR15*, *ScYCF1*, *ScYOR1*, and *ScSNQ2*, or regulators thereof, *ScPDR3* and *ScPDR1*, in the absence and presence of fluconazole (37). Remarkably, expression of most of these genes was significantly lower in the mutant strains than in the wild-type strain under both conditions (Fig. 3 and S3). This indicates that impairment of vesicular transport affects drug efflux gene expression, drug efflux, and, possibly, fluconazole susceptibility in this manner. We cannot exclude the possibility, however, that other processes also contribute to the process.

***Candida albicans* PEP5 is involved in tolerance to fluconazole.** *C. albicans* is a commonly isolated human-pathogenic fungus. To verify whether vesicular transport could similarly affect susceptibility to fluconazole in this fungus, as we described for *S. cerevisiae*, we tested the *C. albicans vps11*Δ/Δ (*CaVps11*Δ/Δ) strain (kindly provided by Glen Palmer) on medium containing fluconazole (38). *CaVPS11* is the orthologue of the *ScPEP5* gene of *S. cerevisiae*. Neither the Etest assay nor the broth microdilution assay showed a decrease in the MIC_{FLC} for the deletion mutant compared to that for the wild

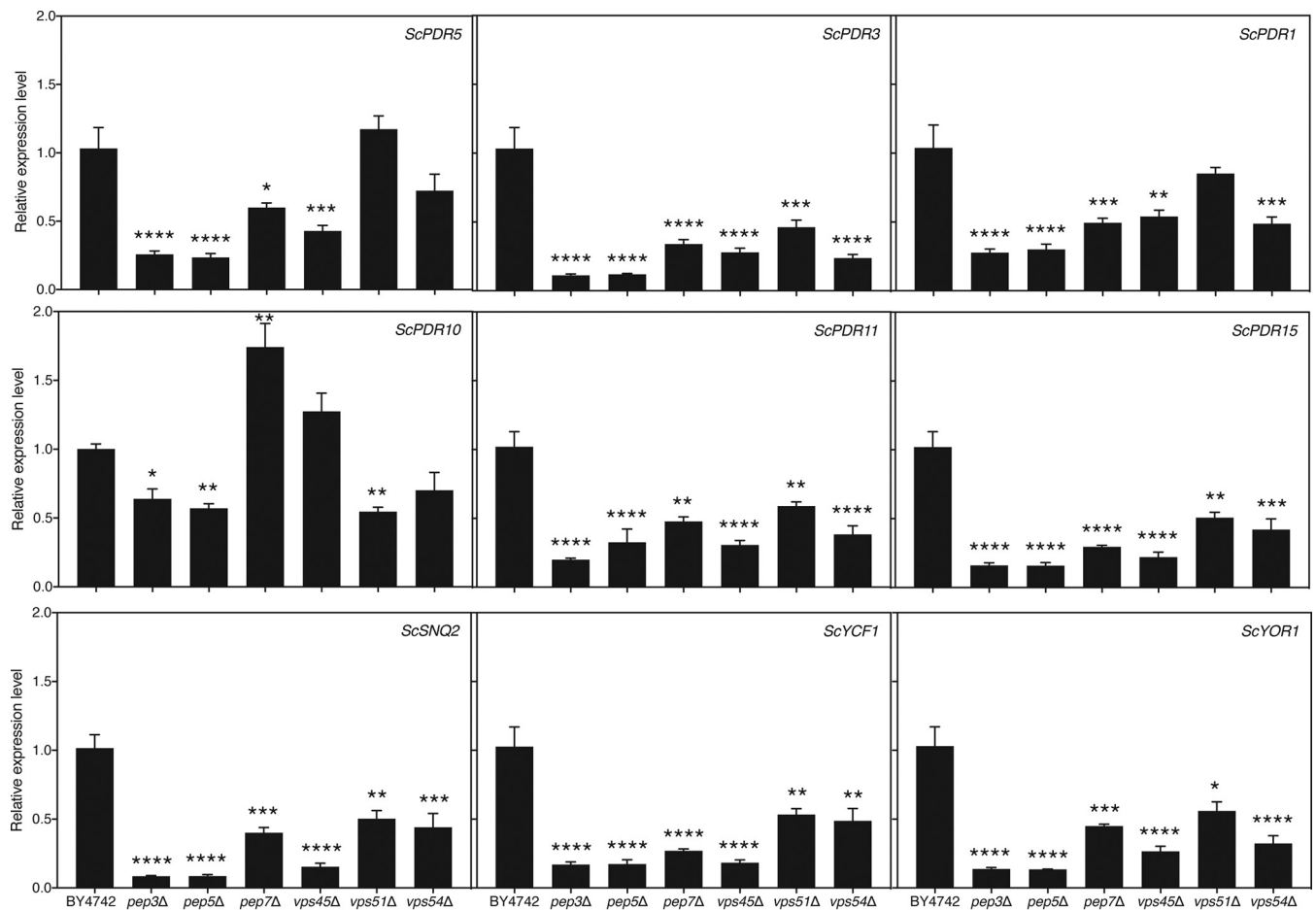


FIG 3 Expression of drug efflux-related genes is downregulated in *S. cerevisiae* vesicular transport mutant strains. The deletion strains, as well as the wild-type strain, were incubated in SD_{glu} medium for 24 h at 30°C in the absence of fluconazole. Gene expression was analyzed using quantitative reverse transcriptase PCR. Average results relative to those for the wild-type control together with the SEM are displayed. Statistical analysis was conducted on log₂(y)-transformed values using one-way analysis of variance with the Bonferroni correction. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

type, indicating that different processes might be involved in both organisms (Table 1 and Fig. S4). Despite the absence of an altered MIC_{FLC}, we could, however, show that the tolerance to fluconazole or growth in the presence of supra-MIC_{FLC}s of the drug was lower for the *Cavps11Δ* mutant strain than for the background strain (Fig. 4). The starting inoculum of approximately 1,830 cells per ml dropped to 0 at higher fluconazole concentrations in the mutant, while it doubled in the wild-type strain. The complete lack of survival at higher fluconazole concentrations indicates that the mutation renders the drug fungicidal in this organism, as cells initially inoculated do not survive treatment. To determine whether vesicular transport also affects expression of drug efflux-related genes in *C. albicans*, we tested the expression of four genes encoding drug efflux pumps. Figure S5A shows the effect of *CaVPS11* deletion on drug efflux gene expression. *CaCDR1*, *CaMDR1*, and *CaSNQ2* gene expression was decreased in the mutant, while *CaCDR2* expression was increased.

