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Article

Novel ABC Transporter Associated with Fluconazole Resistance in Aging of *Cryptococcus neoformans*

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Abstract: *Cryptococcus neoformans* causes meningoencephalitis in immunocompromised individuals, which is treated with Fluconazole (FLC) monotherapy when resources are limited. This can lead to azole resistance, which can be mediated by overexpression of ABC transporters, a class of efflux pumps. ABC pump-mediated efflux of FLC is also augmented in 10-generation old *C. neoformans* cells. Here, we describe a new ABC transporter Afr3 (CNAG_06909), which is overexpressed in *C. neoformans* cells of advanced generational age, that accumulate during chronic infection. The *Δafr3* mutant strain showed higher FLC susceptibility by FLC E-Test strip testing and also by a killing test that measured survival after 3 h FLC exposure. Furthermore, *Δafr3* cells exhibited lower Rhodamine 6G efflux compared to the H99 wild type cells. Afr3 was expressed in the *Saccharomyces cerevisiae* ADΔ strain, which lacks several drug transporters, thus reducing background transport. The ADΔ + Afr3 strain demonstrated a higher efflux with both Rhodamine 6G and Nile Red, even though the FLC MICs were not changed. Characterization of the *Δafr3* mutant revealed unattenuated growth but a prolongation (22%) of the replicative life span. In addition, *Δafr3* exhibited decreased resistance to macrophage killing and attenuated virulence in the *Galleria mellonella* infection model. In summary, our data indicate that a novel ABC pump Afr3p, which is upregulated in *C. neoformans* cells of advanced age may contribute to their enhanced FLC tolerance, by promoting drug efflux. Lastly, its role in macrophage resistance may also contribute to the selection of older *C. neoformans* cells during chronic infection.

Keywords: drug resistance; ABC transporter; efflux pump; cryptococcosis; aging

1. Introduction

Cryptococcus neoformans is an opportunistic yeast that infects immunocompromised individuals, causing meningoencephalitis. Most recent global data estimates that Cryptococcosis affects approximately 223,100 people annually, resulting in 181,100 fatalities. Globally, this invasive fungal infection is the cause of 15% of all AIDS-related deaths [1]. The standard treatment includes amphotericin B (AMB) and 5-fluorocytosine (5-FC) as induction therapy, followed by prolonged treatment with fluconazole (FLC) for maintenance therapy [2]. In countries with limited resources, however, FLC monotherapy is used as an alternative treatment. High mortality, treatment failure, and fluconazole resistance have been described in association with FLC monotherapy [3-5].

FLC is a triazole that inhibits lanosterol 14α-demethylase, an enzyme encoded by the *ERG11* gene, which is a rate-limiting step for ergosterol biosynthesis [6,7]. Azole resistance emerges through different mechanisms. Overexpression and mutations in *ERG11* decrease the susceptibility to azoles, while overexpression of efflux pumps decreases intracellular drug concentration [8-11]. Efflux of azoles is facilitated by the ATP-binding cassette (ABC) transporters, a class of pumps that use ATP as an energy source to drive

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transport [12]. Three efflux pumps have been characterized in *C. neoformans*. Afr1 is the primary azole efflux pump and deletion of the *AFR1* gene leads to lower FLC minimum inhibitory concentrations (MICs), while its overexpression causes increased FLC resistance [13]. Single deletions of another two ABC transporters, Afr2 and Mdr1, cause no change in FLC MICs. Deletion of all three transporters (Afr1, Afr2, and Mdr1) increases susceptibility to FLC compared to Afr1, suggesting that Afr1 acts as the major efflux pump, while Afr2 and Mdr1 augment Afr1 function [14]. Of note is that, when these ABC transporters were expressed in *Saccharomyces cerevisiae,* increased resistance to multiple azoles was observed. Furthermore, a *S. cerevisiae* mutant that expresses these *C. neoformans* transporters showed lower accumulation of radiolabeled FLC [11]. Additionally, a previous study demonstrated increased expression of these transporters in response to FLC indicating that expression of Afr1 and Afr2 was induced by FLC [14]. A search in the *Cryptococcus* genome database revealed a total of 41 ABC transporters in *C. neoformans.* Most transporters are not characterized, and it is not understood if their expression contributes to FLC resistance in *C. neoformans*.

Replicative aging is a conserved trait of eukaryotic organisms and in *C. neoformans* it is linked closely with resilience in the host environment and increased FLC tolerance in 10-generation-old cells [15,16]. Replicative aging is the result of asymmetric cell divisions. In the course of these divisions, the mother cells progressively age and accumulate age-associated phenotypic changes. Interestingly, in *S. cerevisiae* ABC transporters are asymmetrically distributed in the course of replicative aging [17]. Aged mother cells exhibit increased resistance to phagocytosis and macrophage-mediated killing [15,18] as well as increased tolerance to anti-fungal drugs, including FLC. A cell can undergo a finite number of divisions before senescence and the total number of divisions constitutes their replicative life span (RLS) [19], which varies greatly among clinical *C. neoformans* strains.

Transcriptome analysis of young and old *C. neoformans* cells have demonstrated differential expression of the gene *CNAG_06909*. This gene was identified based on homology to encode an ABC transporter. Expression was 16-fold upregulated in 10-generation old *C. neoformans* cells compared to young 0-3 generation-old cells. Due to similarities of the CNAG_06909 efflux pump to the previously described ABC transporters Afr1 and Afr2, we renamed the CNAG_06909 protein Afr3.

Here, we investigated the role of Afr3 in virulence and FLC resistance. We investigated virulence and FLC sensitivity of an *Δafr3* mutant strain and measured efflux function. To exclude compensation by other efflux pumps we expressed Afr3p in the *S. cerevisiae* strain (ADΔ), which lacks other ABC transporters [6] [11]. The present study demonstrates that Afr3 is an ABC transporter that is upregulated in old cells where it effluxes FLC, which may contribute to the age-dependent FLC tolerance in *C. neoformans*.

2. Materials and Methods

2.1. Strains and Media

C. neoformans strains H99 and *Δafr3*, *Δafr1*, and *Δafr2* mutants were cultured in synthetic media (SM; 1.7 g yeast nitrogen base without amino acids (BD), 1 g drop out mix (USBiological Life Sciences, Salem, MA, USA), 4 mL ethanol, 5 g $(NH_4)_2SO_4$, 3.3 g NaCl, 20 g glucose). Calorie restriction synthetic media was prepared as follows (CR, 1.7 g yeast nitrogen base without amino acids (BD), 1 g drop out mix (USBiological Life Sciences, Salem, MA, USA), 4 mL ethanol, 5 g $(NH_4)_2SO_4$, 3.3 g NaCl, 0.5 g glucose). The *Δafr3* strain was derived from the Madhani knockout collection, which is managed by the Fungal Genetics Stock Center. The *Δafr1* and *Δafr2* strains were gifted by Dr. Kwon-Chung Lab [14]. The mutant *S. cerevisiae* strains were cultured in complete supplement medium without uracil (CSM –ura (MP Bio); 20 g galactose, 1.7 g YNB, 5 g $(NH_4)_2SO_4$, 0.77 g CSM –ura). *S. cerevisiae* ADΔ strain, in which several ABC transporters and the URA3 locus were deleted, was previously described [20]. ADΔ strain and pYES2 plasmid were obtained as a gift from Dr. Theodore C. White at the University of Missouri, Kansas City.

