

Supplements – Oliveira, R.K.M. Base Excision Repair AP-Endonucleases-Like Genes Modulate DNA Damage Response and Virulence of the Human Pathogen *Cryptococcus neoformans*

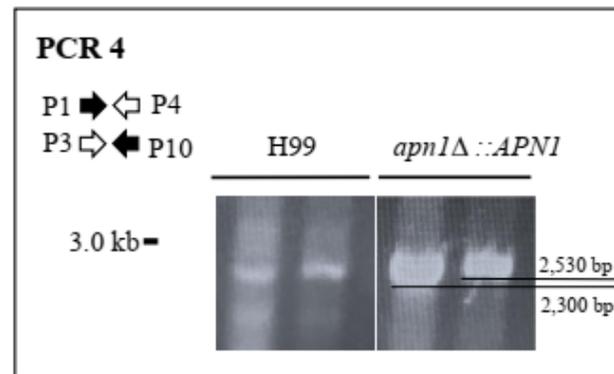
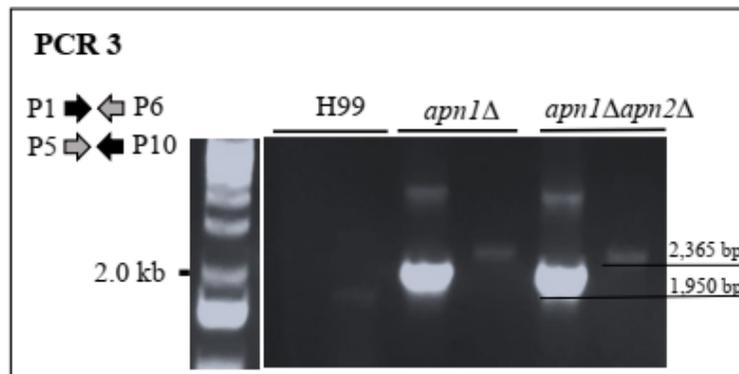
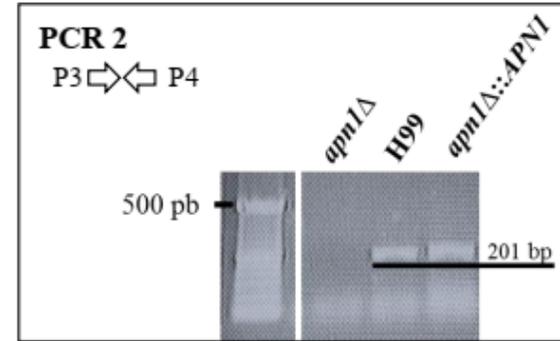
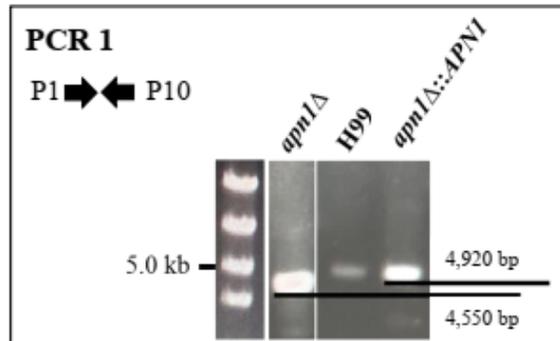
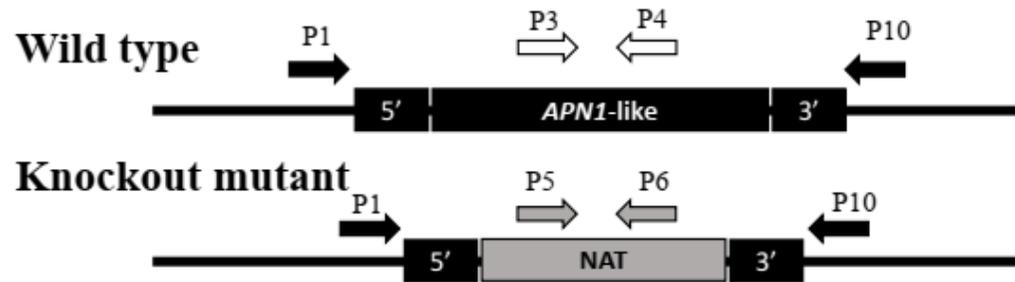
Table S1. Primers used in this study