***Candida glabrata* VPS45 is involved in resistance and tolerance to fluconazole.**

As described above for *C. albicans*, we tested a *C. glabrata* mutant with a mutation in vesicular transport for altered growth in the presence of fluconazole. The *C. glabrata* *VPS45* (*CgVPS45*) gene is the orthologue of the *ScVPS45* gene in *S. cerevisiae*. Both the Etest analysis and the broth microdilution assay showed a reduced MIC_{FLC} for the deletion mutant compared to that for the wild-type strain (Table 1 and Fig. S6). Moreover, we found that the tolerance to fluconazole was also decreased in the *vps45Δ* strain (Fig. 5). This indicates that susceptibility to fluconazole, encompassing

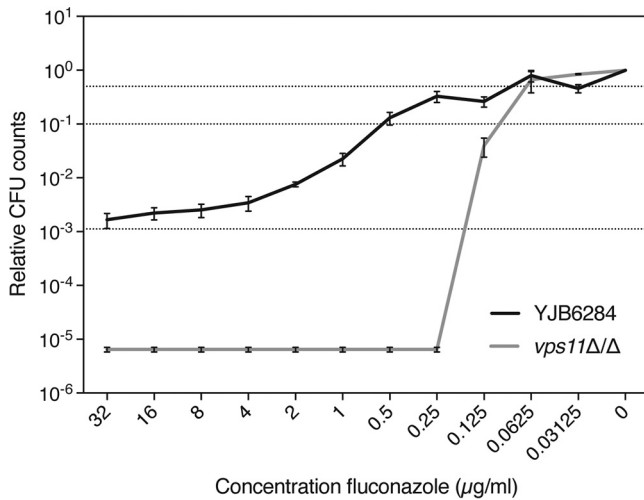


FIG 4 Tolerance to fluconazole is reduced in a *C. albicans* *vps11Δ/Δ* mutant compared to the wild-type control. Tolerance assay results for the *vps11Δ/Δ* mutant and the YJB6284 wild-type control strain are displayed as dose-response curves after 48 h of incubation at 37°C. Relative CFU counts compared to those achieved under the 0-μg/ml fluconazole condition are shown. Dotted lines indicate 50% (upper line) and 90% (middle line) growth inhibition and the initial inoculum (lower line).

both resistance and tolerance, is affected by vesicular transport in *C. glabrata*. To verify if vesicular transport also affects expression of drug efflux-related genes in *C. glabrata*, we tested the expression of genes encoding drug efflux pumps by the background strains and the *Cgvps45Δ/Δ* strain (Fig. S5B). Only *CgCDR2* expression was slightly decreased in the mutant strain compared to the background strain.

An inhibitor of vesicular transport exhibits synergistic interactions with fluconazole against *C. albicans* and *C. glabrata*. Identifying chemical compounds that selectively inhibit vesicular transport similarly to the mutants and, as such, that mimic their phenotypic interaction with fluconazole would hold great potential for combination therapy. We selected two compounds that were earlier identified to be modulators of vacuolar trafficking, termed sortins, for sorting inhibitors (30). Although the exact mechanism is not yet known, it has been shown in *S. cerevisiae*, as well as in *Arabidopsis thaliana*, that *sortin2* and *sortin3* affect vacuolar trafficking, potentially by inhibiting

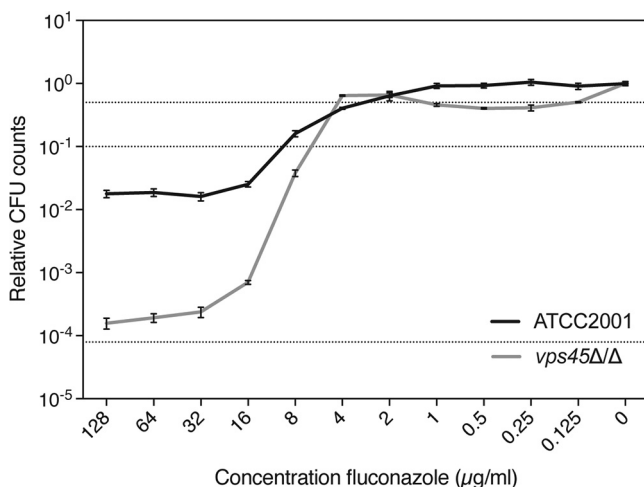


FIG 5 Tolerance to fluconazole is reduced in a *C. glabrata* *vps45Δ/Δ* mutant compared to the wild-type control. Tolerance assay results for the *vps45Δ/Δ* mutant and the ATCC 2001 wild-type control strain are displayed as dose-response curves after 48 h of incubation at 37°C. Relative CFU counts compared to those achieved under the 0-μg/ml fluconazole condition are shown. Dotted lines indicate 50% (upper line) and 90% (middle line) growth inhibition and the initial inoculum (lower line).

