Genome-wide functional analysis of phosphatases in the pathogenic fungus Cryptococcus neoformans

Phosphatases, together with kinases and transcription factors, are key components in cellular signalling networks. Here, we present a systematic functional analysis of the phosphatases in Cryptococcus neoformans, a fungal pathogen that causes life-threatening fungal meningocerehalitis. We analyse 230 signature-tagged mutant strains for 114 putative phosphatases under 30 distinct in vitro growth conditions, revealing at least one function for 60 of these proteins. Large-scale virulence and infectivity assays using insect and mouse models indicate roles in pathogenicity for 31 phosphatases involved in various processes such as thermotolerance, melanin and capsule production, stress responses, O-mannosylation, or retromer function. Notably, phosphatases Xpp1, Ssu72, Siw14, and Sit4 promote blood-brain barrier adhesion and crossing by C. neoformans. Together with our previous systematic studies of transcription factors and kinases, our results provide comprehensive insight into the pathobiological signalling circuitry of C. neoformans.
Aspergillus fumigatus phosphatases and functionally characterized 24 of them in genome-wide analyses of phosphatases have identified functional phosphatome data in several fungal pathogens annually3; of the 155 and 129, respectively, functionally characterized kinases were identified in in vitro and in vivo. Indeed, 178 TFs and 183 of fungal kinases and transcription factors (TFs) provide even more comprehensive insights into how fungal signalling pathways modulate infection and virulence. Indeed, 178 TFs and 183 kinases were identified in Cryptococcus neoformans, which causes life-threatening meningoencephalitis mainly in immunocompromised patients and is responsible for more than 180,000 deaths annually3; of the 155 and 129, respectively, functionally characterized in vitro and in vivo4,5 TFs and 63 kinases are involved in its pathogenicity. Nevertheless, the regulation of these signalling components and their coordination in pathogenicity remain elusive.

To better understand TF and kinase networks, co-analysis of the phosphatase networks as signalling counterparts is essential. Historically, phosphatases have received less attention than kinases, probably because kinases, which possess high substrate specificity, are generally considered to be better drug targets than substrate-promiscuous phosphatases6. However, recent systematic functional phosphatome data in several fungal pathogens indicate that phosphatases play critical roles in maintaining cellular homeostasis by controlling growth and cell cycle, differentiation, stress response, and metabolism. In ascomycete fungi, genome-wide analyses of phosphatases have identified 32 protein phosphatases and functionally characterized 24 of them in Aspergillus fumigatus, the majority of which contribute to stress response, iron assimilation, and toxin production and resistance7. Furthermore, in the wheat scab fungus Fusarium graminearum, Yun et al. identified 82 phosphatase genes, disrupted 71 of them, and functionally analysed these mutants across 15 phenotypic traits (e.g., growth, nutrient response, and virulence), finding that 25 of these phosphatases are involved in virulence of the phytopathogenic fungus8. Among phosphatases, calcineurin is a well-established drug target and a globally conserved fungal virulence factor9.

The goal of this study was to systematically analyse the functions of the C. neoformans phosphatase network and interplay with its kinase and TF networks to better understand its pathobiological signalling. To this end, we constructed a high-quality library of 219 signature-tagged gene-deletion mutant strains representing 109 phosphatases out of 139 putative phosphatases identified in C. neoformans, in addition to 11 signature-tagged mutants representing six phosphatases that we previously constructed10,11. Using a total of 230 signature-tagged mutants representing 114 phosphatases, we analysed their phenotypic traits under 30 distinct in vitro conditions and performed a large-scale virulence assay using two model host systems (insect and murine). This entire phosphatome phenome data set is freely available to the public through the C. neoformans Phosphatase Phenome Database (http://phosphatase.cryptococcus.org).

Results

Identification of phosphatases in C. neoformans. To select putative phosphatase genes, we surveyed a curated annotation of phosphatases in the FungiDB C. neoformans (H99 strain) genome database (http://fungidb.org/fungidb) and validated the presence of phosphatase-related domains using protein sequence analysis and classification (Interpro). Through these analyses, we retrieved a total of 139 putative phosphatase genes in C. neoformans (Fig. 1a, b and Supplementary Data 1). We made three notable findings. First, phosphatases are generally less evolutionarily conserved than kinases, but more conserved than TFs (Supplementary Fig. 1 and Supplementary Data 2). Second, despite a paucity of tyrosine kinases [only three tyrosine kinase-like (TKL) proteins], C. neoformans contains 21 protein tyrosine phosphatases (PTPs). Third, compared to other non-pathogenic and pathogenic yeasts, C. neoformans contains a similar number of putative phosphatases (Fig. 1c and Supplementary Data 3).

Construction of the C. neoformans phosphatase mutant library. To obtain comprehensive insights into the C. neoformans phosphatome networks and their biological functions, we constructed gene-deletion mutants for each phosphatase gene and analysed their in vitro and in vivo phenotypic traits. Among the 139 putative phosphatase genes, 15 have been functionally characterized previously by gene-deletion studies (PTP110, PTP210, YSA12, CNA113, CAC111, TPS214, CCR415, HAD116, EPP117, XPP17, APH118, ASP119, ISC20, PPG121, and PPH221). In addition, we previously deleted two phosphatase genes with kinase domains (OXK1 and FBP26) for construction of the kinase mutant library2 and one phosphatase gene with a DNA-binding domain (AP2) for the TF mutant library4. Besides the 11 signature-tagged mutant strains (two for each PTP1, PTP2, OXK1, FBP26, and APN2 and one for CAC1) that we previously constructed, we performed a large-scale homologous recombination-based gene deletion using nourseothricin-resistance markers containing a series of unique oligonucleotide signature tags that we previously employed for construction of the TF and kinase mutant libraries4,5 (Supplementary Data 4). To obtain a high-quality phosphatase mutant library, we constructed more than two independent mutants for each gene and verified their genotypes by diagnostic PCR and Southern blot analysis. As a result, here, we report 219 new mutant strains representing 109 phosphatases and the analysis of a total of 230 mutant strains representing 114 phosphatases (Supplementary Data 5). Disruption strategies, primer sequences, Southern blot results, and mutant phenome data are available in the Cryptococcus neoformans Phosphatase Phenome Database (http://phosphatase.cryptococcus.org) we constructed for this study. For the remaining 25 phosphatase genes, we failed to obtain any viable transformants or obtained only potential aneuploid mutants possessing both wild-type and mutant alleles after repeated attempts, suggesting these genes may be essential (Supplementary Data 6). We also developed the Cryptococcus neoformans Phenome Gateway Database (http://www.cryptococcus.org/), in which every TFs, kinases, and phosphatases studied4,5 are linked to the most widely used fungal genome database “FungiDB” (https://fungidb.org/fungidb/) to maximize connectivity between research data.

Phenotypic and in vivo expression profiling of phosphatome. To elucidate the functions of C. neoformans phosphatases deleted in this and previous studies, we examined phenotypic traits under...
30 distinct in vitro conditions: growth at different temperatures (25, 30, 37, and 39 °C), mating efficiency, virulence factor production (capsule, melanin, and urease), stress responses (osmotic/cation salt, oxidative, genotoxic, ER, cell membrane/wall, and heavy metal stresses), and antifungal drug susceptibility (Supplementary Data 7). The whole phenome data set of the phosphatase mutant collection was qualitatively illustrated with a colour scale (Fig. 2 and Supplementary Data 7). This systematic phenotypic analysis revealed that ~53% of the phosphatase mutants (60/114) showed at least one discernible phenotype (Fig. 2); 72% of these (43/60) have not been previously functionally analysed. When we compared our phosphatase phenome data with their corresponding BLAST matrix data (Supplementary Fig. 2 and Supplementary Data 2), we found that the putative essential phosphatases that we could not disrupt and the phosphatases showing multiple phenotypic traits were generally more evolutionarily conserved. Phenotypic clustering of phosphatase mutants revealed groups of phosphatases that could be directly or indirectly correlated with regards to cellular function (Fig. 2).

In addition, we monitored how each of the 139 phosphatase genes were transcriptionally regulated during murine infection by assessing expression levels of each phosphatase gene in recovered tissues (lungs, brain, spleen, and kidneys) after 3, 7, 14, and 21 days post-infection (dpi) with strain H99S using the nCounter gene expression profile (NanoString, Seattle, WA, USA) with 139 novel phosphatase probes. The in vivo expression levels of each phosphatase were normalized to average in vivo expression levels of novel phosphatase probes. The in vivo expression levels of each phosphatase gene-deletion mutants that we constructed.}

Fig. 1 Classification of phosphatases in C. neoformans and other fungal species. a Phenylogenetic analysis of phosphatases utilized a protein sequence-based alignment in ClustalX2 (Science Foundation Ireland, Dublin, Ireland). Red letters represent pathogenicity-related phosphatases in C. neoformans identified from 114 phosphatase gene-deletion mutants that we constructed. b Pie chart indicating the classification and distribution of 139 phosphatases based on phosphatase-related domain, as analysed by using the InterPro Database. c Pie chart indicating the distribution of putative phosphatase genes in the fungal species S. cerevisiae, S. pombe, C. albicans, and U. maydis surveyed and classified using the same strategies as for C. neoformans.
Fig. 2 Phenotypic clustering and in vivo expression profiling of phosphatases in *C. neoformans*. In vitro phenotypic traits were examined under 30 different growth conditions and scored on a 7-point scale (−3: strongly reduced/susceptible, −2: moderately reduced/susceptible, −1: weakly reduced/susceptible, 0: wild-type like, +1 weakly enhanced/tolerant, +2: moderately enhanced/tolerant, +3: strongly enhanced/tolerant). All phenotypic data are available in the Cryptococcus neoformans Phosphatase Phenome Database (http://phosphatase.cryptococcus.org). More than three biologically independent experiments were performed for each phenotypic trait. Hierarchical phenotypic clustering of 60 phosphatases showing at least one phenotypic trait was performed with one minus Pearson correlation in Morpheus (https://software.broadinstitute.org/morpheus). The right panel shows the corresponding in vivo gene expression profiles for each phosphatase gene determined by NanoString nCounter platform analysis during intranasal murine infection with *C. neoformans*. Red letters represent pathogenicity-related phosphatases. The sdp101Δ mutant, which did not show any in vitro phenotypes but exhibited reduced infectivity, was also included.