Number	Identification	Sequence (5'→3')
APN1		
P1(APN1)	5'ext_APN1F	CGAGGAGCATAAGGAAGGAGA
P10(APN1)	3'ext_APN1R	GCATGACAACCTTCCCCTAGC
P3 (APN1)	5'APN1F	GAGTTAAGGGGTGGGAGGAAT
P4 (APN1)	3'APN1R	TCGCTGGAGTCTGTAGCTTGA
P5 (APN1)	nat_F	AGCAAGACCCATCAAAGCTCTA
P6 (APN1)	nat_R	CTCCAGAACATTCGTCGCTTAC
P11(APN1)	M13_5'APN1R	ATCATGTCATAGCTGTTTCCTGTCTGGGCCTTCACTCAAGAC
P12(APN1)	M13F_3'APN1F	GCACTGGCCGTCGTTTTACCAGACACGGCAGCTATCATTC
APN2		
P1(APN2)	5'ext_APN2F	CAAGCACCTTCTTACCCTCCT
P10(APN2)	3'ext_APN2R	CCACTTACCAAAGCTCACTGG
P3 (APN2)	5'APN2_F	GAATCGGACGATGATGCTGT
P4 (APN2)	3'APN2R	CAAACCCCATTCCTACTGAC
P5 (APN2)	hyg_F	GCGGGAGATGCAATAGGTCAG
P6 (APN2)	hyg_R	AGCTCTCGGAGGGCGAAGAAT
P11(APN2)	M13_5'APN2R	ATCATGTCATAGCTGTTTCCTGTGTGTAGTGTCCCGATCCAT
P12(APN2)	M13F_3'APN2F	GCACTGGCCGTCGTTTTACCCCTTCTGGTTTAGCTGACG

Table S2. Strains used in this study

Strain	Genotype	Source
H99	<i>MAT</i> α	Perfect et al. (1980) [1]
<i>apn1</i> Δ	<i>apn1::nat MAT</i> α	This study
<i>apn2</i> Δ	<i>apn2::hyg MAT</i> α	This study
<i>apn1</i> Δ <i>apn2</i> Δ	<i>apn1::nat apn2::hyg MAT</i> α	This study
<i>apn1</i> Δ ::APN1	<i>apn1::nat apn1::neo MAT</i> α	This study
<i>apn2</i> Δ ::APN2	<i>apn2::hyg APN2::neo MAT</i> α	This study