TABLE 2 Analysis of interaction between fluconazole and sortins for the three species^a

Strain	Sortin	MIC ($\mu\text{g/ml}$)		Sortin		FIC		ΣFIC
		FLC	FLC + sortin	Sortin	Sortin + FLC	FLC	Sortin	
MIC ₅₀								
<i>S. cerevisiae</i>	2	16	16	200	10	1	0.05	1.05
	3	16	16	200	25	1	0.125	1.125
<i>C. albicans</i>	2	0.0625	0.125	200	10	2	0.05	2.05
	3	0.125	0.5	200	200	4	1	5
<i>C. glabrata</i>	2	32	8	200	5	0.25	0.025	0.275
	3	32	16	200	200	0.5	1	1.5
MIC ₉₀								
<i>S. cerevisiae</i>	2	16	16	200	200	1	1	2
	3	16	16	200	25	1	0.125	1.125
<i>C. albicans</i>	2	4	1	200	50	0.25	0.25	0.5
	3	4	64	200	200	16	1	17
<i>C. glabrata</i>	2	64	32	200	25	0.5	0.125	0.625
	3	128	128	200	200	1	1	2

^aThe MIC was determined at 50 or 90% inhibition. The fractional inhibitory concentration (FIC) was determined by dividing the MIC of the drug in the combination by the MIC of the drug alone. The FIC index (ΣFIC) was determined by adding the FIC for fluconazole to the FIC for sortin.

members of the endomembrane complex (27, 28). We tested the effect of the two small compounds both on the MIC_{FLC} and on tolerance to fluconazole in *C. albicans* and *C. glabrata* and calculated the fractional inhibitory concentration (FIC) values of the drugs as well as the FIC index (ΣFIC) of the interaction (Table 2). Figures 6A and 7A show the results of the checkerboard assay carried out for both pathogenic fungi. Sortin3 did not seem to have any effect on the MIC for fluconazole in *C. glabrata* (Fig. 6A), yet in *C. albicans* increasing concentrations gave rise to increased MIC_{FLC} levels, indicating an antagonistic relationship (Fig. 7A). According to the CLSI guidelines, the ΣFIC for *C. glabrata* of 1.5 or 2 for the MIC₅₀ and MIC₉₀, respectively, indicates an indifferent interaction (39). The ΣFIC for *C. albicans* of 5 and 17 for the MIC₅₀ and MIC₉₀, respectively, indicates a clear antagonistic relationship. This apparent antagonism was also visible in the tolerance assay, where higher levels of sortin3 clearly stimulated survival in the presence of higher concentrations of fluconazole specifically in *C. albicans* (Fig. 7B) and not in *C. glabrata* (Fig. 6B). Sortin2, in general, acted synergistically with fluconazole against both fungi. This was especially visible in the tolerance assay (Fig. 6B and 7B). The MIC_{FLC} of *C. glabrata* was also mildly lower with higher concentrations of the compound, while this was not clearly visible in *C. albicans*. The ΣFIC values confirmed this relation. For *C. glabrata*, the ΣFIC for the MIC₅₀ was 0.275, indicating synergism, and the ΣFIC for the MIC₉₀ was 0.625, indicating an indifferent yet nearly synergistic relationship. For *C. albicans*, the ΣFIC for the MIC₅₀ was 2.05, indicating indifference, and the ΣFIC for the MIC₉₀ was 0.5, indicating synergism. These data confirm, at least for *C. albicans*, that the synergistic effect of sortin2 with fluconazole becomes clearer at higher fluconazole concentrations. We can conclude that sortin2 and sortin3 clearly distinguishably act with fluconazole against different species. Sortin2 acted synergistically with fluconazole at higher concentrations of the latter drug, as such inhibiting tolerance. Specifically for *C. glabrata*, an effect on the MIC_{FLC} could also be observed. Sortin3 did not elicit any clear effect in *C. glabrata*, yet for *C. albicans*, a clear antagonistic relation both on tolerance and on resistance could be observed. We also tested the effect of sortin2 and sortin3 against our model system, *S. cerevisiae*, yet found effects less significant than those against the pathogenic fungi (Table 2). Figure S7 shows that sortin2 increased the susceptibility to fluconazole. Remarkably, we saw that although sortin2 did lower the tolerance to fluconazole, this effect was restored at higher concentrations of the sorting inhibitor. While sortin3 did not alter the MIC_{FLC}, it did lower the tolerance to fluconazole mildly. Taken together, these data indicate that fine-tuning the exact role of vesicular transport in fluconazole susceptibility is of pivotal importance to further explore its potential as a target in combinatorial therapy with fluconazole.