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All strains used in this study are maintained as 30% glycerol stocks and stored at -80°C for future use.

2.2. Construction of S. cerevisiae Strain Expressing Efflux Pumps

First, RNA was extracted from exponentially growing H99 cells using RNAeasy Plus kit (Qiagen) following manufacturer's guidelines. Next, 250 ng of RNA was converted to cDNA using Verso cDNA Kit (Thermo Scientific) in a 20 µl reaction. *AFR3* was amplified from the cDNA using oligos that partially overlapped with the pYES2 plasmid (Table S1), using a thermocycler (Biorad). The forward oligonucleotide was designed to contain 40bp homologous sequence of GAL1 promoter (primer *AFR3* + p*GAL1*) while the reverse oligonucleotide was designed to contain 40bp homologous sequence of CYC1 terminator (primer *AFR3* + *CYC1* tt*)*. The plasmid contains an *URA3* auxotrophic selection marker. pYES2 was first digested with HindIII and then transformed into ADΔ strain with the addition of the *AFR3* cassette, as previously described [21]. The ADΔ strain lacks seven ABC transporters (*Δyor1*, *Δsnq2*, *Δpdr10*, *Δpdr11*, *Δycf1*, *Δpdr5*, and *Δpdr15*), a transcription factor (*Δpdr3*), and the *URA3* gene (*Δura3*). Homologous recombination was used to integrate the *AFR3* cassette into pYES2. Undigested plasmid pYES2 without the cassette was transformed into ADΔ for positive control. Transformants were selected on CSM – ura agar plates after incubation at 37°C for 4 days. To screen for proper integration, 12-15 colonies were selected and replicated into fresh selective plates three consecutive times. Transformants were confirmed by plasmid extraction with QIAprep Spin Miniprep Kit (Qiagen) and PCR for *AFR3* cassette using oligos that amplified the whole gene sequence (primers *AFR3* F and *AFR3* R) (Table S1, Figure S1A). Expression was measured using the RNAeasy Plus kit (Qiagen) to extract RNA from ADΔ and ADΔ + Afr3 and RNA was converted to cDNA using Verso cDNA Kit (Thermo Scientific). cDNA was diluted 1:5 and analyzed with qPCR analysis (Roche) using Power Sybr Green Master Mix (Applied Biosystems) following manufacture's protocols (primers qPCR Afr3 F and qPCR Afr3 R, Table S1). House-keeping gene encoding β-actin (primers Sc *ACT1* F and Sc *ACT1* R) was used as an internal control (Table S1, Figure S1B). Furthermore, samples were sent for sequencing to ensure that the *AFR3* sequence did not undergo mutations.

2.3. Isolation of Old C. neoformans Strains

Isolation of 10-generation-old *C. neoformans* cells was performed following previously published protocol [22]. Briefly, the cells from the strains H99, *Δafr1*, and *Δafr3* were incubated overnight at 37 °C in SM media. The next day, the overnight cultures were washed three times with 1X PBS and were diluted 1:50 times. The diluted cells were then exponentially grown for 6-8 hours. After the exponential growth, the cells were washed three times with 1X PBS and counted with a hemocytometer. 10^8 cells from each strain were then labeled with 8mg/ml sulfosuccinimidyl-6-[biotin-amido] hexanoate (Sulfo-NHS-LC-LC-Biotin, 21338, ThermoFisher Scientific) for 30 minutes at room temperature (RT). The labeled cells were then washed three times in 1X PBS and the washed cells were grown in fresh SM media for 5 generations (12-15 h). After 5 generation cell growth for each strain, the cells were washed three times with 1X PBS and labeled with 100 µl of streptavidin microbeads (130-048-101, Miltenyi Biotec) at a final concentration of 10^8 cells/mL. The streptavidin labeling was done for 15 minutes at 4°C. The labeled cells were washed three times with 1X PBS to remove any unbound streptavidin. The biotin-streptavidin labeled 5 generation cells were then separated by passing the mixed population through AutoMACS® Pro Separator (Miltenyi Biotec). The positively labeled cells were retained in the column attached to the magnet in the pro-separator machine. These cells were retrieved once the magnetic field was removed. These 5 generation old cells were then further grown in fresh SM media for another 5 generations (12-15 h). The grown cells were again washed and passed through AutoMACS® Pro Separator (Miltenyi Biotec) to retrieve the 10 generation old cells. The purity of the population was verified by

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microscopy. As a control, the young generation cells, washed-off from the magnetic columns, from the second separation were used.

2.4. Antifungal Susceptibility Testing

The Minimum Inhibitory Concentration (MIC) was determined as per a previously published protocol [23]. Briefly, the *C. neoformans* and *S. cerevisiae* strains were cultured overnight at 37 °C, and cells were adjusted to 10^5 cells/well for *C. neoformans* and $5x10^5$ cells/well for *S. cerevisiae*. FLC was two-fold serially diluted in a flat-bottom 96-well plate (Costar) with a starting FLC concentration of 64 µg/mL. The plates were incubated at 37 °C for 4 days and the OD_{600} was measured (SpectraMax i3x, Molecular Devices). A row with no drugs was used as a growth control while a row with no cells was used as contamination control in the MIC assay. MICs were defined as the minimum drug concentration that inhibits 80% of the cell growth (MIC_{80}) or 50% of the cell growth (MIC_{50}). The assay was performed in triplicate. For analysis of MICs under CR, the same conditions were performed, in which the cells were cultured in synthetic media with 0.05% glucose. FLC E-Test was also performed to determine FLC susceptibility. 10^6 cells from both H99 and *Δafr3* mutant strains were plated in YPD media containing FLC E-Test Strip (Biomerieux) and incubated at 37 °C for 4 days.

FLC Killing assay was performed using previously published protocol [24]. Briefly, the young and the 10 generation-old cells were isolated as described above. After isolation, the cells were washed three times with 1X PBS and 10^4 cells per well were seeded in 96 well plates (Costar) containing FLC at concentrations of 50, 25, 12.5, 6.25, and 3.125 µg/mL. Cells were also plated in wells containing no drug. Next, the 96 well plates were incubated at 37 °C for 3 h without shaking. After incubation, the cells were diluted 50 times and plated in YPD agar plates. The agar plates were incubated for 48 h and colony-forming units (CFUs) were counted. Percent killing was analyzed by the following formula:

$$\% \text{ killing} = \frac{CFUs\ in\ no\ drug\ well - CFUs\ in\ drug\ well}{CFUs\ in\ no\ drug\ well} x100$$

The assays were done in triplicate. All YPD plates and drug concentrations were made fresh on the day of the experiment.

2.5. Rhodamine 6G Efflux Assay

Rhodamine 6G assay was performed as previously described [25]. Briefly, $5x10^7$ *C. neoformans* and $2x10^7$ *S. cerevisiae* cells were starved for 2 h in phosphate buffer saline (PBS; pH 7.4) buffer at room temperature. Rhodamine 6G (Sigma) was added to a final concentration of 10 µM and the cells were incubated at 37 °C for 30 min. Following the incubation, cells were washed 3x in PBS, and efflux was initiated by the addition of 2% glucose. Samples were collected at 0 min, 10 min, 20 min, and 30 min timepoints, and fluorescence of the supernatants was measured at 525 nm excitation and 555 nm emission wavelengths. The experiment was performed independently on three different days.