A



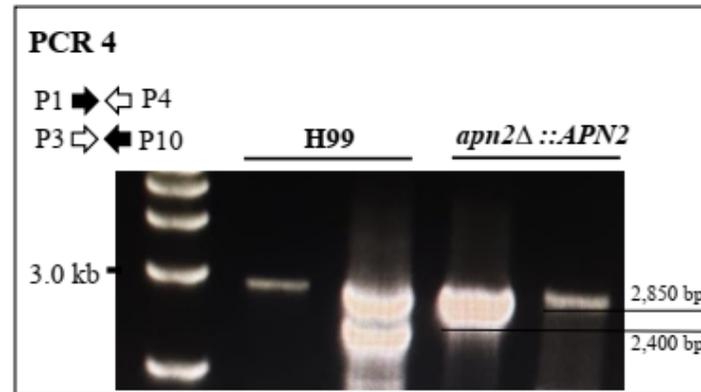
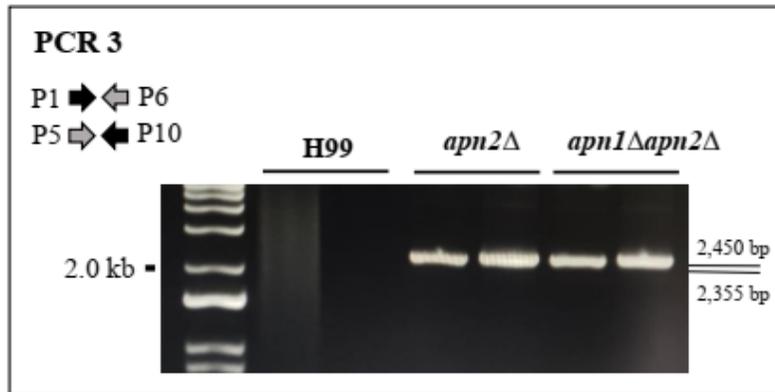
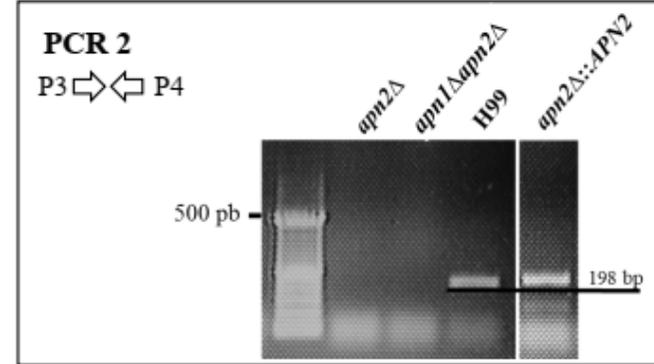
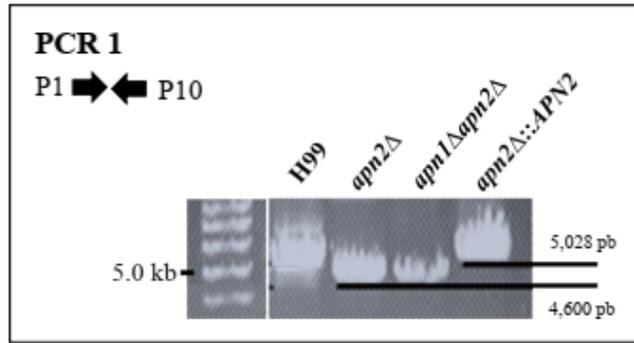
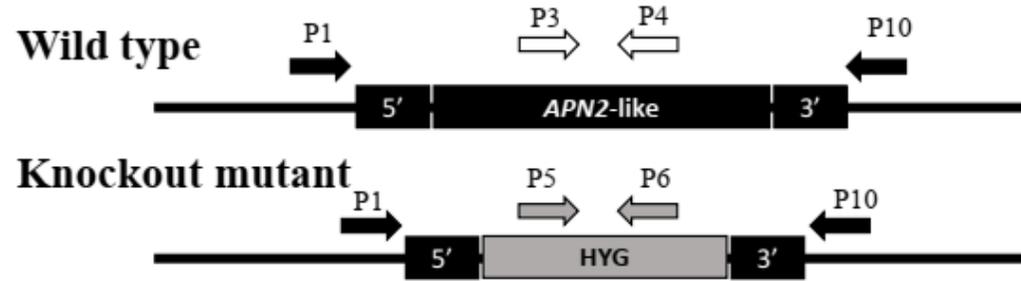
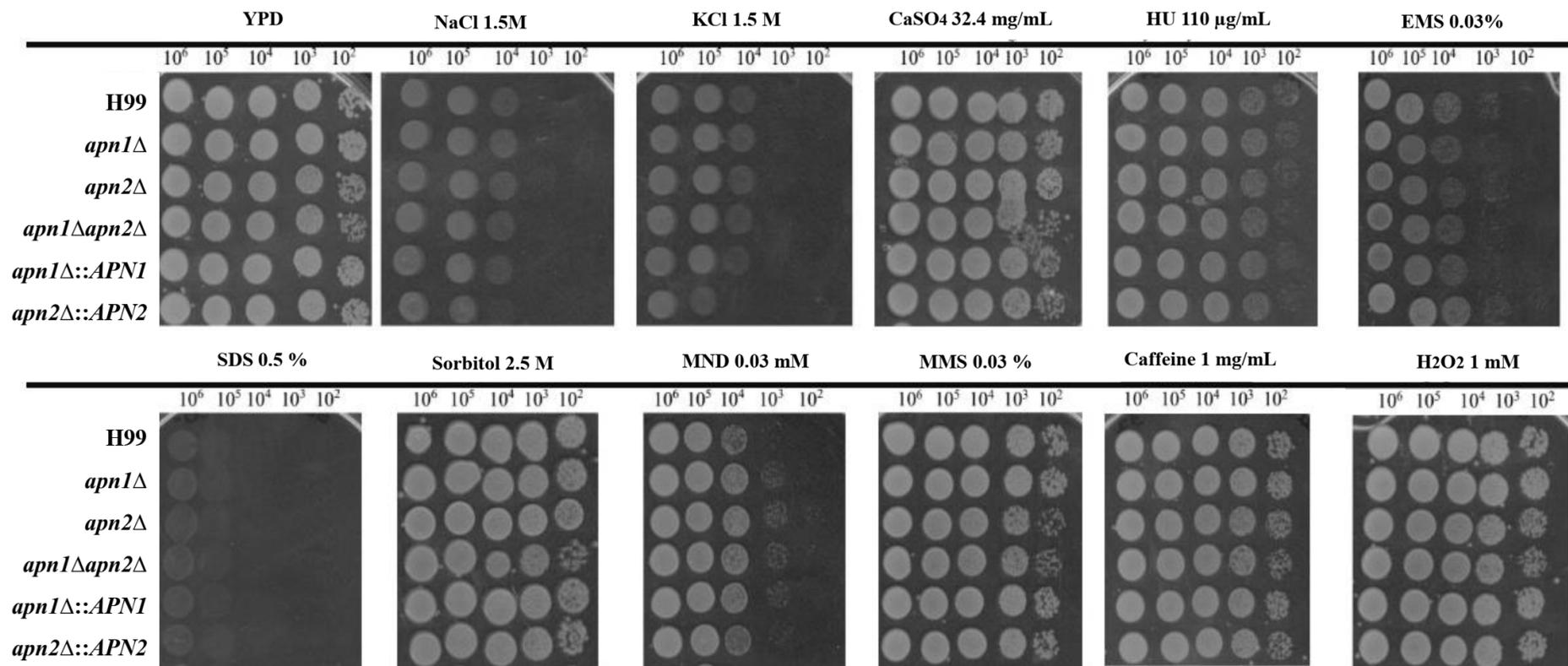
B