**Abbreviations**: 25 25 °C, 30 30 °C, 37 37 °C, 39 39 °C, CAP capsule production, MEL melanin production, URE urease production, MAT mating, HPX hydrogen peroxide, TBH tert-butyl hydroperoxide, MD menadione, DIA diamide, MMS methyl methanesulphonate, HU hydroxyurea, 5FC 5-flucytosine, AMB amphotericin B, FCZ fluconazole, FDX fluodoxonil, TM tunicamycin, DTT dithiothreitol, CDS cadmium sulfate, SDS sodium dodecyl sulfate, CR Congo red, CFW calcofluor white, KCR YPD + 1.5 M KCl, NCR YPD + 1.5 M NaCl, SBR YPD + 2 M sorbitol, KCS YP + 1 M KCl, NCS YP + 1 M NaCl, SBS YP + 2 M sorbitol.
Phosphatases governing \( C. \) neoformans pathogenicity. To identify phosphatases required for the pathogenicity of \( C. \) neoformans, we performed two large-scale infectivity and virulence assays: (1) a virulence assay using the insect larval model system \( Galleria \) mellonella and (2) signature-tagged mutagenesis (STM)-based lung and brain infectivity assays using a murine inhalation model. Both methods have been successfully used for identifying virulence-related genes from a large-scale gene-deletion mutant set in previous studies\(^4,5,24\).

Using the insect-killing virulence assay, we identified 23 phosphatase genes involved in virulence (Fig. 3a and Supplementary Fig. 4): CNA1, TPS2, CAC1, PTP2, VPS29, PPH3, GDA1, YVH1, SSU72, PHS1, SIW14, DBR1, PSR1, YND1, INP5201, INP5202, HAD1, SIT4, PPG1, GUA1, NEM1, FBP26, and OCA101. STM-based murine lung and brain infectivity revealed 24 infectivity-related phosphatase genes, TPS2, CNA1, VPS29, PHS1, SIW14, CAC1, SSU72, PPG1, INP5201, CCR4, DBR1, GUA1, YMR1, NEM1, OCA1, PTP2, FBP26, PSR1, MRE11, CDC1,
Fig. 3 Pathogenicity-related phosphatases and their phenotypic traits in C. neoformans. a Virulence-regulating phosphatases were identified by a Galleria mellonella insect killing assay (n ≥ 15). P values were calculated using the log-rank (Mantel–Cox) test to measure statistical differences between the WT strain (H995) and phosphatase mutants. b Infectivity-regulating phosphatases identified by the signature-tagged mutagenesis (STM)-based murine infectivity assay (n = 6, summary of Supplementary Fig. 5). STM scores were calculated by quantitative PCR. The steΔΔ and ireΔΔ mutants were used as virulent positive control and avirulent negative control strains, respectively. The statistically significant was calculated by one-way ANOVA analysis with Bonferroni’s multiple comparison test. Data are presented as mean values ± standard error of mean (SEM). Red letters represent pathogenicity-related phosphatase mutants identified by both murine and insect models. BLAST matrix comparative search for pathogenicity-related phosphatases was performed using the Comparative Fungal Genomics Platform (http://cfgp.niceblast.snu.ac.kr). Abbreviations: Pi Phytophthora infestans, AI Aspergillus fumigatus, An Aspergillus nidulans, Bg Blumenia graminis, Bc Botrytis cinerea, Cn Coccidioides immitis, Cg Colletotrichum graminicola, Fg Fusarium graminearum, Fs Fusarium oxysporum, Hc Histoplasma capsulatum, Mm Magnaporthe oryzae, Mg Mycophthora graminicola, Nc Neurospora crassa, Pa Podospora anserine, Ca Candida albicans, Sc Saccharomyces cerevisiae, Sp Schizosaccharomyces pombe, Cn Cryptococcus neoformans, Ht Heterobasidion irregular, Lb Laccaria bicolor, Psc Phanerochaete chrysosporium, Sr Sphingomonas lacrymans, Ml Melampsora lariis-papulina, Pg Puccinia graminis, Um Ustilago maydis, Am Allomyces macrognosus, Bd Batrachochytrium dendrobatidis, Ec Encephalitozoon cuniculi, Pb Phytomyces blakesleeanus, Ro Rhizopus oryzae, Dr Dorosphila melanogaster, Hs Homo sapiens, Ce Caenorhabditis elegans, At Arabidopsis thaliana, Os Oryza sativa.

XPP1, SDP102, SDP101, and OCA101 (Fig. 3b and Supplementary Fig. 5), 67% (16/24) of which overlapped with those identified by the insect-killing assay (TPS2, CNA1, VPS29, PHS1, SIW14, CAC1, SSU72, PPG1, INPS201, DBR1, GUAI1, NEM1, PTP2, FBP26, PRS1, and OCA101; Fig. 3b, red). Among these, CNA1 (the catalytic subunit of calcineurin)13, Cac1 (adenylyl cyclase)11, and Ptp2 (phosphotyrosine phosphatase)10 have previously been reported as requisite for C. neoformans virulence, further corroborating our findings.

According to phenotype data, 30 out of the 31 pathogenicity-related phosphatase mutants exhibited at least one phenotypic trait (Fig. 2 and Supplementary Data 7), and a majority showed higher expression in the lungs than other infected tissues (Supplementary Fig. 6). The following in vitro phenotypes were related to temperature. Collectively, these data suggest that C. neoformans pathogenicity is not related to temperature. However, we used this technique to identify the functional correlation between Sdp101 and Sdp102, we tried to construct the sdp101Δ sdp102Δ double mutant strains but failed, even after repeated attempts (data not shown). Similarly, although Inp5201 is paralogous to Inp5202, we failed to generate inp5201Δ inp5202Δ double mutants (data not shown), likely because the inp5201Δ mutant alone showed severe growth defects even at 30 °C (Figs. 2 and 4a). Therefore, Sdp101/Sp102 and Inp5201/Inp5202 may have a synthetic lethal relationship. Conversely, although Oca1 and Oca101 are also paralogous, we successfully obtained an oca1Δ oca101Δ double mutant, but we did not find any additive or synergistic phenotypic traits in the oca1Δ oca101Δ double mutant compared to either single mutant (Supplementary Fig. 7), suggesting that Oca1 and Oca101 may independently contribute to the pathogenicity of C. neoformans.

Of the 31 pathogenicity-related phosphatases identified, five do not have evident orthologues in humans (HS in Fig. 3c): Tps2, Siw14, Had1, Oca101, and Oca1. Therefore, these five pathogenicity-related phosphatases could be excellent anticytotoxic targets. Tps2, Had1, and Oca1 are also required for the virulence of C. albicans23–25, thus drugs that target these phosphatases could have broad antifungal activity.

Phosphatases involved in growth at mammalian body temperature. We next focused on the pathobiological functions of the 31 pathogenicity-related phosphatases in C. neoformans. First, because thermotolerance for mammalian body temperatures is a critical virulence factor for most human fungal pathogens, we quantitatively measured growth of each mutant at 30 and 37 °C. The gua1Δ, yvh1Δ, fbp26Δ, siw14Δ, dbr1Δ, ccr4Δ, ppg1Δ, nem1Δ, and inp5201Δ mutants showed impaired growth at both 30 and 37 °C (Fig. 4a and Supplementary Fig. 8). Of these, the ccr4Δ, ppg1Δ, nem1Δ, dbr1Δ, and inp5201Δ mutants exhibited more growth defects at 37 °C than 30 °C. The ssu72Δ, phs1Δ, mre11Δ, pps2Δ, and cna1Δ mutants showed impaired growth at 37 °C but not at 30 °C. A total of 14 phosphatase mutants showed impaired growth at 37 °C relative to WT (Fig. 4a and Supplementary Fig. 8) and showed reduced murine infectivity or insect virulence relative to WT (Fig. 3a and b). The ppg1Δ, cna1Δ, and pps2Δ mutants exhibited the most significant growth defects at 37 °C and did not grow to the level of the WT even after an extended incubation period (Fig. 4a). Concordantly, ppg1Δ, cna1Δ, and pps2Δ mutants exhibited highly reduced lung and brain STM values (<;5; Fig. 3b). The oca101Δ mutant showed impaired growth relative to WT at 30 °C but not at 37 °C (Supplementary Fig. 8) suggesting that the role of Oca101 in C. neoformans pathogenicity is not related to temperature. Collectively, these data suggest that growth at 37 °C is a critical for virulence of C. neoformans.