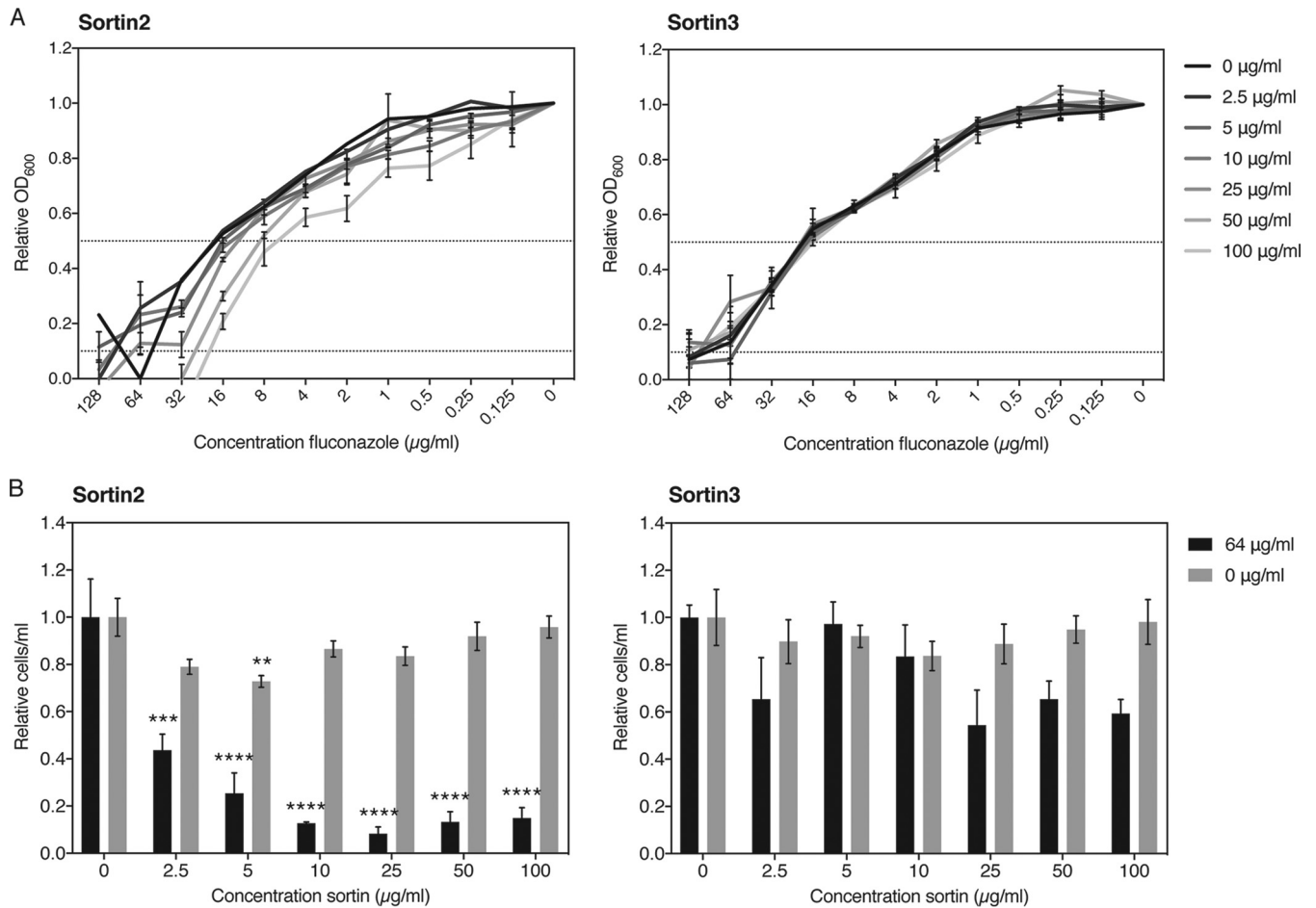


FIG 6 Sortin2 acts synergistically with fluconazole against *C. glabrata*. (A) Broth microdilution assay results for the BG2 wild-type strain in the presence of several concentrations of sortin2 or sortin3 after 48 h of growth at 37°C. Relative OD₆₀₀ values compared to those achieved under the 0-µg/ml fluconazole condition are shown. Dotted lines indicate 50% (upper line) and 90% (lower line) growth inhibition. The MIC₅₀ and MIC₉₀ values can be deduced from the cross-section between the respective dotted lines and the data curves. (B) Tolerance assay results for the BG2 wild-type strain in the presence of several concentrations of sortin2 or sortin3. CFU counts were compared in the absence and the presence of fluconazole (64 µg/ml) relative to those achieved under the 0-µg/ml sortin condition. Statistical analysis was conducted using one-way analysis of variance with the Bonferroni correction. **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

Sortin2 lowers the tolerance of *C. glabrata* and *C. albicans* in clinically relevant isolates.

To explore the clinical relevance of synergy between sortin2 and fluconazole, we tested the effect of this drug combination on a number of clinically retrieved isolates of *C. albicans* and *C. glabrata*. The effect of sortin2 both on the MIC of fluconazole and on tolerance was monitored. The MIC_{FLC} values of the various isolates in the presence (100 µg/ml) and absence of sortin2 are depicted in Table 3. We show that, overall, the MIC₅₀ of fluconazole in the presence of sortin2 remained similar to that when this compound was not added, while the MIC₉₀ mostly decreased. Figure S8 depicts the effect of sortin2 on overall growth in the presence of various concentrations of fluconazole. Figure 8 confirms that, especially in *C. glabrata* isolates, the tolerance to fluconazole dropped dramatically when vesicular transport was inhibited by sortin2. In *C. albicans* isolates, a similar trend could be observed, yet the difference was not always significant due to larger variation between biological repeats.

DISCUSSION

In this study, we found that impairment of vesicular transport in fungal cells potentiates the antifungal effect of fluconazole.

In the model organism *S. cerevisiae*, six deletion mutants affected in vesicular transport were identified to have increased susceptibility to this widely used drug. We showed that upon treatment, these strains retained significantly less ergosterol than

