Supplemental Data

Materials and Methods

Strains plasmids and media:

C. neoformans wild-type strain ATCC 208821 (H99, serotype A, mating type α) was a gift from J. Perfect. H99-derived *ura5* mutant strain H99FOA was selected on 5-FOA medium as described (8). *E.coli* XL-1 Blue MRF' (Stratagene) was the host strain used for screening the Uni-Zap cDNA library. *E. coli* SOLR (Stratagene) was the recipient strain of the Bluescript phagemid after *in vivo* excision from the Uni-Zap XR vector (Stratagene) containing cDNA clones. *E. coli* DH10B (Life Technologies) was the host strain for the recovery of ligated plasmid. Media is as follows: asparagine salts contains (1g/L asparagine, 10 mM sodium phosphate, pH 6.5, 100mg/L $MgSO_4$,) asparagine nor-epinephrine agar contains asparagine salts plus 100 mg/L nor-epinephrine, 18 g/L bacto-agar. Oligonucleotide sequences are provided in Supplemental Table 2.

Cellular localization of epitope-tagged Vad1 by deconvolution immuno-microscopy

A PCR-amplified fragment of *VAD1* containing *VAD1* promoter elements was inserted upstream of a pBluescript cassette consisting of a GC-rich green fluorescent protein (GFP)[28], an EF1α cryptococcal terminator sequence and a 1.3-kb fragment of *URA5* previously described.[17] Primers for *VAD1* were VAD-GFP-A-Mun and VAD-GFP-S-BGL, primers for EF1α were:

Ade-termAvr-S and Ade-termAge-A, and primers for GFP were Serg-GFPR1S and Serg-GFP-R1A. The PCR templates were H99 DNA for *VAD1* and EF1α and pGFP (a generous gift of S. Zolotukhin) for GFP. Linearized constructs without plasmid sequence were transformed into the *Δvad1* strain and selected for growth on hygromycin. Transformants were analyzed by Southern blot analysis of uncut genomic DNA hybridized with a fragment of the HgR open reading frame to identify one (VGFP-1) having genomic incorporation of the construct. VGFP was grown on YPD for two days, then

inoculated into asparagine liquid either containing 2% glucose, or identical media without glucose, incubated at 30ºC for 3 h and prepared for immunofluorescence microscopy as previously described (61), except that an IX70 microscope (Olympus) with Slidebook 3 deconvolution software (Intelligent Imaging, Denver, CO) was used instead of confocal microscopy. To identify the expression and cellular location of the Vad1 protein during neuropathogenesis, mice were inoculated intravenously with 10^5 cells of either wild-type or the GFP-Vad1 expressing strain, VGFP-1. Mice were monitored for two weeks at which time they were sacrificed by CO_2 narcosis. Brains were immediately removed and sectioned longitudinally. One half of the brain was homogenized and cultured for colony counts and the other half homogenized in ice cold phosphate buffered saline and subjected to sucrose-gradient centrifugation for 20 min at 4ºC to separate *C. neoformans* cells from brain tissue as described (20) and subjected to fluorescence microscopy as above.

<u>Sizing of epitope-tagged Vad1 by HPLC gel filtration</u>

Cells were mechanically disrupted with glass beads in lysis buffer (150mM NaCl, 1% Triton X-100, 50mM Tris HCl , pH 8.0) with the following protease inhibitor cocktail (Sigma-Aldrich): 10mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 0.22 mM pepstatin A, 0.14mM E-64, and 50mM 1,10-phenanthroline, and centrifuged at 10,000g x 10 min. The supernatant was then applied to a 30cm x 7.8 mm TSKgel G6000 HHR HPLC column (Sigma-Aldrich) equilibrated with 50 mM sodium phosphate, pH 6.5. Exclusion sizes were estimated by reference to equivalent 1mL/min elutions using molecular mass standards (Sigma-Aldrich). 100µL of each 1mL fraction was mixed with 1% SDS, heated at 95oC for 5 min and vacuum blotted to supported nitrocellulose in the presence of 10% methanol. The Vad1-GFP protein was detected using the Super Signal West Pico kit (Pierce) with the following antibody combination: mouse monoclonal antibody 9E10 (anti-c-myc) (Covance Research,

dilution 1:200), horseradish peroxidase anti-mouse Ab (Sigma, dilution 1:1000).