Phosphatases involved in melanin and capsule production. C. neoformans has two major virulence factors: the polyphenol pigment melanin and the polysaccharide capsule, both of which contribute to its antiphagocytic activity. The melanin pigment also serves as antioxidant due to its reactive oxygen species scavenging activity. Among 19 phosphatase mutants defective in melanin production on Niger seed medium, the following 13 mutants also exhibited defective melanin production on l-DOPA and epinephrine media (Fig. 4b): mre11Δ, ccr4Δ, vps29Δ, yvh1Δ, fbp26Δ, inp5201Δ, cna1Δ, ptp2Δ, ptc2Δ, dbr1Δ, ppg1Δ, nem1Δ, and gua1Δ. All of these mutants, except the ptc2Δ mutant, exhibited reduced infectivity or virulence (Fig. 3a and b), suggesting that melanin production is strongly correlated with pathogenicity. In contrast, SIW14 deletion increased melanin production in all melanin-inducing media (Fig. 4b) but attenuated the virulence of C. neoformans (Fig. 3b), suggesting that other cellular functions mediated by Siw14 may promote virulence.

We next addressed whether these phosphatases are directly involved in induction of LAC1, which encodes laccase, a rate-limiting enzyme for melanin production in C. neoformans, under nutrient-starvation conditions. Deletion of PTP2, CCR4, INPS201, CAC1, DBR1, FBP26, GUAI1, NEM1, and PPG1 significantly reduced LAC1 induction upon nutrient starvation (Fig. 4c), with deletion of PTP2, CCR4, CAC1, and PPG1 almost completely abolishing LAC1 induction. Ptp2 has been reported as
a negative feedback regulator of the Hog1 MAPK; Hog1 deletion increases LAC1 induction and melanin production\textsuperscript{10,30}. Recently, we have reported that LAC1 induction is controlled by the following four core TFs: Bzp4, Usv101, Hob1, and Mbs1\textsuperscript{30}. Among these, expression of BZP4 and HOB1 is induced by nutrient starvation, and BZP4 induction itself is governed by Hob1\textsuperscript{30}. Therefore, we examined whether the nutrient starvation, and BZP4 induction is controlled by the Ccr4-Hob1-Bzp4-dependent signalling pathway appears to be critical for LAC1 induction and melanin production in C. neoformans.

We next focused on phosphatases involved in capsule production. When capsule production was quantitatively measured as packed cell volume, Cac1, Ptp2, Psr1, Ndx5, Hpp2, Oca1, Sdp102, Ynd1, NgI3, Ppp5, and Cwh43 positively impacted cell volume, and Inp5201, Dbr1, Phs1, Cdc1, Cna1, Sit4, Ccr4, Nem1, Pcd102, and Fg4 negatively impacted it (Supplementary Fig. 10). For these mutants, we examined actual capsule thickness; among these, six mutants (cac1\textDelta, sdp102\textDelta, ndx5\textDelta, ptp2\textDelta, ynd1\textDelta, and psr1\textDelta) showed reduced capsule production (Fig. 4e and Supplementary Fig. 10) that mirrored their phenotype in cell volume assays, and five of these (cac1\textDelta, ptp2\textDelta, sdp102\textDelta, ynd1\textDelta,
Fig. 4 Phosphatases involved in major virulence traits of C. neoformans. a Growth curves of WT and phosphatase mutants were generated at 30 °C (control) and 37 °C (mammalian body temperature). Fifteen phosphatase mutants had growth defects at 30 and 37 °C. Nine of these phosphatase mutants had more substantial growth defects at 37 °C than 30 °C (additional data in Supplementary Fig. 8). Each curve represents data from two independent experiments (see Supplementary Fig. 8 for data from the individual experiments). Optical density at 600 nm (OD600nm) was measured with a multi-channel bioreactor (Biosan Laboratories, Inc., Warren, MI, USA) for 40–90 h based on growth rate. b Melanin production was measured using three different melanin-inducing media (Niger seed, dopamine, and epinephrine medium). Representative images from three independent experiments are shown here. Each strain was spotted on medium containing 0.1% glucose, incubated at 30 °C, and photographed after 1–3 days. c, d Gene expression of the melanin-regulating genes LAC1, BZP4, and HOB1 were determined by qRT–PCR in both nutrient-rich (R) and nutrient-starvation (S) conditions. RNA was extracted from three biological replicates with three technical replicates of WT and melanin-regulating phosphatase mutants. Expression was normalized to ACT1, and statistical significance was calculated by one-way ANOVA analysis with Bonferroni’s multiple comparison test. Data are presented as mean values ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). e The capsule production assay was performed using capsule-inducing media (FBS agar medium). Capsule thickness (total diameter – cell body diameter) was measured for WT cells (n = 50) and for each phosphatase mutant (n = 50). Statistical significance was calculated by one-way ANOVA analysis with Bonferroni’s multiple comparison test. Data are presented as mean values ± SEM (*P < 0.05, **P < 0.01, ***P < 0.0001). The graph is representative of more than three independent experiments. The images are representative DIC images of WT and phosphatase mutants incubated on FBS agar medium and stained with India ink. Scale bars, 10 μm.

psr1A) also showed reduced virulence (Fig. 3). In contrast, the inp5201Δ mutant again showed highly enhanced capsule production but exhibited dramatically reduced virulence (STM < −7), the latter of which likely resulted from defective melanin production (Fig. 4b). Overall, the ability to produce melanin and the polysaccharide capsule were highly correlated with pathogenic potential in C. neoformans.

The retromer complex promotes C. neoformans virulence. Among the newly identified virulence-related phosphatases in this study, Yps29 (CNAG_00182) is a putative component of the retromer complex, first discovered in S. cerevisiae35. The yeast retromer is a cytosolic, heteropentameric protein complex that mediates the intracellular trafficking of protein cargo from the post-Golgi organelles to the lytic compartment32,33. It consists of the cargo-recognition core (CRC) complex, which contains Yps29, Vps35, and Vps26, and a membrane-deforming sorting nexin (SNX) complex, which contains Vps5 and Vps1732.

To address whether functions of Yps29 resulted from the conserved role of the retromer complex, we functionally characterized other retromer components in C. neoformans. In the C. neoformans genome, we identified all of the remaining retromer component genes encoding proteins orthologous to Yps35 (CNAG_01837), Yps26 (CNAG_01426), Vps5 (CNAG_01315), and Vps17 (CNAG_00508), suggesting that the retromer complex is evolutionarily conserved in this fungal pathogen. We deleted each gene in the H99 strain and performed phenotypic analyses (Supplementary Figs. 11 and 12). Notably, deletion of Vps35 and Vps26 resulted in more dramatic phenotypic changes than that of Yps29 (Fig. 5a), indicating that Vps35 and Vps26 are critical CRC complex components in C. neoformans. In contrast, deletion of SNX components Vps5 and Vps17 resulted in relatively minor phenotypic changes (Fig. 5a). Supporting this finding, CRC complex mutants showed significantly reduced virulence in the insect-killing assay whereas SNX complex mutants remained as virulent as the wild-type strain (Fig. 5b). However, both CRC and SNX complex mutants exhibited markedly reduced lung and brain infectivity in a murine-based STM analysis (Fig. 5c). Collectively, these findings indicate that the retromer CRC and SNX complexes are critical for the pathogenicity of C. neoformans.

Gda1 and Ynd1 modulate O-mannosylation in C. neoformans. In S. cerevisiae, Gda1 and Ynd1 are Golgi membrane-bound apyrases that modulate mannosylation of O-linked and N-linked glycoproteins and glycosphingolipids by affecting the antiport exchange ratio between GDP-mannose and GMP34,35. Gda1 exhibits activity highly specific to GDP whereas Ynd1 shows a much broader spectrum of activity35,36. In accord, double deletion of GDA1 and YND1 results in more severe glycosylation defects than deletion of either gene alone35. To investigate whether C. neoformans gda1Δ and ynd1Δ mutants show defects in O-glycosylation, as observed in S. cerevisiae and C. albicans mutant strains35,37, we compared the O-glycan profiles of wild-type, gda1Δ, and ynd1Δ strains. The O-linked oligosaccharides assembled on cell wall mannoproteins (cwMPs) of C. neoformans cells were obtained by hydrazinolysis, labelled with 2-amino benzoic acid (2-AA), and then analysed using HPLC with fluorescence detection (Fig. 6a). Consistent with a previous report38, O-linked oligosaccharides from the C. neoformans wild-type strain were mostly composed of 2–4 mannose residues (Man2–Man4; M2–M4), with minor O-glycan species containing xylose (X1M2–X1M4) and a minor α1,2-mannosiose (M3α) species (Fig. 6a). In the O-glycan profile of the gda1Δ mutants, the M4 peak was dramatically reduced, and the M1 peak was increased compared to those in the wild-type strain (Fig. 6b). Notably, the minor xylose-containing species became more detectable in the O-glycans of the gda1Δ strain (Fig. 6b). The ynd1Δ mutants also showed a significantly decreased M4 peak with the increased M1 peak, although the M4 peak decreased to a lesser degree than that of the gda1Δ mutants (Fig. 6b). These altered profiles strongly indicate a severe defect in the elongation processes of O-glycans, which is likely due to inefficient GDP-mannose supply from the cytosol to the lumen of the Golgi caused by decreased GDPase activity in the gda1Δ and ynd1Δ strains (Fig. 6c). Thus, the results collectively suggest that GDA1 and YND1 in C. neoformans encode membrane-bound apyrases required for Golgi N- and O-glycosylation (Fig. 6c), consistent with these genes’ function in other yeasts. The more dramatic reduction of major O-glycans in gda1Δ than ynd1Δ indicates that Gda1p is a major GDPase responsible for GDP-mannose supply to the major O-glycan biosynthesis pathway. The reduced capsule size in ynd1Δ (Fig. 4e) suggests that Ynd1p might be also involved in the GDP-mannose supply to capsule biosynthesis. Notably, however, we failed to obtain gda1Δ ynd1Δ double mutants (data not shown), indicating that the two proteins may have a synthetic lethal relationship in C. neoformans. Because both gda1Δ and ynd1Δ mutants exhibited reduced virulence in the insect killing assay (Fig. 3a), these results collectively suggest that O-mannosylation is critical for virulence of C. neoformans.

