



# Unraveling Caspofungin Resistance in *Cryptococcus neoformans*

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**ABSTRACT** *Cryptococcus neoformans* is a basidiomycetous yeast responsible for hundreds of thousands of deaths a year and is particularly threatening in immunocompromised patients. There are few families of antifungals that are available to fight fungal infections, and the unique efficient treatment for the most deadly cerebral forms of cryptococcosis is based on a combination of 5-fluorocytosine and amphotericin B. The toxicities of both compounds are elevated, and more therapeutic options are urgently needed for better management of life-threatening cryptococcosis. The newest class of antifungals, i.e., echinocandins, has initially led to great hope. Unfortunately, *C. neoformans* was rapidly confirmed to be naturally resistant to these molecules, notably caspofungin. In this respect, we discuss here the recent key findings of the Panepinto research group published in *mBio* (M. C. Kalem et al., *mBio* 12:e03225-20, 2021, <https://doi.org/10.1128/mBio.03225-20>) that provide an unprecedented view of how *C. neoformans* regulates caspofungin resistance through a complex posttranscriptional regulation of cell wall biosynthesis genes.

**KEYWORDS** *Cryptococcus neoformans*, caspofungin, antifungal resistance, cell wall, posttranscriptional regulation

Like bacterial or viral infections, fungal diseases represent a major problem in human health (1, 2). These yeast and mold infections are particularly worrisome in the most vulnerable subjects, mainly immunocompromised patients, in whom they usually develop as deadly deep-seated infections. Although the epidemiology of these fungal diseases has substantially changed in the past few decades, *Candida albicans* and *Aspergillus fumigatus* but also *Cryptococcus neoformans* remain the prominent causative agents of the most life-threatening forms and are responsible for 1 million deaths a year worldwide (3). In the field of medical mycology, *C. neoformans* is one of the species that are well documented for standing out biologically from the other pathogenic fungi, which are predominantly Ascomycota, insofar as this yeast is phylogenetically related to the Basidiomycota. This is reflected in its particular ecology, its unique morphology (i.e., a capsulated spherical yeast), its genetics, and in its antifungal susceptibility profile (4–6). Like *Pneumocystis jirovecii*, *C. neoformans* contributed to bleak years when AIDS emerged in the 1980s, the latter killing most of the immunocompromised patients with AIDS. Nowadays, although things are getting better since the introduction of the systematic anti-HIV tri-therapy, this opportunist yeast still accounts for more than 600,000 fatal cases of meningitis a year, with marked and increasing impact in transplant patients (2). While *C. neoformans* is naturally resistant to azole antifungals (e.g., fluconazole, inhibiting ergosterol biosynthesis), the deep-seated forms of cryptococcosis are commonly treated first with a combination of the prodrug 5-fluorocytosine (inhibiting the pyrimidine salvage pathway) and amphotericin B (disrupting ergosterol functions by directly binding this essential sterol) (7). Because of the virulence of this fungal agent and the toxicity of this limited therapeutic option, *C. neoformans* is still responsible for 15% of AIDS-related deaths (7, 8). Yet, from the beginning of the

**Citation** Papon N, Goldman GH. 2021. Unraveling caspofungin resistance in *Cryptococcus neoformans*. *mBio* 12:e00156-21. <https://doi.org/10.1128/mBio.00156-21>.

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For the article discussed, see <https://doi.org/10.1128/mBio.03225-20>.