Deletion of the *PCK1*, *TUF1* and *MPF3* genes in *C. neoformans*:

Deletion of *PCK1* was performed as follows: a 2.1-kb fragment of the *PCK1* gene containing its ORF was amplified by PCR (*PCK1*-3331, *PCK1*-5412) using *pfx* polymerase (Invitrogen) followed by subcloning into plasmid pCR4Blunt TOPO (Zero Blunt PCR cloning, Invitrogen) to generate plasmid pPW7.2. The plasmid DNA was digested with *BamH*I and *Kpn*I to remove a 1-kb segment of the inserted *PCK1* and was replaced with a 1.3-kb fragment of the *URA5* gene with primers containing suitable restriction sites to generate plasmid pPW7.2/URA5. Complementation plasmid pPW7.3/HgR was made by inserting a PCR amplified fragment produced using H99 DNA as template and primers, PCK-2323S and PCK-5686A into plasmid pBSC-HgR described previously (59). The deletion construct was reamplified by primers *PCK1*-3331 and *PCK1*-5412, purified (PCR purification kit from Qiagen), and transformed to H99FOA. Transformants were screened by PCR using suitable primers and subjected to Southern blot analysis of genomic DNA digested with *BamH*I and *Kpn*I using a radiolabeled fragment amplified using primers PCK1-3331 and PCK1-5412. Reconstitution of the *Δpck1* mutant was performed by transformation with plasmid pPW7.3/HgR and selected on hygromycin containing asparagine agar.

Deletion of *MPF3* was performed as follows: Two fragments of *MPF3* were PCR amplified using primers, PW4-2764S-Xba and PW4-3424A-RI for the first fragment and PW4-3904-Bgl and PW4-4488A-Xho for the second fragment. The fragments were digested with suitable restriction enzymes and ligated with a PCR-amplified fragment of the *URA5* gene containing complementary terminal *Bgl*II and *Eco*RI sites into pBluescript SK (Stratagene) digested with *Xba*I and *Xho*I to produce plasmid PW4/URA5. The construct was PCR amplified with *pfx* polymerase using primer PW4-2764S-

Xba and PW4-4488A-Xho and transformed by electroporation into H99FOA strain and selected on minimal media. Transformants were screened by PCR amplification using suitable primers and deletion of *MPF3* was established in one transformant by Southern blot analysis of genomic DNA digested with *Bgl*II and *Eco*RI hybridized with a PCR amplified fragment of DNA from H99 using primers: PW4-2644S and PW4-4102A.

RNAi suppression of *TUF1* was performed by the following method: *TUF1* was PCR amplified from an H99 cDNA mass-excised library using primers TUF1-3114SR and TUF1-3657AX and two fragments of the *TUF1* gene were ligated downstream from an *ACT1* promoter of a pPM8 episomal vector (41) in a sense-antisense fashion on either side of a linker fragment consisting of a 500 bp PCR-amplified fragment of Intron I of *CNLAC1* generated from a *CNLAC1* genomic clone using primer INTRON-XhoS and INTRON-XhoA to produce plasmid EF-TU7A. EF-TU7A was linearized and transformed into H99FOA and transformants selected on minimal media. Control strains were transformed with the identical plasmid EF-TU7B, which contained only one copy of *TUF1* without a second antisense fragment. All transformants exhibited wild-type laccase activity on nor-epinephrine agar.

Supplemental Results:

Molecular characterization of *VAD1* strains.

Wild-type DNA digested with *BamH*I and hybridized with a radiolabeled cDNA probe of *VAD1* shows a single band of 4.5 kb (Sup. Figure 1B and 1E, lane 1). Interruption of the *VAD1* ORF at position 1319 was demonstrated in the *Δvad1* mutant by the presence on Southern blots of an additional band due to a *Bam* HI site within the pBluescript/URA5 insertional plasmid and from sequencing of the

recovered plasmid (Sup. Figure 1C and 1E, lane 2). To complement the *Δvad1* mutation, a 4.5-kb fragment of wild-type *VAD1* was used to transform the mutant using a HgR gene as a selection marker ((59) Sup. Figure 1D). Southern blot analysis showed an additional copy of the wild-type gene migrating as a 9-kb band in the complemented strain (Sup Figure 1E, lane 3). These results were confirmed by digestion of the strains with a second restriction endonuclease, *Stu*I, which yielded the predicted sizes when hybridized with a PCR amplified fragment of the *VAD1* ORF (H99: 2.6, 1.6 kb; *Δvad1*: 1.4, 2.7, 3.9 kb; *Δvad1*:: *VAD1*: 1.4, 2.7, 3.9, 5.0 kb).