Phosphatases involved in the blood–brain barrier (BBB) crossing. The most lethal damage conferred by C. neoformans is brain infection, which generally results in fatal meningencephalitis. Notably, the brain STM scores for all of the phosphatase mutants
were generally similar to their lung STM scores, although some of them exhibited lower brain scores (Fig. 3b; TPS2, YMR1, FBP26, MRE11, CDC1, and XPP1). However, because the phosphatase mutants administered through intranasal inhalation pass through the lungs first, those reaching the brain were less equally distributed than in pooled input mutants, which made direct comparison of lung and brain STM scores difficult. Therefore, to further address the role of these phosphatases in brain infection, we monitored the ability of the pathogenicity-related phosphatase mutants, except those showing reduced growth at 37°C, to traverse the BBB.

Among these, five mutants (xpp1Δ, ssu72Δ, siw14Δ, sit4Δ, and gda1Δ) showed significantly reduced ability to traverse the BBB (Fig. 7a). We recently showed that adhesion to the BBB is a prerequisite for effective BBB crossing39, and, indeed, xpp1Δ, ssu72Δ, siw14Δ, and sit4Δ mutants, but not gda1Δ, showed reduced BBB adhesion (Fig. 7b), suggesting that reduced O-mannosylation of proteins or lipids is required for BBB crossing but not adhesion. We found that C. neoformans does not actively grow at 37°C in the tissue culture medium that we used for the 24 h in vitro BBB crossing and adhesion assays (data not shown), possibly due to the low glucose concentration (0.1% glucose). Therefore, it is unlikely that Cryptococcus replication in the bottom well complicated our data.

We recently reported several TFs that promote BBB adhesion that are highly induced in vitro by host-mimic conditions (HMC; RPMI medium containing 10% FBS at 37°C under 5% CO2): PDR802, FZC31, and GAT20139. Therefore, we next addressed whether these genes, in addition to known brain infection-related genes [inositol transporter genes (ITR1a and ITR3c) and MPRI40,41], were induced by HMC in xpp1Δ, ssu72Δ, siw14Δ, and sit4Δ mutants. Among these, SSU72 deletion most markedly reduced HMC-mediated MPRI induction (Fig. 7c), suggesting that the defects of ssu72Δ mutants in BBB crossing and adhesion are at least partially caused by reduced MPR1 induction. However, because the ssu72Δ mutants were more defective than the mpr1Δ mutants in BBB crossing and adhesion (Fig. 7a and b), other cellular functions of Ssu72 may also be involved in BBB crossing and adhesion. Deletion of SIT4 and GDA1 markedly reduced HMC-mediated ITR3c induction (Fig. 7c). GDA1 deletion also affected FZC31 induction (Fig. 7c). However, induction of GAT201 and PDR802 was not significantly affected by deletion of SIT4, SIW14, SSU72, GDA1, or XPP1 (Fig. 7c).

To obtain insight into the integrated signalling networks governing brain infection by C. neoformans, we generated functional gene networks using STRING analysis by combining data from this and previously published studies on BBB-crossing-related phosphatases, TFs, and kinases (Fig. 7d)39. We found that genes involved in glucose sensing (Sit4-Snf1-Gal83), RNA processing (Ssu72), and purine metabolism (Xpp1-Met3) were critical for BBB crossing by C. neoformans. Collectively, these findings indicate that C. neoformans utilizes complex signalling networks for brain infection.

Comparison of fungal pathogenicity-related phosphatases. By comparing the virulence data of phosphatase mutants in C. neoformans with those available in other plant and animal pathogenic fungi8,23–27,42–63, we found several core fungal pathogenicity-related phosphatases (Fig. 8 and Supplementary Data 9). In the human yeast pathogens C. neoformans and C. albicans, the following 13 phosphatases are considered core pathogenicity-related phosphatases: Cna1/Cmp1, Sit4, Oca1, Yvh1, Sdp101/Cpp1, Ptp2/Ptp3, Cac1/Cyr1, Ccr4, Had1/Rhr2, Tps2, Inp5201/Inp51, Pgp1, and Gua1. Of the 13 phosphatases, CnaA, SitA, and OrlA (a Tps2 ortholog) have been shown to be required for A. fumigatus virulence55–57. Upon comparison with pathogenicity-related phosphatases in F. graminearum, eight
Six phosphatases that we previously constructed \( ^4, ^5, ^10, ^11, \) were synthesised and tested systematically for their ability to control the virulence of fungal pathogens. In this study, we identified a total of 139 phosphatases in \( \textit{C. neoformans} \), including protein phosphatases, lipid/nucleoside/carbohydrate phosphatases, and pyrophosphatases, and constructed a high-quality library of 219 signature-tagged gene-deletion mutant strains representing 114 phosphatases for functional analysis. By incorporating 11 additional signature-tagged mutants representing 109 phosphatases for functional analysis, we were able to systematically analyse the in vitro and in vivo phenotypic traits of a total of 230 signature-tagged mutant strains representing 114 phosphatases (82% of original 139). Under 30 distinct in vitro growth conditions (e.g., temperature-dependent growth, mating, growth in the presence of various sugars), phosphatases have been shown to be required for the virulence of both animal and plant fungal pathogens: \( \textit{Sit4, Yvh1, Sdp2/Msp5} \) (an \( \text{Sdp101} \) orthologue), \( \text{Ptp2, Ac1 (a Cac1 orthologue), Tps2, Inp53 (an Inp5201 orthologue), and Ppg1.} \) \( \text{Sit4} \) is involved in the \( \text{TOR} \) pathway, \( \text{Cac1} \) is involved in the \( \text{cAMP} \) pathway, and \( \text{Ppg1 and Yvh1}^{31, 66-68} \) are involved in cell growth, nutrient sensing, and the stress response in fungal pathogens.\( ^{2, 69} \) The \( \text{Cna1} \) and \( \text{Had1} \)-mediated calcineurin pathway, the \( \text{Tps2}-\text{mediated trehalose} \) pathway, and the \( \text{Msp5} \)-mediated \( \text{Mpk1/Slt2 MAPK} \) pathway are all required for maintaining cell wall integrity. \( \text{Ptp2, which is a major negative feedback regulator of the HOG pathway, is involved in adaptation and the stress response in fungal pathogens.} \) \( \text{Inp51, Inp52, and Inp53 are involved in phosphoinositide signalling, which controls vesicle trafficking, the actin cytoskeleton, and cell wall integrity.}^{70, 71} \) Based on these data, phosphatases and signalling pathways involved in cell growth, nutrient sensing, cell wall integrity, the stress response, and phosphoinositide signalling appear to play pivotal roles in general fungal pathogenicity. Notably, however, deletion of \( \text{PPH3} \) reduces \( \textit{C. neoformans} \) and \( \textit{F. graminearum} \) virulence but enhances \( \textit{C. albicans} \) virulence.\( ^{8, 42} \) In contrast, deletion of \( \text{PTC2} \) and \( \text{PTC3} \) reduces \( \textit{C. albicans} \) and \( \textit{F. graminearum} \) virulence, respectively, but does not reduce \( \textit{C. neoformans} \) virulence.\( ^{27, 72} \) Thus, some phosphatases may play differential roles in controlling the virulence of various fungal pathogens.