2.6. Nile Red Assay

Briefly, the cells from ADΔ and ADΔ+Afr3 were grown in the CSM-ura media. After growth 10^7 cells were used for the Nile Red assay. First, the cells were washed in PBS three times and then starved for 2 hours in PBS to get rid of any residual glucose. After starvation, Nile red was added to the cells at a final concentration of 7µM. The efflux was initiated after the addition of 2% glucose to the starved cells. Accumulation of Nile Red was measured using fluorescence plate reader (SpectraMax I3) at 0 mins and 30 mins using excitation wavelength of 553 nm and emission of 636 nm. Accumulation was calculated in percentage. More accumulation of the dye at the end of 30 mins period signifies lesser efflux. The assay was done in triplicate.

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2.7. Replicative Life Span (RLS)

The RLS was determined by microdissection as outlined elsewhere [22]. Briefly, 20-30 naïve cells were isolated and arrayed in a straight line in SM plates. Every time a mother cell budded (1-2 h), the daughter cell was separated using a 25 µm needle (CoraStyles) under a tetrad dissection Axioscope A1 microscope (Zeiss) at 250X magnification. After each budding event, the plates were incubated at 37 °C. The RLS was determined by the number of times the mother cell buds before dying (24 h without a budding event).

2.8. Galleria mellonella infection

Galleria mellonella infection was performed as previously described [26]. *G. mellonella* larvae were obtained from Vanderhorst Wholesale Inc., St. Mary's, OH, United States. *C. neoformans* cells were washed and diluted to 10^6 cells/mL in PBS. The worms were injected with 10 µL of the cell suspension and PBS was used as a negative control. Twenty worms were used for each group. Survival of the worms was observed for a week. Retention of *C. neoformans* cells in the hemolymph of *Galleria* larvae was analyzed as an independent experiment. The worms were injected with $5x10^4$ *C. neoformans* cells and the hemolymph was extracted after 24 h. The samples were plated in YPD agar plates and the CFU was counted after 48 h incubation at 37 °C.

2.9. Growth Curve

Growth curves for H99, *Δafr3*, *Δafr1*, and *Δafr2* strains were performed in 96-well flat-bottom plates, in which 0.1 OD_{600} cells were used in triplicate for each strain. The growth curve was carried on for 72 h in a SpectraMax i3x (Molecular Devices) at 37 °C with shaking.

2.10. Expression Analysis

Strains were grown overnight in their respective media. For pump compensation analysis, H99, *Δafr3*, and *Δafr1* were grown overnight in SM media. *AFR3* expression in low glucose was performed in H99 cells grown overnight in SM and calorie restriction low glucose media. Finally, for pump analysis under FLC treatment, we grew H99 overnight in SM media, followed by a 2 h treatment under 32 µg/mL of FLC of 10^7 cells. For 10-generation *C. neoformans* quantification, we isolated young and old H99 cells, where we quantified *AFR1, AFR2*, and *MDR1* expression. RNA was extracted using RNAeasy Plus kit (Qiagen) following manufacturer's guidelines. Next, RNA was quantified using a Biospectrophotometer (Eppendorf), in which an absorbance ratio (A260/A280) of 2.0 or higher was considered good quality RNA. 250 ng of RNA was converted to cDNA using Verso cDNA Kit (Thermo Scientific) in a 20 µl reaction. cDNA was diluted 1:5 with RNase/DNase-free water (HyClone Laboratories). qPCR expression analysis (Roche Life Science) was performed using Power Sybr Green Master Mix (Applied Biosystems) following the manufacturer's protocol. Oligonucleotides used to analyze gene expression of *AFR1* and *AFR3* are described in Table S1. House-keeping gene *ACT1* was used as an internal control. Data was normalized and calculated using the $2^{-\Delta\Delta Ct}$ method as previously described [27].

2.11. Macrophage- mediated Killing Assay

Macrophage-mediated killing assay was performed according to previously published protocol [18]. Briefly, $5x10^4$ cells of J774A.1 murine macrophage cell line were seeded in 96 well plates (Costar) in DMEM (Gibco) media containing 10% Fetal Bovine Serum (FBS), 10% NCTC (Gibco), 1% non-essential amino acids, and 1% penicillin-streptomycin. The 96 well plates were incubated at 37 °C with 5% CO_2 for 24 h. After

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incubation, the cells were activated with LPS and IFNγ as described previously. In a separate tube, young and old *C. neoformans* cells were opsonized for 30 mins at 37 °C with 18b7 antibody. The opsonized *C. neoformans* cells were then added to the 96 well plates containing the activated macrophages at an MOI of 1:1. The plates were incubated for 1 h at 37 °C with 5% CO_2 to allow phagocytosis. After phagocytosis, all wells were washed three times with 1X PBS to remove the non-phagocytosed *C. neoformans* cells. After washing, half of the wells of macrophages were lysed using sterile water, and *C. neoformans* cells were plated in YPD to determine the number of *C. neoformans* cells phagocytosed (time 0). Next, to the other half of macrophage-containing wells, fresh DMEM media was added. The macrophages along with the phagocytosed *C. neoformans* cells were incubated for another 1 h at 37 °C with 5% CO_2. This was done to analyze macrophage-mediated killing of young and old *C. neoformans* cells. After 1 h of killing, the wells were washed three times with 1X PBS. The macrophages were then lysed and the surviving *C. neoformans* cells were plated in YPD agar plates. YPD plates were then incubated at 37 °C for 48 h and CFUs were counted. The assay was performed in triplicate. Macrophage-mediated killing was calculated as follows:

$$\% \text{ macrophage-mediated killing} = \frac{CFU\ post\ phagocytosis\ at\ time\ 0 - CFU\ after\ 1h\ killing}{CFU\ post\ phagocytosis\ at\ time\ 0}\ x\ 100$$

2.12. Statistics

Statistical analyses were performed using GraphPad Prism 9.0. The specific analyses are described in the figure legends. The coefficient of variation (CV) is calculated by dividing de standard deviation (SD) by the mean of the strain (CV% = SD/mean x 100).

3. Results

3.1. Afr3 is Similar to Other ABC Transporters

Based on amino acid sequence, CNAG_06909 exhibits 29% and 26% homology to Afr1 (CNAG_00730) and Afr2 (CNAG_00869) respectively, with a high query coverage of above 85% (Table 1). Protein sequence similarity between Afr1 and Afr2 is 38%, which is comparable to the similarity of CNAG_06909 transporter with either Afr1 or Afr2. Furthermore, CNAG_06909 contains an ABC transporter domain, while Afr1 contains two domains. Afr2, similarly to Afr1, also contains two ABC transporter domains. Performing an alignment and analysis employing NCBI Blast Tool exhibits high similarity between the ABC transporter domain of Afr3 (CnAFR3) and the two domains of Afr1 (CNAFR1.1 and CnAFR1.2; 46% positives, 25% identities, and 53% positives, 35% identities, respectively) (Figure 1). Based on the similarity with Afr1 and Afr2, and the below characterized functional overlap we have renamed the CNAG_06909 gene *AFR3* and the protein Afr3.