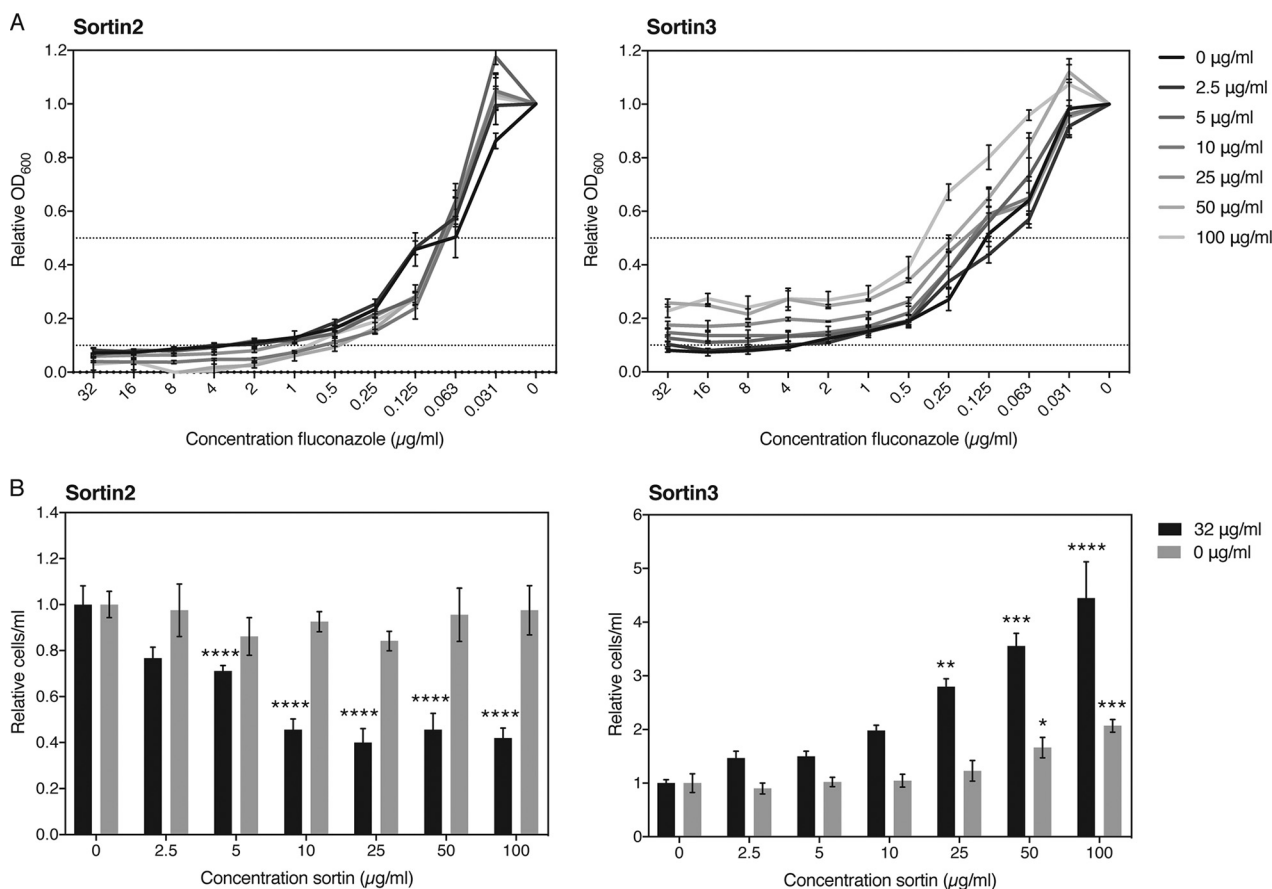


FIG 7 Sortin2 acts synergistically and sortin3 acts antagonistically with fluconazole against *C. albicans*. (A) Broth microdilution assay results for the SC5314 wild-type strain in the presence of several concentrations of sortin2 or sortin3 after 48 h of growth at 37°C. Relative OD₆₀₀ values compared to those achieved under the 0-µg/ml fluconazole condition are shown. Dotted lines indicate 50% (upper line) and 90% (lower line) growth inhibition. The MIC₅₀ and MIC₉₀ values can be deduced from the cross-section between the respective dotted lines and the data curves. (B) Tolerance assay results for the SC5314 wild-type strain in the presence of several concentrations of sortin2 or sortin3. CFU counts were compared in the absence and the presence of fluconazole (32 µg/ml) relative to those achieved under the 0-µg/ml sortin condition. Statistical analysis was conducted using one-way analysis of variance with the Bonferroni correction. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

the wild-type control strain, leading ultimately to a lower MIC_{FLC} and/or tolerance to fluconazole. Remarkably, in the absence of the drug, a slight yet significant increase in ergosterol levels could be observed in the mutants compared to the wild-type strain. This indicates that the mutant genotypes as such do not negatively affect ergosterol

TABLE 3 Effect of sortin2 on MIC_{FLC} of clinical isolates^a

Isolate	MIC (µg/ml)			
	50%		90%	
	FLC	FLC + sortin2	FLC	FLC + sortin2
<i>C. glabrata</i>				
1	16	8	32	16
2	8	8	64	32
3	16	16	64	32
4	8	8	32	16
<i>C. albicans</i>				
1	0.25	0.125	1	0.5
2	16	8	128	16
3	0.125	0.0624	128	1
4	16	8	128	16

^aThe MIC values were determined at 50 or 90% inhibition in the absence and presence of 100 µg/ml sortin2.

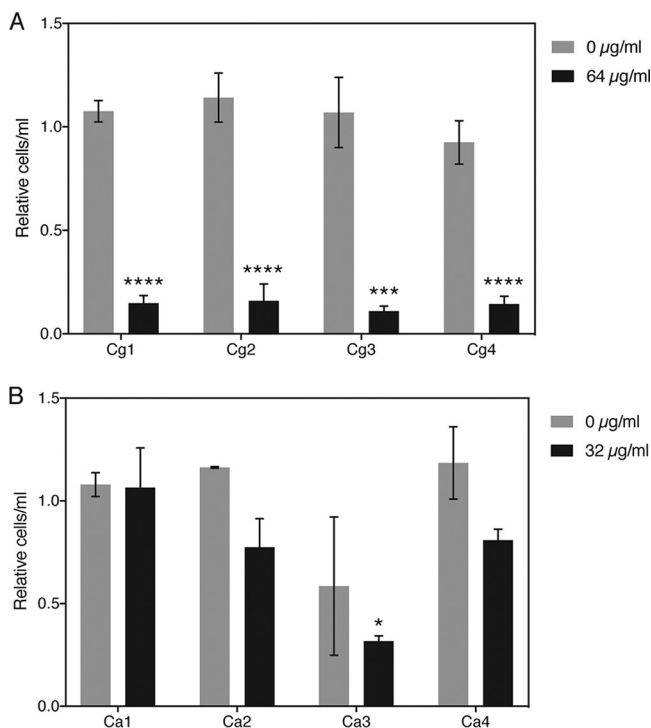


FIG 8 Sortin2 lowers tolerance to fluconazole in clinical *C. glabrata* (A) and *C. albicans* (B) isolates. Tolerance to fluconazole, indicated as CFU counts at 64 and 32 µg/ml fluconazole for *C. glabrata* and *C. albicans*, respectively, was determined in the absence and the presence of 100 µg/ml sortin2. All values were compared to those under the condition without sortin. Statistical analysis was conducted using two-way analysis of variance with the Bonferroni correction. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

metabolism but that the phenotype strictly depends on the presence of the drug. We expect that the inhibition of vesicular transport positively influences the concentration of the drug inside the cell, as such leading to stronger inhibition of the Erg11 enzyme and increased susceptibility. It was reported earlier that an *Scvps3*Δ mutant of *S. cerevisiae* displays lower drug efflux rates than the corresponding wild-type strain (36). *ScVps3* is a member of the CORVET complex, to which *ScPep3* and *ScPep5* also belong, and regulates tethering of vesicles to and from the endosome. To verify whether drug efflux would also be affected in the six deletion mutants resulting from our screening, we decided to measure the expression levels of seven genes encoding drug efflux pumps and two genes encoding regulators thereof. Remarkably, we found that expression of nearly all genes was significantly downregulated in the six deletion mutants in both the absence and the presence of fluconazole. When taking a closer look at the genes which were analyzed, we noticed that the expression of all of them is directly or indirectly regulated by the transcription factor *ScPdr1* (40, 41). *ScPDR1* expression itself is also reported to be a target of regulation by its own gene product in *C. glabrata* (42). Any effect of impaired vesicular transport on nuclear localization or expression of this protein/gene would thereby also likely lead to the observed effects on the expression of genes encoding drug efflux pumps or *ScPDR3*.