**Discussion**

In this study, we identified a total of 139 phosphatases in \( \textit{C. neoformans} \), including protein phosphatases, lipid/nucleoside/carbohydrate phosphatases, and pyrophosphatases, and constructed a high-quality library of 219 signature-tagged gene-deletion mutant strains representing 109 phosphatases for functional analysis. By incorporating 11 additional signature-tagged mutants representing 109 phosphatases for functional analysis, we were able to systematically analyse the in vitro and in vivo phenotypic traits of a total of 230 signature-tagged mutant strains representing 114 phosphatases (82% of original 139). Under 30 distinct in vitro growth conditions (e.g., temperature-dependent growth, mating,
Fig. 7 In vitro BBB transmigration and adhesion assays for C. neoformans phosphatases. a In vitro BBB migration and b human brain microvascular endothelial cell line (hCMEC/D3) adhesion assay of pathogenicity-related phosphatases. Right: y-axis indicates trans-endothelial electrical resistance (TEER). Data plots represent individual data from three independent experiments (n = 3). Data presented as mean values ± SEM. Significant differences were calculated by two-tailed (unpaired) Student’s t-test comparing wild-type (WT) and each phosphatase deletion mutant. c Host-mimic condition (HMC)-mediated induction of brain infection-related genes in wild-type (WT) and phosphatase deletion mutant strains. Gene expression was determined by quantitative RT-PCR with cDNA synthesized from total RNA prepared from cells shifted from basal condition (YPD at 30 °C, grey shaded) to HMC (RPMI with 10% foetal bovine serum at 37 °C under 5% CO2) and further incubated for 3 h (red shaded). Fold-change of gene expression was calculated by quantitative RT-PCR with cDNA synthesized from total RNA prepared from cells shifted from basal condition (YPD at 30 °C, grey shaded) to HMC (RPMI with 10% foetal bovine serum at 37 °C under 5% CO2) and further incubated for 3 h (red shaded). Fold-change of gene expression was calculated relative to basal expression levels of each gene in WT. Data from three independent experiments (black dots) are presented as mean values ± SEM. Statistical significance between basal and HMC was determined by two-tailed (unpaired) Student’s t-test (*P < 0.05; **P < 0.01; ***P < 0.001).

d Functional protein association network analysis of BBB crossing-related TFs, kinases, and phosphatases of C. neoformans predicted by STRING (http://string-db.org) with protein sequences obtained from FungDB (https://fungidb.org/fungidb/). Images were drawn with Cytoscape v3.7.2. Dotted lines: groups of genes involved in purine metabolism (purple), RNA processing (red), and glucose sensing (orange).

capsule/melanin/urease production, stress and antifungal drug resistance), 60 out of 114 mutants (53%) exhibited at least one phenotypic trait. Among the remaining 54 mutants that did not show a phenotypic trait under these conditions, none possessed infectivity and virulence defects, suggesting that these 54 phosphatases may not be involved in the pathobiology of C. neoformans. Among these, however, functionally redundant phosphatases could exist, and evident phenotypes may only result from their combined mutation. In particular, our in vivo expression profiling revealed that the following phosphatase genes were markedly upregulated during host infection: CDC1, GDA1, GEP4, NDX2, NDX3, PTP3, APH4, HAD9, YMR1, INM1, PP11, DPP101, and AKP1 (Supplementary Data 8). Therefore, we recommend that the role of these phosphatases be further characterized in future work.

To identify functional correlations between TFs, kinases, and phosphatases, we attempted to make co-phenotypic clusters of TF, kinase, and phosphatase mutants constructed by this and previous studies, but found that even well-established correlations between known signalling components, such as Hog1 and Ptp2 and Cna1 and Crz1, were not evident in the co-clustering analysis. There could be several explanations for this. Mutation of a phosphatase gene, which functions as a negative feedback regulator of a kinase-dependent pathway, may not lead to a
phenotype opposite to the kinase mutant phenotype. For example, mutations in PTP2, which encodes a major negative feedback regulator of Hog1 MAPK, results in phenotypes similar to hog1Δ mutant phenotypes. In addition, TF mutants generally have milder phenotypes than their upstream kinase and phosphatase mutants. For example, mutations in CRZ1, which encodes a downstream TF activated by Cna1 phosphatase, result in much milder phenotypes than mutations in CNA1. Thus, simple co-phenotypic clustering of TFs, kinases, and phosphatases may lead to misinterpretations of the correlations between the signalling components. Functional and mechanistic relationships between signal components should be further investigated by RNA-seq, ChIP-seq, phosphoproteomics, and protein–protein interaction assays in future works.

Our systematic analysis unveiled both evolutionarily conserved and distinct sets of phosphatases in \textit{C. neoformans}. Among 25 phosphatase genes that we were not able to disrupt, 14 genes (CDC25, SAC1, FCP1, IPC1, CET1, GLC7, TIM50, IDI1, IPP1, RPP1, HIS2, MET22, PAH1, and GPI13) are known to be essential in \textit{S. cerevisiae}, \textit{S. pombe}, or \textit{C. albicans}: (Supplementary Data 6). The following nine genes (CDC25, FCP1, IPC1, CET1, TIM50, IDI1, IPP1, RPP1, and GPI13) are likely to be core essential fungal phosphatases because they are reported to be essential in \textit{S. cerevisiae} and \textit{S. pombe}. Out of 15 reported essential phosphatase genes in \textit{S. cerevisiae}, we successfully deleted PHSI, CYR1 (Cac1 ortholog), CDC1, CDC14, SSU72, and DUT101 in \textit{C. neoformans}. PHSI, CDC1, and DUT101 are also essential in \textit{S. pombe}, suggesting that they are functionally divergent between ascomycete and basidiomycete fungi or have a synthetic lethal relationship with other functionally redundant phosphatases in \textit{C. neoformans}. We have described similar findings in our previous analysis of kinase mutant collections in \textit{C. neoformans}. Therefore, it seems evident that some kinase/phosphatase-mediated signalling networks are functionally divergent among fungi.

Here, we unravelled 31 pathogenicity-related phosphatases that affect a variety of biological processes, including growth, virulence factor production, stress response, carbohydrate metabolism, cell signalling, protein sorting, and vesicular trafficking. Some of these findings are corroborated by previous research, including the role of PTP2, CAC1, CNA1, CCR4, and HAD1 in the virulence of \textit{C. neoformans}. Furthermore, in examining phosphatase mutants in a large deletion mutant analysis, Liu et al. demonstrated that 4 of 14 mutants (cac1Δ, ptc2Δ, sdp102Δ, and mre11Δ) show reduced infectivity in the lungs consistent with our findings in cac1Δ, sdp102Δ, and mre11Δ mutants. Notably, Madhani’s and our studies indicate that the yvh1Δ mutant is as infective as the wild-type strain as quantified by lung STM analysis, but, in our study, the yvh1Δ mutant exhibited attenuated virulence in the insect-killing model. Another deviation from prior research is the secreted acid phosphatase APH1, which was reported by Lev et al. to contribute to the virulence of \textit{C. neoformans}, but we found that deletion of APH1 did not significantly contribute to virulence in either the insect-killing assay or murine infection model. We attribute this discrepancy to the fact that Lev et al. incubated infected \textit{G. mellonella} at 30°C; because when we incubated insect hosts at 37°C, the impact of thermotolerance at mammalian temperatures may have offset any advantages seen at lower temperatures. For the remaining 23 phosphatases reported here, we are the first to describe their role in the pathogenicity of \textit{C. neoformans}.

In this study, we found that a majority of the 31 pathogenicity-related phosphatases were involved in regulation of three major cryptococcal virulence factors: thermotolerance, capsule, and melanin. Fourteen of them (Cna1, Gua1, Yvh1, Phs1, Nem1, Ppg1, Siw14, Inp5201, Ccr4, Dbr1, Sus72, Mre11, Pfp26, and Tps2) promote thermotolerance at 37°C. In addition, 13 phosphatases (Mre11, Ccr4, Vps29, Yvh1, Fbp26, Inp5201, Cac1, Ptp2, Dbr1, Ppg1, Nem1, Gua1, and Siw14) are involved in melanin production. Finally, capsule production was altered by deletion of six phosphatases: Cac1, Sdp102, Ptp2, Ynd1, Psr1, and Inp5201. Therefore, a total of 28 phosphatases are involved in regulation of any of the three major virulence factors, and 12 of them (Ccr4, Yvh1, Fbp26, Inp5201, Cac1, Ptp2, Dbr1, Ppg1, Nem1, Gua1, Mre11, and Siw14) are involved in modulating more than two virulence factors. The remaining 11 phosphatases (Ymr1, Oca1, Cdc1, Xpp1, Sdp101, Oca101, Gda1, Pph3, Had1, Sit4, and Inp5202) were unrelated to thermotolerance or capsule/melanin synthesis. Among these, Sdp101 and Inp5202 are paralogous to Sdp102 and Inp5201, respectively, but we failed to obtain corresponding double mutants, suggesting that Sdp101/Sdp102 and Inp5201/Inp5202 may have a synthetic lethal relationship and play redundant roles in \textit{C. neoformans}.  