3.2. The Afr3 Efflux Pump is Important for C. neoformans FLC tolerance

Given the known role of ABC transporters in mediating azole resistance in fungal cells, we assessed FLC susceptibility in H99 and Δ*afr3* with standard methods with slight modifications. First, we performed a FLC E-Test strip analysis on SM media plates, in

Table 1. Similar Proteins to Afr3 Across Different Fungal Species

Description	Max Score	Total Score	Query Cover	E value	% Identity	Accession length	Accession Code
ATP-binding cassette transporter [Cryptococcus neoformans var. grubii H99]	205	302	86%	3e-55	29.29%	1543	CNAG_00730

ABC transporter [Cryptococcus neoformans var. grubii H99]	199	340	88%	3e-53	31%	1462	CNAG_07799
ATP-dependent permease [Cryptococcus neoformans var. grubii H99]	179	234	77%	1e-44	36.96%	1173	CNAG_06533
ABC transporter family protein [Cryptococcus neoformans var. grubii H99]	170	313	61%	9e-44	30.99%	1241	CNAG_05470
ABC transporter PMR5 [Cryptococcus neoformans var. grubii H99]	165	315	87%	5e-42	25.05%	1421	CNAG_06348
ATP-binding cassette transporter [Cryptococcus neoformans var. grubii H99]	144	271	90%	1e-39	26.06%	1529	CNAG_00869
ABC transporter, putative [Cryptococcus neoformans var. neoformans JEC21]	204	343	86%	3e-56	30.16%	1463	CNL06490
ABC transporter [Cryptococcus gattii VGIV IND107]	1167	1167	100%	0.0	93.45%	625	KIR83217.1
related to ATP-binding cassette protein (ABC) transporter [Ustilago trichophora]	208	413	85%	3e-58	41.35%	669	SPO26649.1
abc transporter [Lasallia pustulata]	671	671	96%	0.0	54.27%	637	KAA6412381.1
hypothetical protein EHS25_004009 [Saitozyma podzolica]	910	910	95%	0.0	73.22%	1711	RSH94206.1

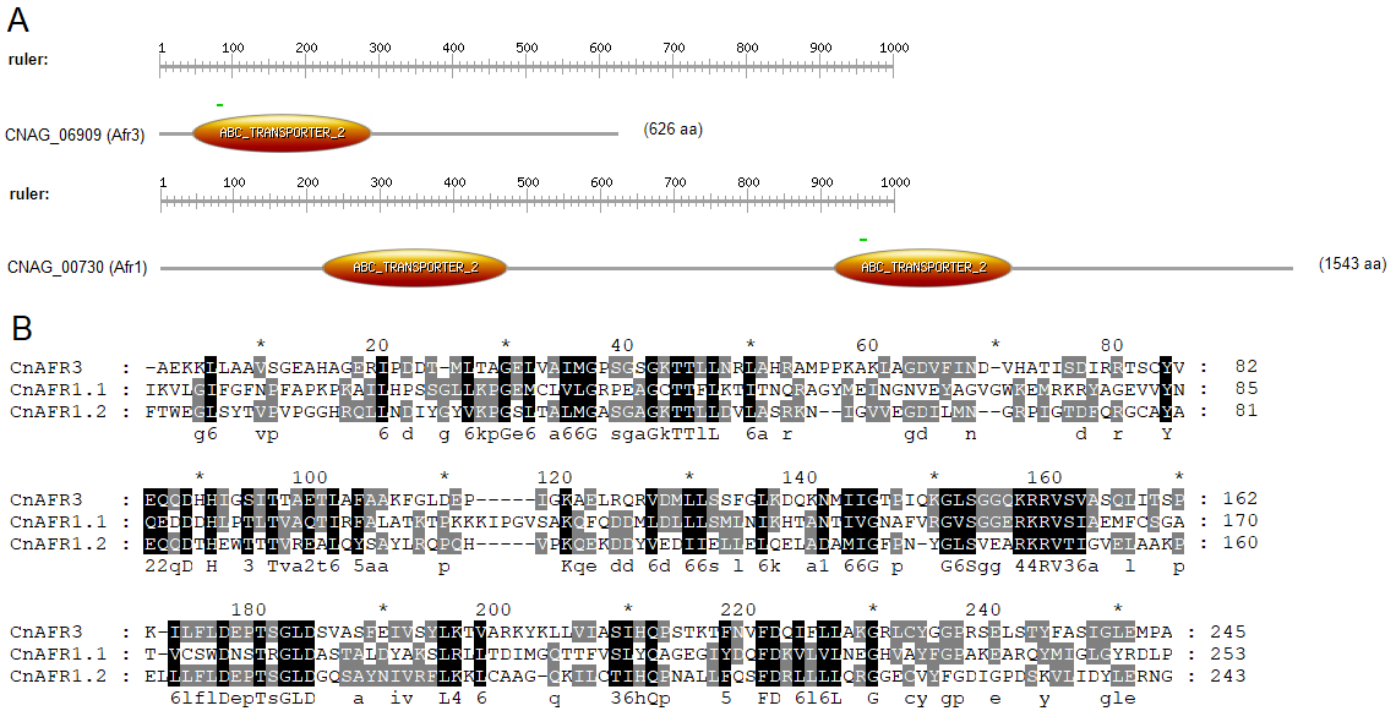

Figure 1. Afr3 (CNAG_06909) Has Similar Motifs to Afr1 (CNAG_00730). (**A**) Afr3 possesses one ABC transporter motif, while Afr1 has two. The search was conducted using Prosite (https://prosite.expasy.org/scanprosite/); (**B**) Alignment analysis between the ABC transporter domain of Afr3 (CnAFR3) and the two domains of Afr1 (CnAFR1.1 and CnAFR1.2) was performed with ClustalW, in the BioEdit program. The figure was generated using GeneDoc. Sequences in black are highly conserved.

which Δafr3 displayed a lower MIC than H99 (0.75 μg/mL vs. 4 μg/mL) (Figure 2A). It is noteworthy, that the Δafr3 mutant lacked heteroresistance whereas individual colonies grew at higher MIC in the wild-type H99 consistent with heteroresistance. Next, we explored whether *AFR3* was overexpressed during FLC treatment. After a 2h FLC treatment, *AFR3* did not show an increase in expression when compared to H99 cells without treatment (Figure 2B).

Given augmented ABC transporter-mediated efflux of FLC is the main mechanism of how ABC pumps mediate FLC resistance in *C. neoformans*, we explored efflux pump

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activity of *Δafr3*. The fluorescent dye Rhodamine 6G was added to *C. neoformans* cells and the cellular efflux was initiated by the addition of glucose, followed by fluorescence measurement in the supernatant. These data demonstrated that *Δafr3* exhibited lower efflux than wild-type in all three time points (10, 20, and 30 min; $p < 0.001$) (Figure 2C), indicating that Afr3 is a relevant efflux pump.