Figure S1. Confirmation of knockout mutants by genomic DNA PCR amplification. Schematic representation of four PCR sets to confirm gene disruption. PCR 1 was performed with primers designed outside the 5' upstream flanking region (5') and the 3' downstream flanking region (3') (black arrows). Knockout mutants were expected to generate a single fragment in PCR1 that differed in size from that of the WT and specific reconstituted strains. PCR 2 consisted of multiplex reactions using specific primers for the target gene (white arrows). The target gene was expected to be amplified from the WT gDNA, whereas no amplicon was expected from the knockout mutants' DNA. PCR3 was performed with primers designed outside the 5' and the 3' regions (black arrows), and for the antibiotic resistance marker (HYG: Hygromycin B; NAT: Nourseotricin; Gray arrows). Amplicons in PCR 3 were expected to be generated only from the knockout mutants' DNA. PCR 4 was performed using a combination of flanking region primers and specific primers for the target gene (white arrows). In PCR4, the target genes were expected to be amplified only from the reconstituted strains DNA. **(A)** Electrophoretic analysis in 0.8% agarose gel stained with 0.5 µg/mL EtBr of *APN1*-like gene knockout and reconstituted strains' amplicons. **(B)** Electrophoretic analysis in 0.8% agarose gel stained with 0.5 µg/mL EtBr of *APN2*-like gene knockout and reconstituted strains' amplicons. The amplicons' size is indicated in base pairs (bp).