**Fig. 8 Pathogenicity-related phosphatases in fungal pathogens.** a Venn diagram showing the distribution of pathogenicity-related phosphatases in the human fungal pathogens \textit{C. neoformans}, \textit{Candida albicans}, and \textit{Aspergillus fumigatus}. b Venn diagram showing the distribution of pathogenicity-related phosphatases in \textit{C. neoformans} and plant-pathogenic filamentous fungi \textit{Fusarium graminearum} and \textit{Magnaporthe oryzae}. 
The pathobiological functions of Ymr1, Cdc1, Had1, and Pph3 remain unclear because mutants of these genes do not demonstrate clear phenotypic traits related to *C. neoformans* pathogenicity. However, the *ymr1Δ, cdc1Δ*, and *had1Δ* mutants showed increased susceptibility to cell membrane disruption, and this instability may have contributed to reduced infectivity or virulence relative to WT. The *had1Δ* mutant also exhibited increased susceptibility to cell wall-destabilizing agents, which is in good agreement with recent reports showing that Had1 is a potential target of calcineurin, which plays a role in regulating cell wall integrity. Had1 was also shown to be required for virulence in a murine model of systemic cryptococcosis. *YMR1* encodes a phosphatidylinositol 3-phosphate (PI3P) phosphatase involved in various protein sorting pathways that functionally overlaps with other lipid phosphatases in *S. cerevisiae*. Therefore, deletion of *YMR1* does not generate evident phenotypes in the yeast model. Cdc1 is a putative mannose-ethanolamine phosphatase phosphoesterase involved in both cell division cycle and GPI-anchor remodelling. The role of Ymr1 and Cdc1 in vacuolar protein sorting and GPI remodelling, respectively, may contribute to membrane stability in *C. neoformans*. In *S. cerevisiae*, Pph3 is a catalytic subunit of the protein phosphatase PP4 complex and governs recovery from the DNA damage checkpoint. Pph3 likely plays a similar role in *C. neoformans* because the only in vitro phenotype observed in the *pph3Δ* mutant was increased susceptibility to DNA damage by methyl methane sulfonate (MMS). Given our previous demonstration that the DNA damage response pathway is critical for the virulence of *C. neoformans*, we hypothesize the role of Pph3 in the DNA damage response likewise contributes to virulence.

We recently identified a group of *C. neoformans* TFs and kinases involved in brain infection processes, including BBB adhesion and crossing. Here we found that the following pathogenicity-related phosphatases are also involved in BBB crossing of *C. neoformans*: Xpp1, Sit4, Ssu72, Siw14, and Gda1. In *S. cerevisiae*, Pxx1 (the orthologue of Xpp1) is a metal-dependent cysteolipid phosphatase and generates inorganic phosphate by cleaving polyphosphate that serves as a major phosphate reservoir and is required for long-term cell survival, gene regulation, cell motility, and stress responses. The functions of Sit4 and Ssu72 are likely related to the target of rapamycin (TOR) pathway involved in nutrient-sensing. In *S. cerevisiae*, Sit4 is regulated by the nutrient-sensing TOR pathway and controls nitrogen catabolite repression genes through the GATA TF Gln3. Prior to transcriptional termination, Ssu72 dephosphorylates the C-terminal domain of the RNA polymerase II, which is degraded through the TOR-signalling pathway in response to rapamycin. Accordingly, our *sit4Δ* and *ssu72Δ* mutants indeed exhibited highly increased susceptibility to rapamycin (Supplementary Fig. 13). Notably, we found that Sit4 and Ssu72 promote induction of the inositol transporter Irr3c and metalloprotease Mp1, respectively, required for BBB crossing of *C. neoformans*. In *S. cerevisiae*, Siw14 hydrolyses the β-phosphate from 5-diphosphoinositol pentakisphosphate (IP5), and its deletion increases IP7 and decreases IP6 levels. Because deletion of the inositol pentakisphosphate kinase IPK1 decreases IP6 levels, abolishing the virulence of *C. neoformans*, it is likely that Siw14 could promote BBB crossing and virulence of *C. neoformans* through modulation of the inositol signalling pathway. Finally, Gda1 is a Golgi membrane-bound asparagine that modulates O-mannosylation in yeast. *C. neoformans* serotypes have a H99s strain background through homologous recombination using gene-disruption cassettes containing the nourseothricin-resistance marker (nourseothricin acetyl transferase; NAT) using NAT-split marker/double joint PCR (DJ-PCCR) strategies and constructs phosphatase regulatory subunit orthologues in *C. neoformans* (Supplementary Data 10). However, further research is needed to characterize the functional and mechanistic relationships between the catalytic and regulatory subunits of phosphatases; this research could lead to the development of more antifungal drugs that target phosphatases.

**Methods**

**Ethics statement.** Animal care and all experiments were conducted in accordance with the ethical guidelines of the Institutional Animal Care and Use Committee (IACUC) of Yonsei University. The Yonsei University IACUC approved all vertebrate studies.

**Construction of the *C. neoformans* phosphatase and retromutant strains.** We constructed phosphatase and retromutant mutant strains in the *C. neoformans* serotype D H99s, *C. neoformans* serotype A 1891, and *C. neoformans* serotype B 1893 strains by introducing the *C. neoformans* gene with each *S. cerevisiae* gene through Cre-lox recombination using gene-disruption cassettes containing the nourseothricin-resistance marker (nourseothricin acetyl transferase; NAT) using NAT-split marker/double joint PCR (DJ-PCCR) strategies (see Supplementary Data 4 for primers). We amplified 5′- and 3′-flanking regions of target genes by PCR with primer pairs L1/L2 and R1/R2, respectively, from H99s genomic DNA. The signature-tagged NAT marker was amplified by PCR with primers M13Fe (M13 forward extended) and M13Re (M13 reverse extended) from a pNAT-STM plasmid containing the NAT gene with each unique signature-tagged sequence. After amplification of the 5′- and 3′-flanking regions and NAT marker in the first round of PCR to generate template DNA, the second round of PCR constructed 5′- and 3′-regions of the NAT-split gene-disruption cassette with primer pairs L1/NSL and R2/NSR, respectively. Bioinformatic transformation introduced the NAT gene-disruption cassettes by incubating the H99s strain in 50 ml YPD medium for 16 h at 30 °C, followed by spin-down, re-suspension in 5 ml distilled water, application on YPD agar medium containing 1 M sorbitol, and further incubation for 3 h at 30 °C. Gene-disruption cassettes were then combined with 600 μg of 0.6-μm gold microcarrier beads (Bio-Rad Laboratories, Hercules, CA, USA) and introduced into cells using a particle delivery system.
system (PDS-100, Bio-Rad). After 4 h incubation at 30 °C for recovery of cell membrane integrity, cells were scraped and spread on YPD agar medium containing 1% (w/v) NaCl. Diagnostic and pathological melanophages were identified as transformants. Southern blot analysis confirmed the genotype of each screened transformant, and we constructed at least two independent mutant strains for each phosphatase gene.

**ncounter in vivo gene expression profiling analysis.** Using previously reported RNA samples, we performed an nCounter gene expression analysis (NanoString) to quantify the expression levels of the 14 phosphatase members. Total RNA samples were obtained from 6-week-old female A/J mice infected with 5 × 10⁸ cells through intranasal inoculation. The expression level of each phosphatase mutant was determined by one-way analysis of variance (ANOVA) with Bonferroni correction. The phosphatase mutants construction in the H99S strain and each phosphatase mutant was used as virulent and avirulent control strains, respectively, as described previously.

Expression analysis. To measure the expression level of known melanin-regulating genes (LAC1, HOB1, MB51, and B2Z4), the H99S strain and phosphatase mutants (mre11A, ccc4A, vsa2PA, yvh1Δ, fbp2Δ, mps201A, ccai1Δ, ptp2Δ, plc25Δ, dib1Δ, ppg1Δ, nem1Δ, and gusαA) were incubated in liquid YPD medium for 16 h at 30 °C and sub-cultured into fresh liquid YPD medium with OD₆₀₀nm = 0.2. When the cells reached the early logarithmic phase (OD₆₀₀nm = 0.6–0.8), half of the cell culture was sampled to prepare a basal sample. The remaining cell culture was washed with PBS and incubated in nutrient-starvation conditions (YNB medium with ammonium sulfate without glucose) for 2 h, followed by immediate pelleting and liquid nitrogen and lyophilized. To measure the expression level of BBB crossing-related genes (ITR1A, ITR3C, MPK1, FZC31, GAT201, and PDR8002), the H99S strain and phosphatase mutants (sit4AΔ, sit4AΔΔ, sit4AΔΔΔ, sit4AΔΔΔΔ, sit4AΔΔΔΔΔ) were incubated in liquid YPD medium at 30 °C for 3 h, sub-cultured into 50 ml of fresh YPD broth, and further incubated until OD₆₀₀nm reached 0.8. The cells were harvested by centrifugation and washed three times with sterile distilled water. One tube was kept in liquid nitrogen to monitor basal expression levels, and the other tube was resuspended with an equal volume of RPMI1640 medium containing 10% FBS. After 3 h incubation at 37 °C in a CO₂ incubator with 120 rpm horizontal shaking, cells were centrifuged and lyophilized, and total RNA was extracted from each sample using a commercial RNA extraction kit (easy-BLUE, iNtRON Biotechnology, Gyeonggi, Korea) and cDNA was synthesized using RTase (Thermo Scientific, Waltham, MA, USA). Quantitative reverse transcription-PCR (qRT-PCR) was performed with target gene-specific primer pairs listed in Supplementary Table 4.