FLC MIC analysis under low glucose media (0.05% glucose) was performed because such conditions are encountered by *C. neoformans* in vivo. These data showed that both strains increased FLC tolerance under low glucose, with no difference between H99 and *Δafr3* strains, indicating that Afr3 is not responsible for the increase in FLC tolerance observed under low glucose conditions (Figure 2D). Of note, the FLC MIC_{80} was the same for H99 and *Δafr3* in a broth microdilution assay. This data was further supported by expression analysis of *AFR3* under low glucose, in which the *AFR3* levels were not significantly increased (Figure 2E). Possible pump compensation between Afr3 and Afr1 was also studied. *AFR1* expression was measured in *Δafr3* strain, while *AFR3* expression was measured in *Δafr1* strain to seek for upregulation. Upregulation of *AFR1* in a *Δafr3* strain would indicate that there is increased production of Afr1 to compensate for the lack of Afr3, and vice-versa. No upregulation was observed in this analysis, which indicates no compensation between Afr1 and Afr3 (Figure S2).

Given that Afr3, as well as Afr1 and Afr2, are overexpressed in 10-generation old cells (Table 2), we assessed the contribution of Afr3 to age-associated FLC tolerance with established high dose FLC killing assays, since growth-based assays can only assess FLC MICs of young *C. neoformans* cells. Killing of young and old *C. neoformans* cells was assessed after 3 h exposure to high doses of FLC ranging from 50 μg/mL to 3.125 μg/mL. As expected, these assays demonstrated that the *Δafr3* mutant was killed more efficiently over a range of FLC doses when compared to the wild-type H99 (*Δafr3* Y vs H99 Y, FLC 50, 25, 12.5 μg/mL: 80 vs. 25 %; FLC 6.25 μg/mL: 48 vs 7 %; FLC 3.125 μg/mL: 60 vs 5 %; ### $p < 0.001$) (Figure 2F). Older *C. neoformans* cells showed higher tolerance to FLC killing than younger *C. neoformans* cells for most FLC concentrations for both H99 (H99 O vs H99 Y; FLC 50 μg/mL: 13 vs 25 %, $p < 0.05$; FLC 25 μg/mL: ~0 vs 23 %, $p < 0.01$; and FLC 12.5 μg/mL: 6 vs 25 %, $p < 0.01$) and *Δafr3* (*Δafr3* O vs *Δafr3* Y; FLC 50 μg/mL: 17 vs 80 %; FLC 25 μg/mL: 35 vs 80 %; and FLC 12.5 μg/mL: 37 vs 79 %; $p < 0.01$). The H99 wild-type data corroborates data previously published for the RC2 strain [19]. The percentage FLC killing of older *Δafr3* cells, however, was higher than the killing of 10- generation H99 cells (*Δafr3* O vs H99 O; FLC 25 μg/mL: 35 vs ~0 %, FLC 12.5 μg/mL: 37 vs 6 %; and FLC 6.25 μg/mL: 38 vs ~0 %; $ $p < 0.05$), indicating that the presence of the Afr3 efflux pump may contribute to age-associated FLC tolerance, but is not the only factor.

Table 2. Efflux Pumps Expression in 10-generation Cells

Strain	qPCR Fold-change	P value	Significant?
Afr1	28.26	0.0415	Yes
Afr2	5.16	0.0026	Yes
Afr3	16.06 *	0.0017	Yes
Mdr1	4.36	0.0108	Yes

* [15]

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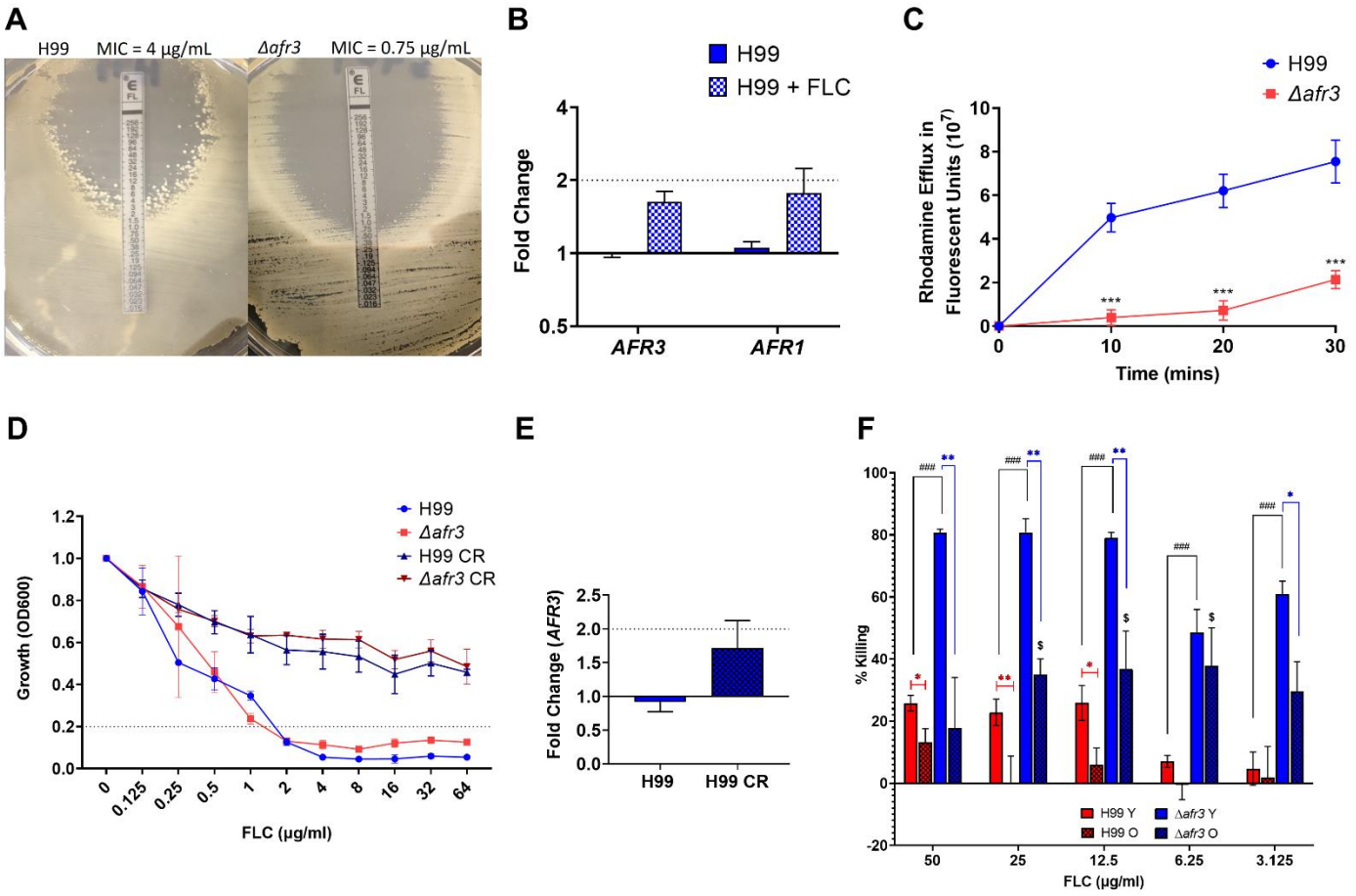