A

30 °C



B

37 °C

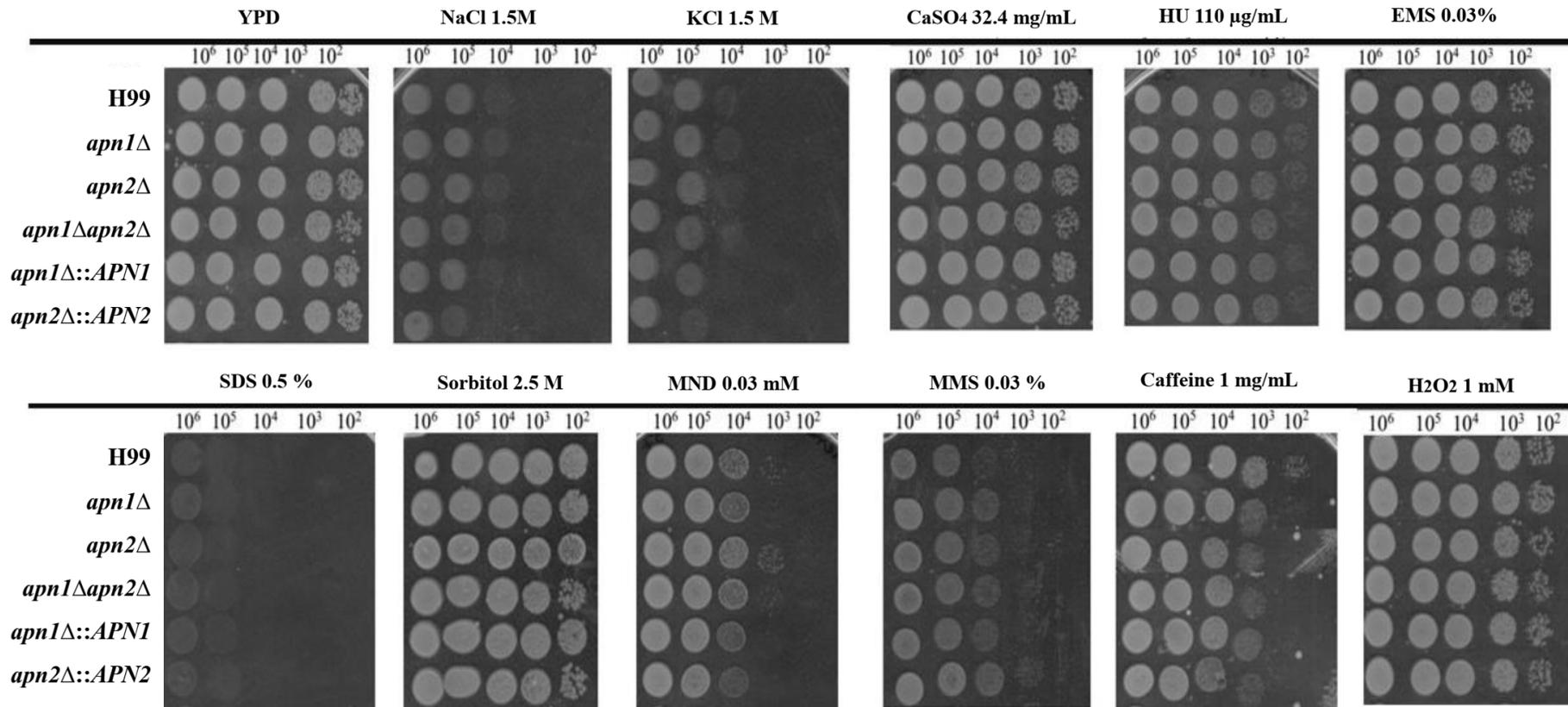


Figure S2. Temperature and chemical cell stress-related phenotypes of the *C. neoformans* APN-like genes' mutants. Ten-fold serial dilutions for the indicated strains were

spotted onto solid YPD supplemented with different compounds at 30 (**A**) and 37 °C (**B**). Growth was assessed after 48 h of incubation. EMS: ethylmethane sulfonate; MMS: methylethane sulfonate; HU: hydroxyurea; MND: Menadione).