Supplemental Figure Legend

Sup. Figure 1. Homology of RCK/p54 relatives of Vad1 and construction of *Δvad1* mutants in *Cryptococcus neoformans*. Panel A: Diagram of RCK/p54 members showing ATPase and RNA interaction sites and poorly conserved C-terminus that includes a poly-glutamine region in Vad1. Panel B: diagram of the *VAD1* ORF with relevant restriction sites. Panel C: Diagram of insertional construct, pMUT8 within the *VAD1* gene. Panel D: Complementation construct of *VAD1*. Panel E: Southern blot analysis of *VAD1* strains.

Supplemental Table 1. *C. neoformans* serotype A strains

Strain	Genotype	Source
H99	wt, Mat α	A gift from J. Perfect
H99FOA	Isogenic to H99, except *ura5*	Erickson et al. (2001)
M049	Isogenic to H99, except *ade2*	
C53	Isogenic to H99FOA, except *vad1*::*URA5*	This work
CC53	Isogenic to H99FOA, except *vad1*::*URA5* + *VAD1*::*HgR*	This work
PK-19	Isogenic to H99FOA, except *pck1*::*URA5*	This work
PK-19-6	Isogenic to H99FOA, except *pck1*::*URA5* + *PCK1*::*HgR*	This work
MPF-1	Isogenic to H99FOA, except *mpf3*::*URA5*	This work
MPF-c	Isogenic to H99FOA, except *mpf3*::*URA5* + *MPF3*::*HgR*	This work
NO20	Isogenic to H99, with P_{GPD1} -*NOT1*-T_{TRP1} ::*HgR*	This work

Supplemental Table 2. Oligonucleotide Sequences

Oligo Name	Oligo Sequence
C53T3:	GGTCACTGAACTTGGCTACTC
C53U5:	TACCATGGCGGGAGCGACATAG
VAD-GFP-A-Mun:	GCCGCCCAATTGAGCCTGTTGGCTCTG
VAD-GFP-S-BGL:	GCCGCCAGATCTTTGGTGTATATATCGGTAA
Ade-termAvr-S:	GCCGCCCCTAGGATCAACGGTTTTATGCTGGT
Ade-termAge-A:	GCCGCCACCGGTGTGAAGTTGGGGGAGAA
Serg-GFPR1S:	GCCCCCAATTCGGGCGAGGAACTGTTCAC
Serg-GFP-R1A:	CGCCGAATTCTCACTTGTACAGCTCGTCCAT
PCK1-3331	CACAACAGTCATCTCCCC
PCK1-5412	GACCTGTTTGACCGTTAG
PCK-2323S:	GGAACACAGCTGTAGTTGCTA
PCK-5686A:	ATTGAGAGAGATACAAAGAGGTC
PW4-2764S-Xba:	GCCGGTCTAGAGTAAATATTGACGTCAGCATG
PW4-4102A:	TTGCTCACTGACCAAAGGATC.
PW4-3904-Bgl:	GCCGCCAGATCTGCTAGTAGCGCTTCTAAC
PW4-4488A-Xho:	GCCGCCTCGAGAGTGATAGACAGAGC
PW4-2644S:	CCAGTTTGACTCGTCTTGGAAC
PW4-4102A:	TTGCTCACTGACCAAAGGATC.
TUF1-3114SR:	GCCGGAATTCTCTATCTCTGGTCGAGG
TUF1-3657AX:	GCCGCTCGAGCGATGTCATGCACAAG

INTRON-XhoS: GCCGCTCGAGATCCTAATCGGTAAATATTTCTTTC

INTRON-XhoA: GCCGCTCGAGCGTCGGTATAGCTAAATTG

iNOT1-F-RI: GGAGGAGAATTCGGTTCGCATGCTCCTAACCTCGC

iNOT1-R-Xh: GGAGGACTCGAGCGTATCGTGTCGCAACAAACGCCG

FL-NOT-F-Xb: GGAGGATCTAGAATGTCAATCCCTCCTCCCGGCCTGG

myc-NOT1-R- Nt: TTCTTCGCGGCCGCTTAGAGGTCCTCCTCGCTTATGAG

CTTCTGCTCCATCTGCAATCCCTGCGCGCCGCG