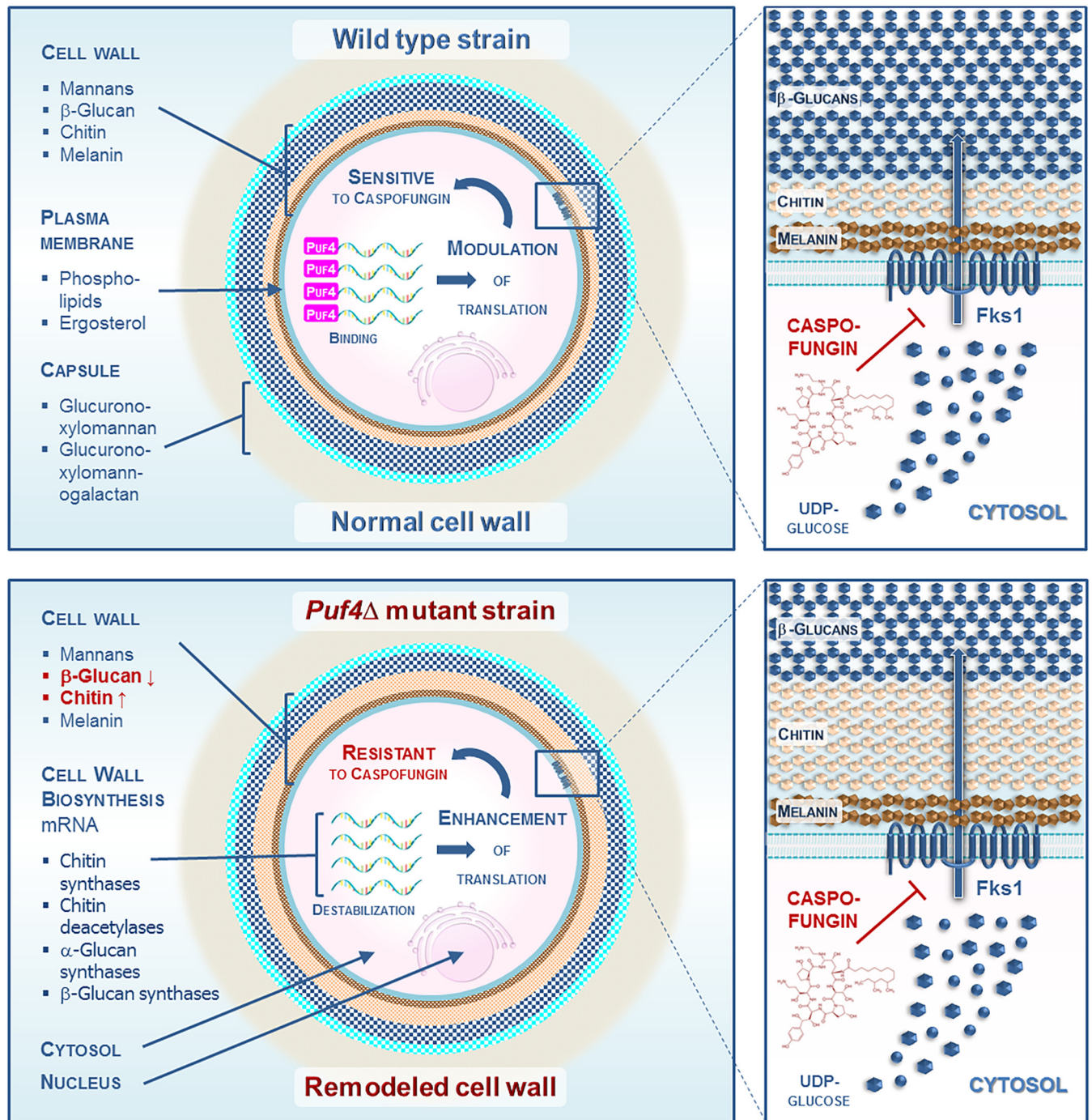
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**Published** 16 March 2021

2000s, unprecedented hopes arose following the availability of a new class of highly active antifungals, namely, echinocandins. Unfortunately, as soon as the 2010s, it became rapidly well-ingrained in the literature that *C. neoformans* is naturally resistant to echinocandins (9). However, and intriguingly, preliminary investigations challenging the protein sequence of the echinocandin fungal target,  $\beta$ -1,3-glucan synthase, encoded by the *FKS1* gene, revealed that resistance may not rely on specific amino acid variations, as previously observed in *Candida* and *Aspergillus* species. *In vitro* experiments showed that *C. neoformans* 1,3- $\beta$ -glucan synthase activity is very sensitive to caspofungin and cilofungin, suggesting that echinocandin resistance may be due to a mechanism unrelated to echinocandin inhibitors (9). Obviously, this led some research groups to the identification of new candidate mechanisms that may underlie echinocandin resistance in *C. neoformans*, such as cell wall remodeling and integrity pathways governing, in particular, chitin contents. In such a perspective, identifying molecular components of these regulatory pathways emerged as the main interest since they may represent new targets whose inhibition may potentiate the activities of echinocandins (10).

In this respect, several studies demonstrated a crucial role of calcineurin signaling pathways in the regulation of echinocandin resistance, intracellular trafficking, cell integrity, and RNA processing (11). These observations progressively propelled calcineurin as a pivotal stress-integrating hub regulating a plethora of cell processes, including drug tolerance in connection with cell wall composition. In this context, the research group of John C. Panepinto previously reported the involvement of the pumilio domain and FBF (PUF) domain-containing RNA-binding protein Puf4 in the regulation of endoplasmic reticulum stress in *C. neoformans* (12). Given that Puf4 has also been shown to act as an effector of calcineurin signaling, this RNA binding protein has cast its spell on the preliminary experiments of Kalem and colleagues.