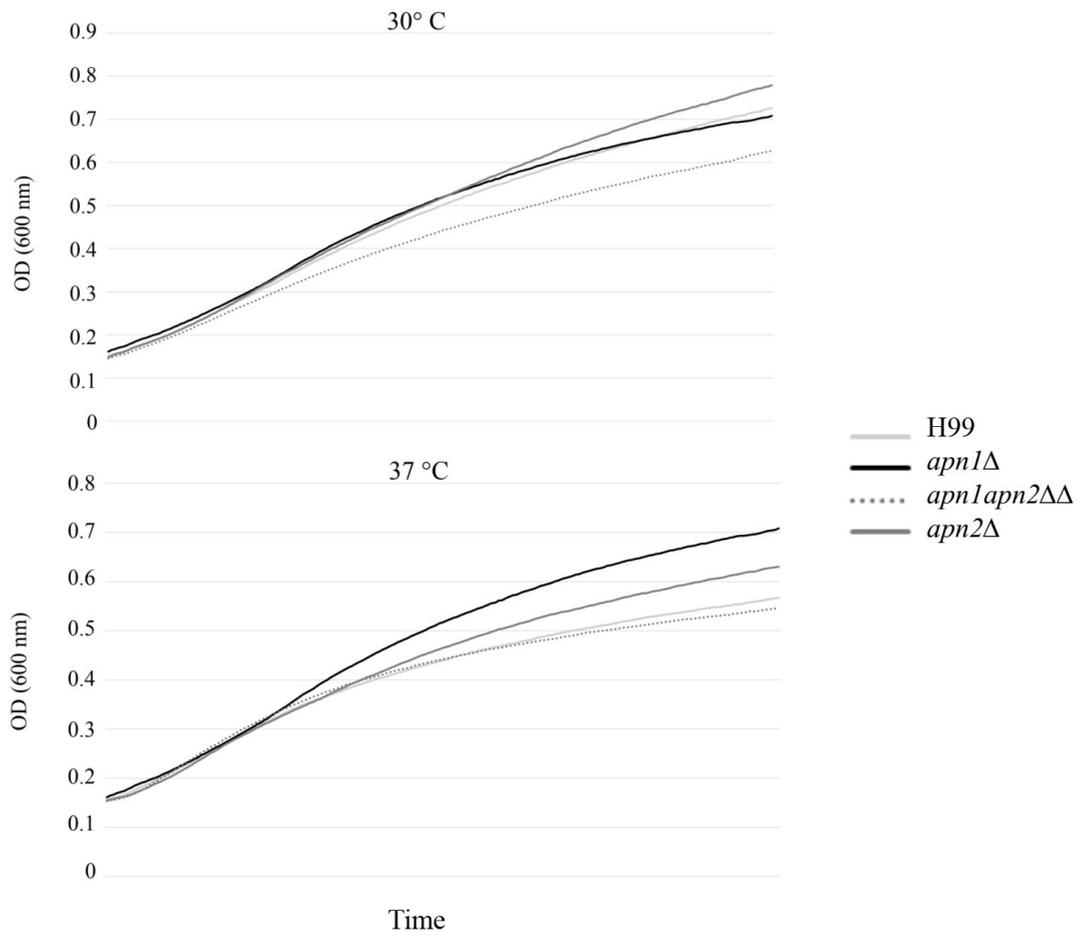


Figure S3. Growth curve of *C. neoformans* WT and mutant strains. Cells were grown overnight in liquid YPD and diluted to new cultures at 10^4 cells/mL per well. The cultures were then incubated at 30 or 37 °C in an automated microbiology growth curve analysis system (BioTek Eon Microplate Spectrophotometers, Winooski, VT, United States) and the absorbance (600 nm) was read every 30 min for 96 h. OD: optical density. The graphs represent the averages of three readings.