**HPLC analysis of O-linked glycans from cwpMs.** Analysis of O-linked glycans from cwpMs was conducted as described previously. The O-linked oligosaccharides were released from the purified cwpMs by modified hydrazinolysis. Dried cwpMs (50 µg) were resuspended in hydrazine monohydrate and incubated at 60 °C for 4 h. After cooling and desiccation of the reactant, the pellets were dissolved in NaHCO₃, mixed with (CH₃CO)₂O, and incubated on ice for 30 min. O-glycans were purified by Dowex 50WX8-400 resins (Sigma-Aldrich, St. Louis, MO, USA) and labelled with 2-AA. The purified O-glycan was analysed using HPLC on a TSKgel Amide-80 column (0.46 × 25 cm, Tosoh Corp., Tokyo, Japan) with 90% solvent A (2% acetic acid and 1% tetrahydrofuran in acetonitrile) and 10% solvent B (5% acetic acid, 3% triethylamine, and 1% tetrahydrofuran in water). After sample injection, the proportion of solvent B increased to 90% over 60 min at a flow rate of 1.0 ml/min and then O-glycans were detected with a fluorescence detector (2475, Waters Corp., Milford, MA, USA) at excitation and emission wavelengths of 360 and 425 nm, respectively. Data were analysed using chromatography software (Empower 2, Waters).

**Insect-based in vivo virulence assay.** At least 15 G. mellonella caterpillars (Vanderhorst Wholesale, Inc., Saint Marys, OH, USA) in the final larval instar with a body weight of 200–300 mg, arriving within 7 days from the day of shipment, were used for in vivo insect virulence assays. Each phosphatase mutant and wild-type H99S strain was incubated at 30 °C overnight, pelleted, washed three times with PBS, and resuspended in PBS at a concentration of 10⁶ cells/ml. Four thousand C. neoformans cells per larvae were injected into the second-to-last prolegs with a 100-µl syringe equipped with a 10-µl needle and repeating dispenser (PB600-1, Hamilton Company, Reno, NV, USA). Negative control G. mellonella received PBS only. Infected larvae were placed in Petri dishes in a humidified chamber, incubated at 37 °C with moderate humidity, and observed with a dissecting microscope every day until they turned black and showed no movement upon touching. Larvae that pupated during the experiment were censored for statistical analysis. Survival curves were illustrated using Prism 8 (GraphPad, San Diego, CA, USA) and analysed with a log-rank (Mantel–Cox) test. We examined two independent strains for each phosphatase mutant.

**STM-based marine infectivity assay.** A set of phosphatase mutants with the 41 signature-tagged NAT selection markers was cultured for 16 h at 30 °C. The steg05A (STM#282) and tre1Δ (STM#169) mutants were used as virulent and avirulent control strains, respectively, as described previously. Mutants and

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In vitro BBB-crossing and BBB-adhesion assays. Human brain microvascular endothelial cells (hCMEC/D3 cell line, Merck & Co., Kenilworth, NJ, USA) were cultured as follows based on the reported methods106,107, Briefly, 5 × 10^4 hCMEC/D3 cells in EGM-2 medium (Lonza Group, Basel, Switzerland) were seeded on collagen (Corning, Inc., Corning, NY, USA)-coated 8-μm porous membranes (BD Biosciences, Franklin Lakes, NJ, USA) for the BBB-crossing assay or 12-well plates (BD Biosciences) for the BBB-adhesion assay. The day after seeding, medium was refreshed with EGM-2 medium supplemented with 2.5% human serum and further incubated for 4 days before C. neoformans inoculation. The medium was replaced with 0.5× diluted EGM-2 medium, and the cells were maintained at 37 °C and 5% CO2. The integrity of tight junctions between cells was confirmed by verifying that the trans-endothelial electrical resistance (TEER) measured ~200 Ω/cm², as assed by an epithelial volt/ohm meter (EVOM2 device, World Precision Instruments, Sarasota, FL, USA). For the BBB-crossing assay, 5 × 10^5 cells of C. neoformans WT (H99), mtp1Δ mutant, and phosphate deletion mutants were added to 500 μl of PBS and inoculated onto the porous membranes. After 24 h incubation at 37 °C in 5% CO2, the number of yeast cells passing through the porous membrane was measured by counting CFU. Tight junction integrity was measured by TEER as described above before and after inoculation of yeast cells, The BBB migration ratio was calculated by dividing the output CFU of each tested strain by that of WT. For the BBB-adhesion assay, 5 × 10^5 yeast cells in 100 μl of PBS were inoculated onto a monolayer of hCMEC/D3 cells grown in a 12-well plate and incubated for 24 h at 37 °C in 5% CO2. Following incubation for 4 days, the adhered cells with PBS, incubated with sterile distilled water for 30 min at 37 °C to burst the host cells, and collected for CFU quantification. The BBB-adhesion ratio was calculated by dividing the adhered CFU of each test strain by that of WT C. neoformans.

C. neoformans phosphatase web-accessible database. We developed the Cryptococcus neoformans Phosphatase Phenome Database (http://phosphatase.cryptococcus.org) and C. neoformans Phosphatome Gateway Database (http://www.cryptococcus.org/) to facilitate public access to the phenomic and genomic data for the C. neoformans phosphatase mutant library. The database was built under the same software development environment as described in our two previous publications14,5. Genome sequences and annotations of the H995 strain were obtained from the standardized genome data warehose in the Comparative Fungal Genomics Platform Database (CFGP 2.0; http://cfgp.riceblast.snu.ac.kr)108. Except reported phosphatases, those first functionally characterized by this study were named based on the published gene nomenclature rules for C. neoformans16 (Fig. 1a).

Statistical analysis. Statistical analyses were performed with GraphPad Prism version 8. For the capsule production assay and expression analysis, ANOVA with Bonferroni’s multiple comparison test was used. For the insect-killing assay, the log-rank (Mantel–Cox) test was used for statistical analysis. For the murine STM analysis, the statistical significance between st50Δ (positive control) and mutants was calculated by one-way ANOVA with Bonferroni’s multiple comparisons test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The phosphate domain and sequence data in Fig. 1 were retrieved from FungiDB (https://fungidb.org/fungidb/) and InterPro (https://www.ebi.ac.uk/interpro/) of the EBI. All data to classify the phosphatases in Fig. 1a and b are available in Supplementary Data 1. Detailed information about phosphatases in S. cerevisiae, S. pombe, C. albicans and U. maydis is listed in Supplementary Data 3. Information about gene IDs and names, strain numbers, and genotypes with signature tag numbers for phosphate mutant strains used in this study is available in Supplementary Data 5. We provide the whole phosphatase mutant collection via Fungal Genetics Stock Center (FGSC, http://www.fgsc.net/) in USA, Korean Culture Center of Microorganism (KCCM, http://www.kccm.or.kr/) and Korean Collection for Type Cultures (KCTC, https://kctc.kribb.re.kr) in South Korea. The whole gene data for phosphate mutants are available in the Cryptococcus neoformans Phosphatome Phenome Database (http://phosphatase.cryptococcus.org). Integrated phenotype data of transcription factor, kinase, and phosphate mutants in C. neoformans is available in the previous studies after deposition in the C. neoformans Phenome Gateway Database (http://www.cryptococcus.org/), in which individuals’ gene/protein information is linked to that of FungiDB. The whole NanoString-nCounter analysis data for in vivo phosphatase gene expression and probe information are available in Supplementary Data 8. The source data underlying Supplementary Figs. 7b, 11b, d, f and h are provided as a Source Data file. Source data are provided with this paper.

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References
1. Bonomi, A. et al. Protein kinases and phosphatases in the control of cell fate. Enzym. Res. 2011, 329098 (2018).
2. Bahn, Y. S. & Jung, K. W. Stress signaling pathways for the pathogenicity of Cryptococcus. Eukaryot. Cell 12, 1564–1577 (2013).
3. Rajasingham, R. et al. Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. Lancet Infect. Dis. 17, 873–881 (2017).
4. Jung, K. W. et al. Systematic functional profiling of transcription factor networks in Cryptococcus neoformans. Nat. Commun. 6, 6757 (2015).
5. Lee, K. T. et al. Systematic functional analysis of kinases in the fungal pathogen Cryptococcus neoformans. Nat. Commun. 7, 12766 (2016).
6. Blaschko, M. A. Drug discovery and protein tyrosine phosphatases. Curr. Med. Chem. 16, 2095–2176 (2009).
7. Winkelstroter, L. K. et al. Systematic global analysis of genes encoding protein phosphatases in Aspergillus fumigatus. G3: Genes Genomes Genet. 5, 1325–1359 (2015).
8. Yun, Y. et al. Functional analysis of the Fusarium graminearum phosphatase. PLoS Pathog. 7, e1001119 (2011).
9. Park, H. S., Lee, S. C., Cardenas, M. E. & Heitman, J. Calcium-calmodulin-calcineurin signaling: a globally conserved virulence cascade in eukaryotic microbial pathogens. Cell Host Microbe 26, 453–462 (2019).
10. Lee, K. T. et al. Distinct and redundant roles of protein tyrosine phosphatases Ptp1 and Ptp2 in governing the differentiation and pathogenicity of Cryptococcus neoformans. Eukaryot. Cell 13, 796–812 (2014).
11. Alsopugh, J. A. et al. Adenyl cyclase functions downstream of the Gαlph protein Gpa1 and controls mating and pathogenicity of Cryptococcus neoformans. Eukaryot. Cell 1, 75–84 (2002).
12. Lee, K. T., Kwon, H., Lee, D. & Bahn, Y. S. α nudix hydrolase protein, Ysa1, regulates oxidative stress response and fungal drug susceptibility in Cryptococcus neoformans. Mycobiot 42, 52–58 (2014).
13. Odom, A. et al. Calcineurin is required for virulence of Cryptococcus neoformans. EMBO J. 16, 2576–2589 (1997).
14. Petzold, E. W. et al. Characterization and regulation of the trehalose synthesis pathway and its importance in the pathogenicity of Cryptococcus neoformans. Infect. Immun. 74, 5877–5887 (2006).
15. Panepinto, J. C. et al. Binding of serum mannann binding lectin to a cell integrity-defective Cryptococcus neoformans ccrAΔ mutant. Infect. Immun. 75, 4769–4779 (2007).
16. Jung, W. H. et al. Had1 is required for cell wall integrity and fungal virulence in Cryptococcus neoformans. G3: Genes Genomes Genet. 8, 643–652 (2018).
17. Kretschmer, M. et al. Defects in phosphate acquisition and storage in the central nervous system. Infect. Immun. 82, 2697–2712 (2014).
18. Lev, S. et al. Identification of Aph1, a phosphate-regulated, secreted, and vacuolar acid phosphatase in Cryptococcus neoformans. mBio 5, e01649–01614 (2014).
19. Lev, S. et al. Fungal inositol pyrophosphate IP5 is crucial for metabolic adaptation to the host environment and pathogenicity. mBio 6, e00531–00515 (2015).
20. Shea, J. M., Keichichian, T. B., Luberto, C. & Del Poeta, M. The cryptococcal enzyme inositol phosphohydrolipid-phospholipase C confers resistance to the antifungal effects of macrophages and promotes fungal dissemination to the central nervous system. Infect. Immun. 74, 5977–5988 (2006).
21. Gerik, K. J. et al. Cell wall integrity is dependent on the PKC1 signal transduction pathway in Cryptococcus neoformans. Mol. Microbiol. 58, 393–408 (2005).
22. Fanning, S. et al. Divergent targets of CANDA allicins biofilm regulator Bcr1 in vitro and in vivo. Eukaryot. Cell 11, 896–904 (2012).
23. Xu, W., Solis, N. V., Filler, S. G. & Mitchell, A. P. Pathogen gene expression profiling during infection using a NanoString nCounter platform. *Methods Mol. Biol.* 1361, 57–65 (2016).