Intriguingly, the investigators first observed that the *puf4* $\Delta$  mutant displayed a marked resistance to the echinocandin drug caspofungin compared to the wild-type strain. In addition, *PUF4* transcript and protein expression was shown to be decreased in the presence of caspofungin, suggesting the indirect influence of genetic interactions occurring between components of posttranscriptional gene regulatory networks on the drug resistance phenotypes. Then, the authors detailed the mechanism by which Puf4 directly binds and stabilizes the *FKS1* mRNA (encoding  $\beta$ -1,3-glucan synthase) by triggering a specific 5' sequence called PBE (Puf4 binding element). Further experiments allowed Kalem and colleagues to nicely show that Puf4 may play a role in modulating not only *FKS1* expression but also the cell wall  $\beta$ -1,3-glucan contents. They provided molecular evidence that an increased translation of the *FKS1* mRNA is proportionally correlated with Fks1 protein abundance in a mutant lacking the *PUF4* gene. This means that Puf4 may act as a repressor of *FKS1* mRNA translation in *C. neoformans*. Interestingly, beyond binding *FKS1* mRNA, the Puf4 protein was also shown to directly interact with PBE, predicted to be within the sequence of a series of additional cell wall biosynthesis mRNAs, such as those encoding chitin synthases, chitin deacetylases, and  $\alpha$ -glucan and  $\beta$ -glucan synthases. More specifically, the results shed light on multiple interactions that modulate the stability of cell wall biosynthesis mRNA, triggering either a positive or a negative effect on their translation during caspofungin treatment. In sum, all these results are consistent with a role of Puf4 as a master posttranscriptional regulator of cell wall remodeling that tunes caspofungin susceptibility. Finally, the structural relevance (i.e., cell wall composition changes) deriving from this complex Puf4-mediated molecular interplay was nicely revealed thanks to specific staining coupled with microscopy and flow cytometry approaches. In particular, this gave evidence that the absence of Puf4-mediated gene regulation creates a cell wall enriched in chitin and exposed chito-oligomers, while being devoid of  $\beta$ -1,3-glucan. Overall, this enlightening article provides an unprecedented view of how *C. neoformans* regulates caspofungin resistance through a complex posttranscriptional regulatory network (Fig. 1).



**FIG 1** Cell wall biosynthesis genes and cell wall remodeling are posttranscriptionally regulated by Puf4 and govern caspofungin resistance in *C. neoformans*. In the wild-type strain, Puf4 binds cell wall biosynthesis mRNA and thus controls its stability and abundance. This participates in maintaining the cell wall composition and architecture. In the *puf4*Δ mutant strain, cell wall biosynthesis transcripts are destabilized. The lack of gene regulation by Puf4 induces a cell wall harboring more chitin and less  $\beta$ -1,3-glucan. All these posttranscriptional regulations of the cell wall biosynthesis transcripts influence caspofungin resistance in cells lacking Puf4.

For more than 3 decades, the identification of fungal pathogenesis factors as new targets, the search for new biological and synthetic compounds with interesting antifungal activities, and deciphering molecular mechanisms involved in primary or acquired resistance toward antifungals have been part of a global strategy for antifungal drug development aiming ultimately at improving the management of deep-seated fungal infections (13, 14). Indeed, in the same way as antibiotic resistance in



bacterial pathogens—but of course to a lesser extent—antifungal resistance has progressively become a serious clinical issue (15, 16). We know now that in medical mycology, this relies on two main features, namely, the extensive use of antifungal drugs for the prevention and treatment of an increasing number of immunocompromised patients (e.g., more patients with cancers or receiving transplants, etc.) and the emergence of naturally multiresistant fungal agents (e.g., Mucorales, *Candida auris*). A rapid overview of the literature teaches us that, globally, acquired resistances deriving from antifungal monotherapies usually involve a single to a couple of single nucleotide polymorphisms in the genomes of resistant isolates, suggesting that a few point mutations are sufficient to dynamically select resistant populations for surviving in a treated host organism. However, the context is sharply different in the case of primary resistance. While not deriving from drug exposure, underlying molecular mechanisms of primary resistance are mostly of multifactorial origins and were likely developed during the stepwise evolution and specialization of fungal lineages (17). As a consequence, unravelling the molecular determinants of intrinsic resistance in fungal pathogens is often laborious work. With such a perspective, we must recognize this excellent report by the research group of John C. Panepinto, additionally because of the contribution of posttranscriptional gene regulation in drug resistance phenotypes, which has hitherto remained largely unexplored in pathogenic fungi due to the substantial complexity of these cell processes. However, there is no need to argue that a better understanding of such complex regulatory networks may lead to the identification of key components that could be targeted by adjunctive therapies for improving the efficacies of currently available drugs (10, 18–20).

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