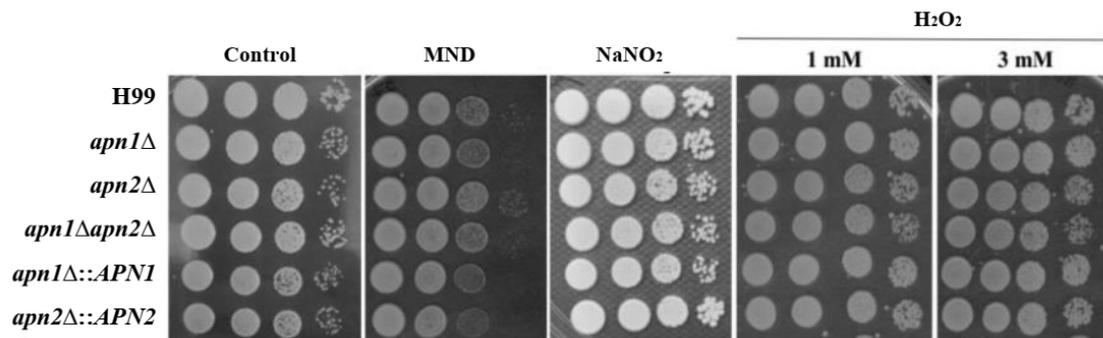


Figure S4. The *apn1*Δ, *apn2*Δ and *apn1*Δ*apn2*Δ mutants are not susceptible to oxidizing agents. *C. neoformans* WT, mutant and reconstituted strains of yeasts were ten-fold serially diluted and spotted onto YPD agar supplemented with 0.03 mM menadione (MND), 4 mg/mL sodium nitrite (NaNO₂) or 1 or 3 mM hydrogen peroxide (H₂O₂). Growth was assessed after 48 h of incubation, at 30 °C, protected from light. The results are representative of at least three independent experiments.