Figure 2. Afr3 is an Important Pump for Drug Resistance. (**A**) *C. neoformans Δafr3* is more sensitive to FLC than H99 in an FLC E-Test in a YPD plate; (**B**) H99 cells that underwent FLC treatment with 32 µg/mL for 2h (checkered blue/white bar) do not increase expression of *AFR3* and *AFR1* compared to H99 wild-type (blue bar); (**C**) *Δafr3* (red line) decreases efflux compared to H99 (blue line) in a Rhodamine 6G assay. Statistical analysis was performed with multiple unpaired Student's t-test, *** $p < 0.001$; (**D**) FLC tolerance observed under CR conditions (SM 0.05% glucose) (H99 CR: dark blue line, *Δafr3* CR: dark red line) is independent of Afr3 presence, as shown by the susceptibility under normal conditions (SM 2% glucose) (H99: blue line, *Δafr3:* red line); (**E**) Expression of *AFR3* is not increased under CR conditions (checkered blue bar) when compared to normal glucose conditions (blue bar); (**F**) The *Δafr3* young cells (*Δafr3* Y, blue bar) are more susceptible to FLC killing than H99 young cells (H99 Y, red bar. Furthermore, *Δafr3* old cells (*Δafr3* O, checkered blue bar) lose FLC killing tolerance when compared to H99 old cells (H99 O, checkered red bar). Statistical analysis was performed with multiple unpaired Student's t-test, * $p < 0.05$, ** $p < 0.01$, ### $p < 0.001$, and $ $p < 0.05$; Error bars represent the standard deviation between biological triplicates.

3.3. S. cerevisiae Expression of Afr3 Increases Drug Efflux

To exclude compensation by other ABC transporters when the Afr3 pump is deleted, we expressed Afr3p in the *S. cerevisiae* ADΔ strain, which lacks all seven of the main ABC transporters, thus reducing background transport. We expressed Afr3 in pYES2 plasmid under a *GAL1* promoter (Fig 3A) that permits the expression of Afr3 only in presence of galactose. FLC MICs for ADΔ control and ADΔ expressing Afr3 (ADΔ + Afr3) showed no difference between the strains for MIC_{80} (Figure 3B). This might be due to the longer period of incubation used for the assay, which could lead to a decrease in protein activity.

Next, we sought to evaluate the efflux activity of the *S. cerevisiae* strains. Rhodamine 6G efflux was increased in the ADΔ + Afr3 strain when compared to ADΔ after 20 min ($p < 0.01$) and 30 min ($p < 0.0001$) (Figure 3C). We also performed a Nile Red Assay in which cells were incubated with Nile Red and glucose was added to initiate transport. The percentage of Nile Red accumulation inside the fungal cells was quantified by measuring the fluorescence after 30 min. ADΔ + Afr3 mutant exhibited decreased intracellular Nile Red

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accumulation when compared to the ADΔ strain ($p < 0.01$) (Figure 3D). These results confirmed Afr3 efflux activity and showed that this pump exhibits efflux pump activity independent from other ABC pumps.

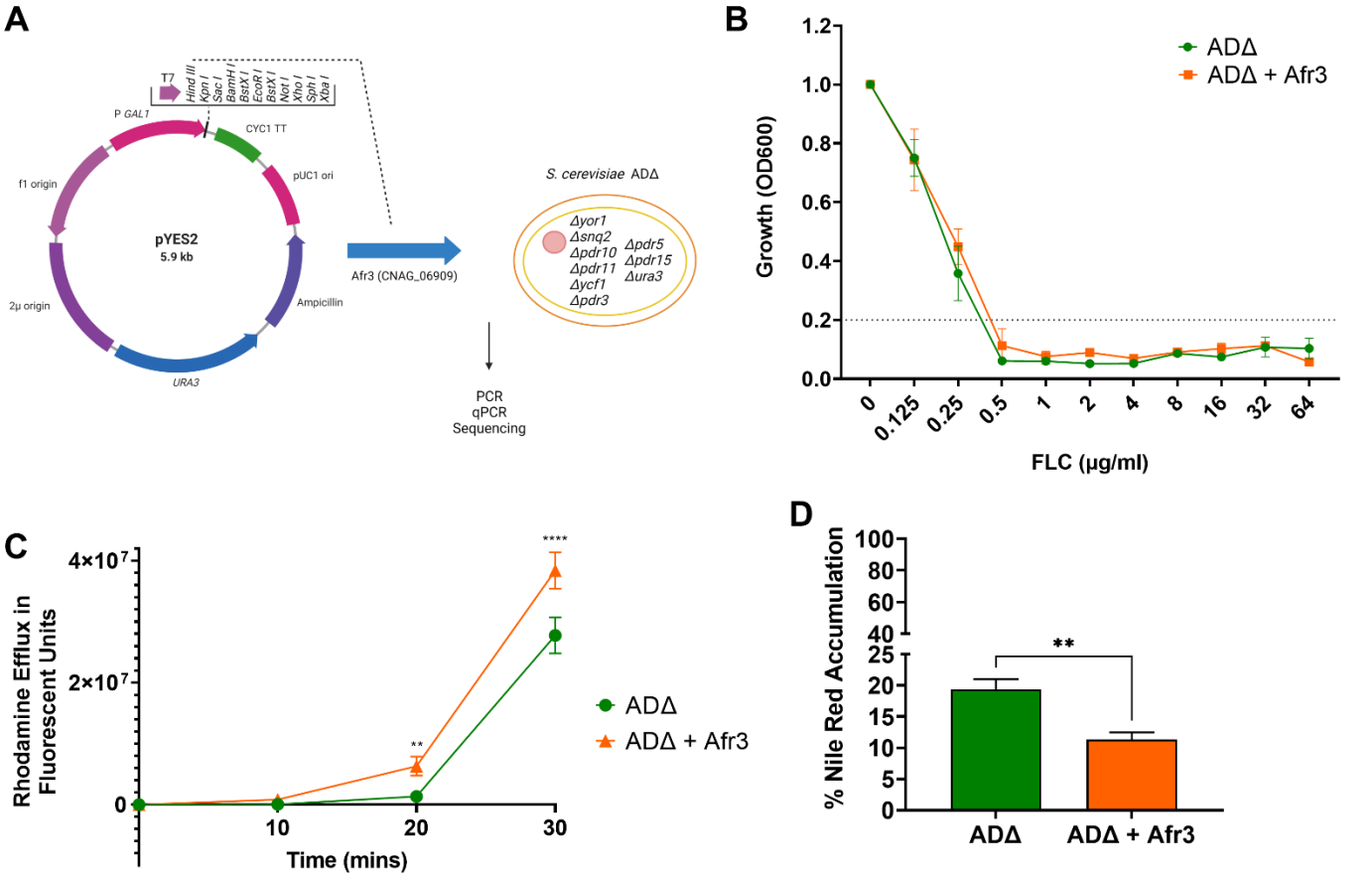

Figure 3. Afr3 Expression in *Saccharomyces cerevisiae* Increases Efflux. (**A**) Schematic diagram of Afr3 expression in *S. cerevisiae* ADΔ strain. The pYES2 plasmid contains a *URA3* auxotrophic marker gene, a 2μ origin of replication, a *GAL1* promoter, and a *CYC1* terminator. Hind III was used as the cloning site between the *GAL1* promoter and *CYC1* terminator. *AFR3* (*CNAG_06909*) cassette was inserted in this cloning site when transformed into *S. cerevisiae* ADΔ strain. The transformation was confirmed through plasmid PCR, qPCR for *AFR3* expression, and sequencing of the *AFR3* cassette. The figure was designed with BioRender; (**B**) There is no significant difference in FLC MICs between ADΔ (green line) and ADΔ expressing Afr3 (ADΔ + Afr3, orange line). Error bars represent the standard deviation between biological triplicates; (**C**) ADΔ + Afr3 (orange line) shows increased efflux when compared to ADΔ (green line). Statistical analysis was performed with multiple unpaired Student's t-test, ** $p < 0.01$, **** $p < 0.0001$. Error bars represent the standard deviation between biological triplicates; (**D**) ADΔ + Afr3 (orange bar) has lower Nile Red intracellular accumulation when compared to ADΔ (green bar). Statistical analysis was performed with Student's t-test, ** $p < 0.01$. Error bars represent the standard deviation between biological triplicates.