We confirmed a role for vesicular transport in fluconazole susceptibility in *C. albicans* and *C. glabrata*. Apart from the small and potentially insignificant effect on the MIC_{FLC} , homozygous deletion of the *CaVPS11* gene, the orthologue of the *ScPEP5* gene, caused the nearly complete absence of trailing growth at supra- MIC_{FLC} s of fluconazole. The *Cgyps45*Δ/Δ strain in a *C. glabrata* background similarly showed a decrease in tolerance yet also a lowered MIC_{FLC} . This indicates that the observations that we made in *S. cerevisiae* are, at least to some extent, relevant for other fungi as well. Expression of drug efflux genes seems altered to a lesser extent in these fungal pathogens than in *S. cerevisiae*. In both organisms, the expression of some efflux pumps was mildly de-

creased, while that of others was not. We hypothesize that the stronger effect of vesicular transport inhibition on the MIC_{FLC} in *S. cerevisiae* correlates specifically with altered levels of drug efflux gene expression.

Sortins are small compounds that interfere with vesicular trafficking to or from the vacuole and endosomes (30). Based on our observations on the role of vesicular transport in fluconazole susceptibility in *C. albicans* and *C. glabrata*, we verified whether these chemicals could synergize with fluconazole in these two organisms. For both *C. albicans* and *C. glabrata*, we observed a synergistic effect of sortin2 with higher concentrations of fluconazole. The level of tolerance or trailing growth at supra- MIC_{FLC} s of fluconazole was significantly reduced with higher levels of sortin2. Specifically, in *C. glabrata*, the MIC_{FLC} also dropped mildly when higher concentrations of sortin2 were added to the culture medium. We also showed that sortin2 lowered the tolerance to fluconazole in our model organism, *S. cerevisiae*, yet this effect seemed to be concentration dependent. These results indicate that inhibition of vesicular transport indeed synergizes with fluconazole against several fungi and, thus, emphasizes the potential of targeting this process in combination therapy. Contrary to the results obtained with sortin2, sortin3 did not affect fluconazole efficacy in *C. glabrata* and even antagonized its effect in *C. albicans* regarding both the MIC_{FLC} and tolerance. Contrary to what we found in *Candida* species, sortin3 acted mildly synergistically on tolerance with *S. cerevisiae*, yet only at high concentrations of sortin. No effect on the MIC_{FLC} could be distinguished. It thus seems to be of pivotal importance to elucidate how both sortins function.

Another aspect that needs to be considered when proposing sortin2 or an analogous compound for combination therapy with fluconazole is its potency against clinically relevant isolates. Therefore, we tested the effect of sortin2 against several clinical *C. glabrata* and *C. albicans* isolates with various MIC_{FLC} values. For nearly all isolates, sortin2 lowered the tolerance to fluconazole and the MIC_{90} of this drug. Especially in *C. glabrata*, the reduction was dramatic. The effect of the compound on the MIC_{50} of fluconazole was minimal, confirming that sortin2 specifically alters the growth of the pathogens at higher fluconazole concentrations. Although the reduction of growth upon treatment with sortin2 strictly depended on the presence of fluconazole, further in-depth analysis is necessary to confirm the absence of toxicity in mammalian cells, as it was shown that the effect of these sorting inhibitors is not limited to yeast cells (30).

In conclusion, we identified specific components of the vesicular transport pathway which are important for the development of resistance and/or tolerance to fluconazole in *Candida* species. In addition, we show here that inhibition of vesicular transport in a targeted way leads to synergism with fluconazole against several fungal species, as summarized in Fig. 9. Further analysis is necessary to elucidate the full potential of vesicular transport-inhibiting compounds, such as sortin2, in combination therapy with fluconazole. Apart from its potential clinical relevance, we show that sortin2 acts rather specifically against tolerance to fluconazole. We thus propose that this compound will be a valuable tool in further elucidation of the relevance and modes of action of this phenomenon in pathogenic fungi.

MATERIALS AND METHODS

Strains and growth conditions. All *S. cerevisiae* strains used in this study were isogenic to the BY4742 laboratory wild-type strain and are listed in Table S3 in supplemental material. The deletion strains used in further assays originated from the Yeast Knockout (YKO) collection and were recreated in this study to obtain at least three biological replicates of each genotype. The primers used to generate these strains are listed in Table S4. The *C. albicans vps11Δ/Δ* strain was used in combination with its wild-type strain, YJB6284 (38). For the wild-type and mutant *C. albicans* strains, three colonies were considered biological repeats. The *Cgvps45Δ/Δ* strain in the ATCC 2001 background (43) was created using the pYC44 plasmid (44). For sortin-fluconazole interaction assays, the BG2 clinical isolate was used as the wild-type strain (45), and for *C. albicans*, the SC5314 strain was used as the wild-type strain (46).