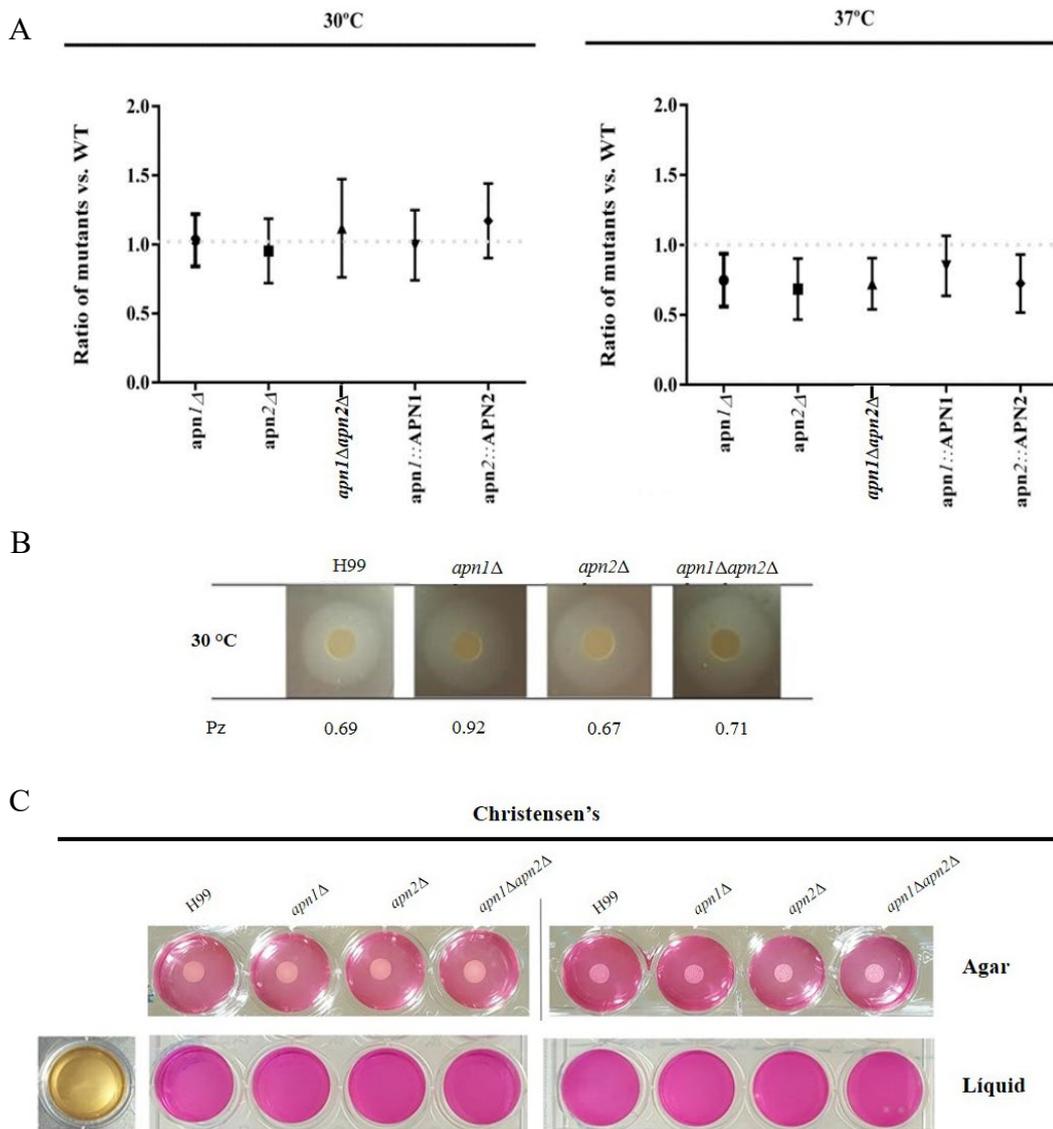


Figure S5. The capsule expansion, phospholipase and urease activities were unaffected by the disruption of *Apn*-like genes. (A) Determination of the capsule size. The strains were inoculated in minimum media (MM) at 30 or 37 °C for 72 h to induce capsule formation. Capsule size was visualized by India ink counterstaining and quantified by assessing the packed cell volume of normalized cell suspensions, expressed as a ratio to the WT strain. Data points represent averages of triplicate samples (+/- standard error). Statistical test: One-way ANOVA with Dunnett's post-test (B) Phospholipase activity assay. The yeast cells were spotted on Agar Egg Emulsion medium and incubated at 30 °C for 72 h. Hydrolysis halo sizes were measured according to the Price et al., (1982) protocol⁸⁴. The phospholipase activity (Pz) was measured by the ratio of the colony diameter and the colony plus precipitation zone diameter after 72 h of growth at 30 °C. (C) Urease activity assay. Strains were cultivated for 48 h at 30 or 37 °C in liquid and in solid Christensen's urea medium. The shift from yellow to pink in the medium color is indicative of urease activity.

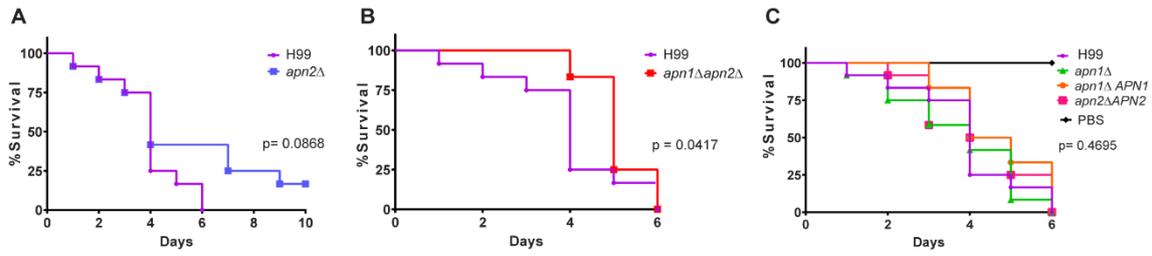


Figure S6. Evaluation of virulence of *C. neoformans* APN mutants using *G. mellonella* infection model.

Larvae were infected in the last left proleg with 10 μ L of 10^6 cell suspension of the indicated *C. neoformans* strains (WT, *apn1* Δ , *apn2* Δ , *apn1* Δ *apn2* Δ mutants) or 10 μ L of PBS (mock control). After infection, they were incubated at 37 $^{\circ}$ C and daily monitored for survival. (A) Comparison between survival curves of larvae infected with H99 and *apn2* Δ . (B) Comparison between survival curves of larvae infected H99 and *apn1* Δ *apn2* Δ mutant. C) Survival curves of the larvae infected with WT, *apn1* Δ , *apn2* Δ , *apn1* Δ *apn2* Δ and the PBS control. Survival curves were analyzed using Log-rank (Mantel-Cox) test.

References

1. Perfect J.R., Lang S.D., Durack D.T. Chronic cryptococcal meningitis: a new experimental model in rabbits. *Am J Pathol.* 1980;101:177–194.