3.4. Afr3 Affects Cryptococcal Virulence

Next, we assessed if Afr3 plays a role in virulence. First, we documented that the mutant strain exhibited no growth defect at 37 °C when compared to the wild type (Figure S3). Next, we analyzed phagocytosis and macrophage killing of *Δafr3* and compared the percentages to the H99 wild type. These data indicated that the phagocytosis of *Δafr3* was comparable to that of H99. However, murine J774 macrophages more successfully killed the *Δafr3* mutant strain after phagocytosis when compared to H99 wild-type (64% vs. 6.6%, $p < 0.01$) (Figure 4A). Last, virulence of the mutant strain was assessed in a *G. mellonella* infection model. The larvae infected with the *Δafr3* strain exhibited increased survival when compared to the

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larvae infected with H99 (8 vs. 6 d median survival, $p < 0.0001$), indicating that the Afr3 pump plays a role in virulence (Figure 4B). This data is further supported by the lower number of *C. neoformans* cells circulating in the hemolymph of larvae infected with *Δafr3* after 24 hrs when compared CFU from the hemolymph of larvae infected with H99 (29 vs. 52 % cells retained, $p < 0.05$) (Figure 4C). These data support the notion that Afr3 is also important for *C. neoformans* virulence.

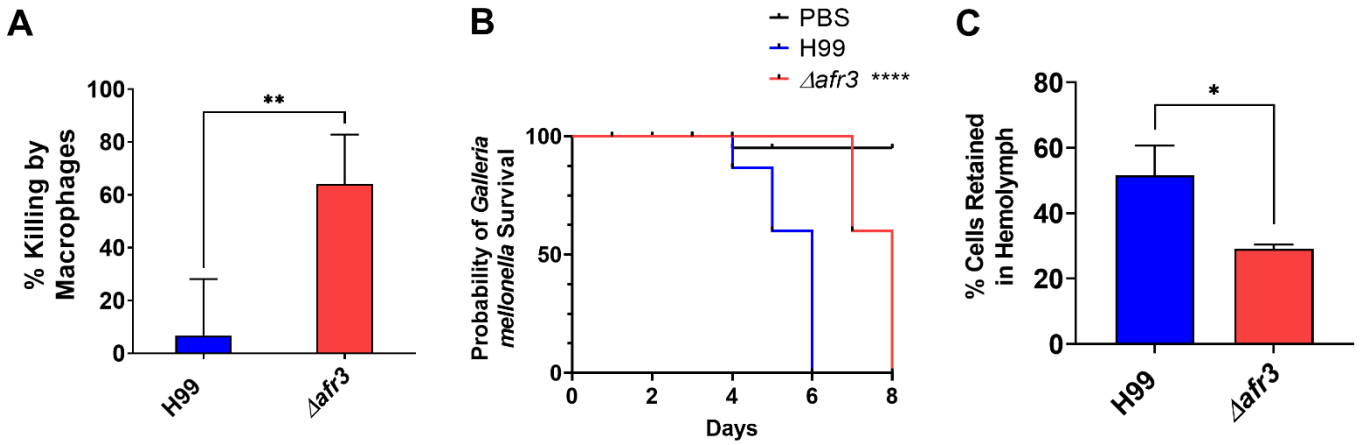

Figure 4. Afr3 Plays a Role in Virulence. (**A**) Phagocytosed *Δafr3* (red bar) cells are better killed by J774A.1 murine macrophages than wild-type H99 cells (blue bar). Statistical analysis was performed with Student's t-test, ** $p < 0.01$; (**B**) *Galleria mellonella* larvae survived longer when infected with mutant strain *Δafr3* (red line) when compared to the survival of the larvae infected with wild-type H99 (blue line). Black line represents PBS uninfected controls. Statistical analysis was performed with Log-rank (Mantel-Cox) test, **** $p < 0.0001$; (**C**) *Δafr3* (red bar) has lower retention of *C. neoformans* cells in the larvae hemolymph than H99 (blue bar). Statistical analysis was performed with Student's t-test, * $p < 0.05$; Error bars signify standard deviations between the biological triplicate.

3.5. Afr3 Affects Cryptococcal Life Span

Given that Afr3, as well as Afr1 and Afr2, are overexpressed in 10-generation old cells, we first investigated whether Afr1, Afr2, and Afr3 affect RLS. The RLS of *Δafr3*, *Δafr1*, and *Δafr2* were determined by micro-dissection and the total number of divisions the respective mutant and wild-type *C. neoformans* cells undergo before their death is recorded. These experiments showed that loss of *AFR3* had a moderate prolongevity effect and extended the median RLS by 29% compared to wild-type H99 strain (22 vs. 17, $p < 0.001$) (Figure 5). In contrast, loss of *AFR1* and *AFR2* did not alter the RLS (15 and 17 median RLS, respectively). The RLS of *Δafr1* and *Δ*afr2 mutants exhibited high variability whereas RLS of *Δafr3* was characterized by lower stochasticity. The coefficient of variation measures the amount of variation between individual cells within the strain data set. H99 showed 35% of variation between individual cells, while *Δafr3* had only 14% variation, in accordance with its lower stochasticity. The mutant strains *Δafr1* and *Δ*afr2 had a much higher variation, with 55% and 65%, respectively.

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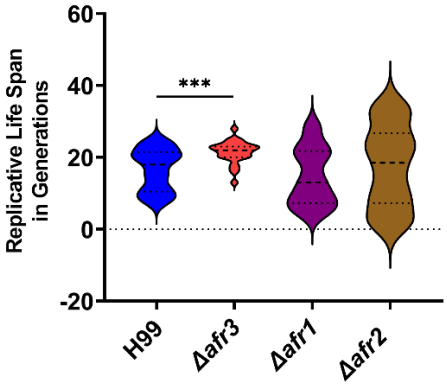

Figure 5. Afr3 Plays a Role in Aging. The *C. neoformans Δafr3* mutant strain (red) has an expansion of the median lifespan compared to the H99 wild-type strain (blue), while *Δafr1* (purple) and *Δafr2* (brown) mutant strains do not show significant difference. Statistical analysis was performed with Student's t-test, *** $p < 0.001$.