S. cerevisiae strains were grown in synthetic dextrose medium, consisting of 0.17% Difco yeast nitrogen base without amino acids or ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose (SD_{glu} medium). The pH of the medium was adapted to 5.5 for liquid medium and 6.5 for solid medium. After addition of 1.6% agar, the medium was autoclaved. Appropriate amino acids and nucleotides were

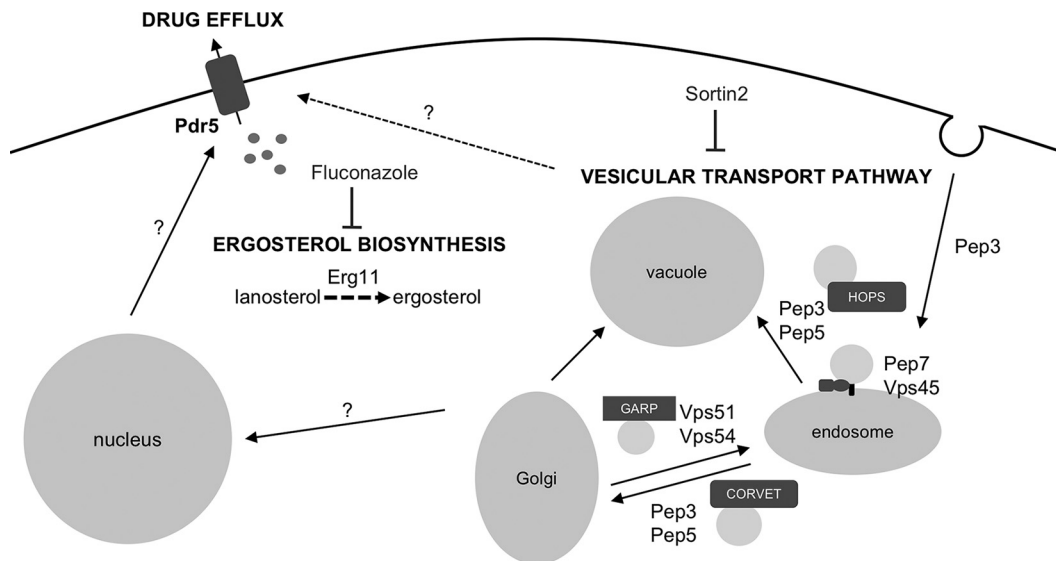


FIG 9 Schematic overview of the role of vesicular transport in susceptibility to fluconazole. The six proteins identified to be essential for retaining resistance and/or tolerance to fluconazole in *S. cerevisiae* are depicted according to their function in vesicular transport. We hypothesize that a signal originating from this process induces expression of drug efflux pump-encoding genes in the nucleus, thereby increasing the efflux of fluconazole and lowering the intracellular concentration of the drug. This would result in the increased production of ergosterol and reduced susceptibility to fluconazole. Sortin2 acts synergistically with fluconazole in *Candida* fungi by inhibiting vesicular transport. HOPS, homotypic fusion and vacuole protein sorting; GARP, Golgi-associated retrograde protein; CORVET, class C core vacuole/endosome transport complex.

added before use. In certain experiments, fluconazole (catalog number F8929; Sigma) and doxycycline (catalog number D9891; Sigma) were added, obtaining concentrations of, respectively, 10 or 20 $\mu\text{g/ml}$ and 50 or 100 $\mu\text{g/ml}$ (31). Cultures containing fluconazole and/or doxycycline were always kept in the dark.

C. albicans and *C. glabrata* strains were pregrown in SD_{glu} medium. Assays were carried out in filter-sterilized RPMI 1640 medium with L-glutamine (catalog number R6504; Sigma) and buffered with 0.165 M morpholinepropanesulfonic acid at pH 7. Depending on the assay, autoclave-sterilized and precooled agar and 1.8% glucose were added to the medium.

Screening the yeast knockout collection for enhancers of fluconazole susceptibility. The deletion strains from the *MAT α* Yeast Knockout (YKO) collection were screened according to a protocol adapted from one described previously (32, 34). The strains were initially spotted on solid SD_{glu} medium containing 10 or 20 $\mu\text{g/ml}$ fluconazole and 50 or 100 $\mu\text{g/ml}$ doxycycline (47). Addition of doxycycline reduced the background growth, thereby easing the visual inspection of the growth reduction. Enhancer strains of fluconazole susceptibility, which were deletion strains that showed increased susceptibility to the drug, were selected by visually monitoring reduced growth after 3 days of incubation at 30°C compared to the growth of the BY4742 wild-type laboratory strain and were stored in 96-well microtiter plates. The genes deleted in all enhancer strains were clustered, using the online tool FunSpec (33). These enhancers of fluconazole susceptibility were later retested on the same medium, using at least three biological replicates.

Determination of fluconazole susceptibility: spot assays, MIC_{FLC} evaluation, and tolerance assay. Spot assays were conducted by adapting the optical density at 600 nm (OD_{600}) to 1 and making serial 1/5 dilutions. The cultures were spotted on solid SD_{glu} medium containing different concentrations of fluconazole and/or doxycycline. After incubation of the plates at 30°C for 72 h, they were scanned.

To determine the MIC of fluconazole (MIC_{FLC}) of the strains, both the Etest (bioMérieux) and broth microdilution assay (BDA) were used (48, 49). For the Etest assay, overnight *S. cerevisiae* cultures were adjusted to an OD_{600} of 0.5 in water and spread on SD_{glu} agar medium. After placing the strip onto the cells, the plates were incubated for 72 h at 30°C. The MIC_{FLC} was determined by identifying the concentration of fluconazole on the strip where the latter intersects the halo of growth inhibition/retardation (49). For *C. albicans* and *C. glabrata*, the same protocol was used, but the culture was diluted to an OD_{600} of 0.2 instead of 0.5 and plated on RPMI 1640 agar medium containing 2% glucose (32). The plates were scanned after 48 h of incubation at 37°C. The Etest method was used in parallel with the broth microdilution assay. This technique was performed using a protocol based on both CLSI (39) and EUCAST (50) standard methods. According to the CLSI protocol, cells were loaded in round-bottom, UV-sterilized 96-well microtiter plates to obtain a concentration of 0.5×10^3 to 2.5×10^3 cells per milliliter (39). Fluconazole was added to the wells, together with SD_{glu} or RPMI 1640 medium, at concentrations ranging from 0 to 128 $\mu\text{g/ml}$ or 32 $\mu\text{g/ml}$, in the case of *C. albicans*, in 1/2 dilutions. According to CLSI, one can determine breakpoints after 24 or 48 h of static growth. We determined the growth after 48 h of incubation at 30°C or 37°C for *S. cerevisiae* and the *Candida* strains, respectively, in

order to achieve clearer breakpoints (32). The OD_{600} of the resuspended cultures was measured objectively using a spectrophotometer and plotted to obtain dose-response curves, according to EUCAST measures (50). We earlier showed the proper concordance of both CFU counts and spectrophotometric measurements to determine the MIC_{FLC} (32). The MIC_{FLC} was calculated by subtracting the background OD of the growth medium from all data points and normalization to the condition with 0 $\mu\text{g/ml}$ fluconazole. When this relative OD drops below 50 or 10% of the initial OD with no drug, the MIC_{50} and MIC_{90} , respectively, can be determined.