4. Discussion

This paper describes a novel ABC transporter in *C. neoformans*, which can efficiently efflux FLC and render *C. neoformans* cells more resistant to FLC. The present study was initiated because in 10-generation-old cells [15], which are more tolerant to FLC, the *AFR3* gene is markedly upregulated, similarly to the upregulation of *AFR1* and *AFR2* genes. Based on similarities with known *C. neoformans* efflux pumps, Afr1 (CNAG_00730, 29.29% identity), Afr2 (CNAG_00869, 26% identity) and Pmr5 (CNAG_06348, 25% identity), as well as ABC transporters from the environmental fungi, *Ustilago trichophora* (41.35% identity), *Lasallia pustulata* (54.27% identity), and *Saitozyma podzolica* (73.22% identity), these data characterize Afr3 as member of the conserved family of ABC transporters.

Overexpression of efflux pumps, leading to decreased cellular drug concentration is a major mechanism of drug resistance [28]. During treatment of chronic cryptococcosis, this can lead to persistence of infection, despite appropriate treatment, which translates into failure to clear the fungal cells [29]. Deletion of *AFR3* resulted in increased sensitivity to FLC in E-Test strip analysis and increased CFU killing when higher FLC concentrations were used. However, *Δafr3* did not exhibit differences in FLC sensitivity using the standard MIC_{80} assay. E-Test and microdilution methods do not always yield the same results [30], and agreement between these two tests can vary in 70 – 96% of the times [31]. It is conceivable that the observed difference could be due to "trailing growth", a phenomenon where fungal cells exhibit reduced but persistent growth at FLC concentrations above the MIC. This effect is more commonly observed when microdilution methods are performed [32], because in liquid assays slow growth of subpopulations may eventually dominate [33]. Interestingly, the E-Test assay indicated a lower formation of heteroresistant colonies in the *Δafr3* mutant strain when compared to H99. Heteroresistance signifies the presence of a sub-population that manifests higher FLC tolerance when compared to the majority of the population [34,35]. Loss of heteroresistance was also observed with deletion of *AFR1* [14]. However, the role that Afr3 plays in heterotolerance mechanisms is still to be determined.

Furthermore, the decreased Rhodamine 6G efflux, which mimics alterations in drug accumulation, corroborates the importance of Afr3 in driving drug resistance, especially since transcriptome data also suggests that there is no compensation by the other main efflux pump Afr1. Afr1 is the most well-characterized ABC transporter in *C. neoformans.* Deletion of *AFR1* increases drug susceptibility to FLC, which is corroborated by data from a mouse infection model that indicates that Afr1 also plays a role in FLC resistance and fungal virulence [13,34,36]. Single deletions of Afr2 and Mdr1 did not influence susceptibility to FLC, which was only seen in a triple-deletion of Afr1, Afr2, and Mdr1 [14]. Compensatory upregulation of the transporters by qPCR in the *Δafr3* and *Δafr1* mutant strains

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was evaluated to assess if the lack of Afr3 would be compensated by Afr1. The data did not indicate overexpression of any transporter when the other was deleted.

Heterologous protein expression in yeast model systems, like *S. cerevisiae*, has enabled functional analysis of specific proteins and has been previously employed to study efflux pumps in a variety of fungi [6,20,37]. Although expression of *AFR3* in *S. cerevisiae*, did not compromise viability of *S. cerevisiae* or its growth, Afr3 protein function appeared to decline rapidly within days without overt cell toxicity or loss of the plasmid. The presence of proper gene integration into the pYES2 plasmid, lack of mutations, and robust expression under the GAL1 promoter suggest that the cryptococcal Afr3 protein may not be stable in *S. cerevisiae*. Similar problems have been encountered with other heterologous proteins, which were found to be unstable in *S. cerevisiae* [38-41]. This may explain the lack of difference in the FLC MIC between ADΔ and ADΔ + Afr3, as it requires several days of incubation. The Rhodamine 6G and Nile Red experiments, however, clearly demonstrated the increase of efflux when Afr3 was expressed. In contrast to MIC experiments, these experiments were done on the same day the transformants were recovered.

Moreover, since both drug resistance and heteroresistance have been linked to increased virulence [34], the decreased cryptococcal virulence of *Δafr3* in a *Galleria mellonella* survival model further confirms the important role of this efflux pump. The efflux pumps may play a role not only in the decrease of FLC concentration but also in detoxification of the cell by extruding metabolites and other components that may be detrimental [42]. Accumulation of toxic metabolites can lead to loss of cell fitness and impact fungal virulence. Other fungal pumps have been shown to impact virulence, including Atm1, a mitochondrial ABC pump in *C. neoformans*, Mlt1, a vacuolar ABC transporter in *Candida albicans*, and AbcB, an efflux pump of *Aspergillus fumigatus* [43-45]. As efflux pumps can aid in the process of detoxification of the cell, we anticipated a decrease in RLS in the *Δafr3* as have been described for other efflux pumps in *S. cerevisiae* [17]. Instead, we documented a moderate increase in RLS of the Δafr3 strain was also associated with a loss of variability of lifespan which may indicate a specific role in stress response, while the *Δafr1* and *Δafr2* mutants maintained high stochasticity, similar to the wild-type strain. Stochasticity of life span is still poorly understood in *C. neoformans*.

Exposure to stress, such as glucose deprivation, leads to an increase in FLC tolerance, as the change is only transient and does not become intrinsic to the strain [46]. Low glucose conditions lead to an increase in efflux through increased pump activity dependent on Afr1 [46]. Thus, we explored whether Afr3 contributes to increased FLC tolerance under low glucose growth conditions. We found *AFR3* deletion did not affect the enhanced FLC tolerance under glucose starvation, nor did we observe upregulation of AFR3 during glucose deprivation. *C. neoformans* can achieve resistance to FLC by the duplication of chromosome 1 and rarely chromosome 3 in response to prolonged exposure to FLC. Chromosome 1 is most commonly duplicated, increasing the copy number of genes *ERG11* and *AFR1* [47]. The observed increased FLC tolerance in low glucose growth conditions, however, is not associated with a change in gene copy number of AFR1 or AFR2. [46]. *AFR3* is located on chromosome 3 and the gene copy number was unchanged under low glucose stress (data not shown). It was previously hypothesized that the increase in efflux pump expression is being driven by stress-induced upregulation of transcription factors, as many transcription factors correlated with FLC resistance are upregulated in low glucose [14,46,48]. Afr3, as well as the other characterized ABC transporters (Afr1, Afr2, and Mdr1), are upregulated in 10-generations old cells. Additionally, the results from the modified killing assay showed that deletion of *AFR3* in older cells leads to a partial loss of resistance, indicating that Afr3 could aid in the FLC tolerance observed in 10- generation cells.

In summary, these data identify a novel ABC transporter that contributes to FLC tolerance in *C. neoformans* older cells. This ABC transporter promotes drug efflux from the fungal cell, influencing the susceptibility to treatment and the virulence of the cryptococcal cells. Our data encourage further efforts to understand its role in relation to other efflux pumps and which mechanisms the aging cells employ to increase drug resistance.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1; Figure S1: Confirmation of *Saccharomyces cerevisiae* Transformants; Figure S2: Pump Compensation between Afr1 and Afr3; Figure S3: Growth Curves; Table S1: List of Primers.

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Conflicts of Interest: The authors declare no conflict of interest.

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