To evaluate drug tolerance, we generated dose-response curves based on CFU counts for the control and/or mutant strain as described by CLSI (39) and as established earlier (32). In short, we loaded the cells in round-bottom, UV-sterilized 96-well microtiter plates to obtain a concentration of 0.5×10^3 to 2.5×10^3 cells per milliliter (39). Fluconazole was added to the wells, together with SD_{glu} or RPMI medium, at concentrations ranging from 0 to 128 $\mu\text{g/ml}$ or 32 $\mu\text{g/ml}$, in the case of *C. albicans*, in 1/2 dilutions. After 48 h of growth at 30 or 37°C, the contents of the wells of the broth microdilution assay plate were resuspended, and each culture was diluted and plated. The drug tolerance was determined by checking the CFU counts under the conditions seen with the two highest fluconazole concentrations. All experiments were conducted with at least three biological replicates.

Fluconazole interaction analysis: checkerboard assay. The interaction between fluconazole and sortins was determined by checkerboard analysis, generally as described previously (31). *S. cerevisiae* strains were incubated in SD_{glu} medium, and *Candida* strains were incubated in RPMI medium supplemented with 2% glucose. Cells were added in round-bottom, UV-sterilized 96-well microtiter plates to obtain a concentration of 0.5×10^3 to 2.5×10^3 cells per milliliter. Fluconazole was added to the wells at concentrations ranging from 0 to 128 $\mu\text{g/ml}$ in 1/2 dilutions in the appropriate medium. In addition, sortins were first dissolved in 20% dimethyl sulfoxide (DMSO) and were added at concentrations of 0, 2.5, 5, 10, 25, 50, and 100 $\mu\text{g/ml}$ in the appropriate medium containing a final concentration of 1% DMSO. After 72 h of static growth at 30°C for *S. cerevisiae* or 48 h of growth at 37°C for the *Candida* strains, the OD_{600} of the resuspended cultures was measured using a spectrophotometer and plotted to obtain dose-response curves. The MIC_{FLC} was calculated by subtracting the background OD of the growth medium with sortins from all data points and normalization to the condition with 0 $\mu\text{g/ml}$ fluconazole and 0 $\mu\text{g/ml}$ sortin. Additionally, for each concentration of sortin, CFU were plated in one of the highest and lowest concentrations of fluconazole on yeast extract-peptone-dextrose plates to determine tolerance. ΣFIC values were determined using the following formula, based on the CLSI guidelines (39) and according to the Instructions to Authors of *Antimicrobial Agents and Chemotherapy* (51): $(MIC_{FLC} + \text{sortin})/MIC_{FLC} + (MIC_{\text{sortin} + FLC}/MIC_{\text{sortin}})$. Based on these guidelines, a ΣFIC of ≤ 0.5 implies synergy, a ΣFIC of >4 implies antagonism, and the values in between denote indifference. Both the ΣFIC for an MIC_{50} and a ΣFIC for an MIC_{90} were determined, based on MIC_{50} and MIC_{90} data.

Gene expression analysis by quantitative reverse transcriptase PCR. We performed gene expression analysis according to the protocol described previously (32). The *S. cerevisiae* deletion strains, as well as the wild-type strain, were incubated in SD_{glu} medium for 24 h at 30°C in the absence or the presence of 20 $\mu\text{g/ml}$ fluconazole. The *C. albicans* and *C. glabrata* strains were inoculated in RPMI 1640 medium with 2% glucose and 8 $\mu\text{g/ml}$ fluconazole and incubated for 8 h at 37°C. The cells were washed with cold Milli-Q water, frozen in liquid nitrogen, and kept at -80°C as long as necessary. The pellets were resuspended in the TRIzol reagent (Thermo Fisher), followed by breaking of the cells with glass beads using a FastPrep machine. RNA was extracted using chloroform and isopropanol and was washed with 70% ethanol afterwards. To break down the residual DNA, DNase enzyme (New England Biolabs) was added. The RNA was converted into cDNA using an iScript cDNA synthesis kit from Bio-Rad. The actual quantitative PCR was performed with the GoTaq polymerase from Promega and a StepOnePlus device from Thermo Fisher. Data analysis was performed using the qBasePlus software from Biogazelle. Statistical analysis was performed using GraphPad Prism software. Prior to that, all data points were $\log_2(y)$ transformed in order to allow the use of standardized statistical methods.

Sterol measurement. Sterols were measured as described previously (35, 52). The strains under study were incubated in SD_{glu} medium for 48 h at 30°C in the absence or the presence of 20 $\mu\text{g/ml}$ fluconazole. Cells were washed with cold Milli-Q water, frozen in liquid nitrogen, and kept at -80°C as long as necessary. The cell pellets were resuspended in 3 ml saponification medium, consisting of KOH, H_2O , and ethanol, and vortexed for 1 min. After incubation of the samples for 1 h at 80°C, 1 ml of water and 4 ml of hexane were added. After another 3 min of vortex mixing, the two layers were allowed to separate. The $OD_{281.5}$ and OD_{230} of the upper layer were measured using a spectrophotometer and UV-transmittable 96-well microtiter plates (Costar; Corning). The percentage of ergosterol relative to the wet weight was measured using the formula introduced in reference 35.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01998-18>.

SUPPLEMENTAL FILE 1, PDF file, 3.2 MB.

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L.D. and K.V.D. conceived of and performed all experiments. All authors contributed to discussion of the results.

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