24. Liu, O. W. et al. Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. *Cell* 135, 174–188 (2008).

25. Zaragoza, O., de Virgilio, C., Ponton, J. & Gancedo, C. Disruption in *Cryptococcus neoformans* of the *TPS2* gene encoding trehalose-6-phosphate phosphatase affects cell integrity and decreases infectivity. *Microbiology* 148, 1281–1290 (2002).

26. Desai, J. V. et al. Coordination of *Candida albicans* invasion and infection functions by phospholipid-phosphosphate Rh2. *Pathogens* 4, 573–589 (2015).

27. Hanaoka, N. et al. Identification of the putative protein phosphatase gene *PTC1* as a virulence-related gene using a silkworm model of *Candida albicans* infection. *Eukaryot. Cell* 7, 1640–1648 (2008).

28. Yang, Y., Aisen, P. & Casadevall, A. *Cryptococcus neoformans* melanin and virulence: mechanism of action. *Infect. Immun.* 63, 3131–3136 (1995).

29. Kozel, T. R. & Gotschlich, E. C. The capsule of *Cryptococcus neoformans* passively influences phagocytosis of the yeast by macrophages. *J. Immunol.* 129, 1675–1680 (1982).

30. Lee, D. E. & Bahn, U. Unraveling melanin biosynthesis and signaling networks in *Cryptococcus neoformans*. *mBio* 10, e02267–19 (2019).

31. Seaman, M. N., McCaffery, J. M. & Emr, S. D. A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *J. Cell Biol.* 142, 665–681 (1998).

32. Bonifacino, J. S. & Hurley, J. H. Retromer. *Curr. Opin. Cell Biol.* 20, 427–436 (2008).

33. Seaman, M. N. Recycle your receptors with retromer. *Curr. Opin. Cell Biol.* 20, 427–436 (2008).

34. Abeijon, C. et al. Guanosine diphosphatase is required for protein and sphingolipid glycosylation in the Golgi lumen of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 274, 21450–21456 (1999).

35. Yanagisawa, K., Resnick, D., Abeijon, C., Robbins, P. W. & Hirschberg, C. B. A guanosine diphosphatase enriched in Golgi vesicles of *Saccharomyces cerevisiae*. Purification and characterization. *J. Biol. Chem.* 265, 19331–19335 (1990).

36. Herrero, A. B., Ucelletti, D., Hirschberg, C. B., Dominguez, A. & Abeijon, C. The Golgi GDPase of the fungal pathogen *Candida albicans* affects morphogenesis, glycosylation, and cell wall properties. *Eukaryot. Cell* 1, 420–431 (2002).

37. Lee, D. J., Bahn, Y. S., Kim, H. J., Chung, S. Y. & Kang, H. A. Unraveling the novel structure and biosynthetic pathway of O-linked glycans in the Golgi apparatus of the human pathogenic yeast *Candida albicans*. *J. Biol. Chem.* 290, 1861–1873 (2015).

38. Lee, K. T. et al. Fungal kinases and transcription factors regulating brain infection in *Cryptococcus neoformans*. *Nat. Commun.* 11, 1521 (2020).

39. Vu, K. et al. Identification of the central nervous system by *Cryptococcus neoformans* requires a secreted fungal metalloprotease. *mBio* 5, e01101–e01114 (2014).

40. Liu, T. B. et al. Brain inositol is a novel stimulator for promoting Cryptococcus penetration of the blood-brain barrier. *PLoS Pathog.* 9, e1003247 (2013).

41. Feng, J. et al. The N-terminal pY3XXL motif of CpsPy2 is critical for the function of protein phosphatase 4 in *C. albicans* deactivation. DNA damage-induced filamentation and virulence in *Candida albicans*. *Int. J. Med. Microbiol.* 307, 471–480 (2017).

42. Bader, T., Bodendorfer, B., Schroppel, K. & Morschhäuser, J. Calcineurin is essential for virulence in *Candida albicans*. *Infect. Immun.* 71, 5344–5354 (2003).

43. Lee, C. M., Nantel, A., Jiang, L., Whiteway, M. & Shen, S. H. The serine/threonine protein kinase *SIT4* modulates yeast-to-hypha morphogenesis and virulence in *Candida albicans*. *Mol. Microbiol.* 51, 691–709 (2004).

44. Hanaoka, N. et al. A putative dual-specific protein phosphatase encoded by *YHV1* controls growth, filamentation and virulence in *Candida albicans*. *Microbiology* 151, 2223–2232 (2005).

45. Csanak, C. et al. Derepressed hyphal growth and reduced virulence in a VH1 family-related protein phosphatase mutant of the human pathogen *Candida albicans*. *Mol. Biol. Cell* 8, 2539–2551 (1997).

46. Noble, S. M., French, S., Kohn, L. A., Chen, V. & Johnson, A. D. Systematic screens of a *Candida albicans* homologous deletion library decouple morphogenetic switching and pathogenicity. *Nat. Genet.* 42, 590–598 (2010).

47. Rocha, C. R. et al. Signaling through adenyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans*. *Mol. Biol. Cell* 12, 3631–3643 (2001).

48. Bagley, M. J. et al. Cell wall integrity is linked to mitochondria and phospholipid homeostasis in *Candida albicans* through the activity of the post-transcriptional regulator Ger1-Pop2. *Mol. Microbiol.* 79, 968–989 (2011).
76. Parrish, W. R., Stefan, C. J. & Emr, S. D. Essential role for the myotubulin-related phosphatase Ymr1p and the synaptopin-like phosphatases Sfl2p and Sfl3p in regulation of phosphatidylinositol 3-phosphate in yeast. Mol. Cell. Biol. 15, 3567–3579 (2004).

77. Hartwell, L. H., Culotti, J. & Reed, B. Genetic control of the cell-division cycle in yeast. I. Detection of mutants. Proc. Natl Acad. Sci. USA 66, 352–359 (1970).

78. Vazquez, H. M., Vionnet, C., Roubaty, C. & Conzelmann, A. Cdc1 removes the phosphate at O-1 of the first monophosphate of GPI anchors and thus facilitates the integration of GPI proteins into the yeast cell wall. Mol. Cell. Biol. 25, 3375–3388 (2014).

79. Keogh, M. C. et al. A phosphatase complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery. Nature 439, 495–501 (2006).

80. O’Neill, S. M. et al. Pph3-Psy2 is a phosphatase complex required for Rad53 dephosphorylation and replication fork restart during recovery from DNA damage. Proc. Natl Acad. Sci. USA 104, 9290–9295 (2007).

81. Jung, K. W. et al. Unraveling fungal radiation resistance regulatory networks through the genome-wide transcriptome and genetic analyses of Cryptococcus neoformans. mBio 7, e01483-16 (2016).

82. Jung, K. W. et al. Rad53- and Chk1-dependent DNA damage response pathways cooperatively promote fungal pathogenesis and modulate antifungal drug susceptibility. mBio 10, e01726-18 (2019).

83. Srivastav, R., Sharma, R., Tandon, S. & Tandon, C. Role of DHH superfamily proteins in nucleic acids metabolism and stress tolerance in prokaryotes and eukaryotes. Int. J. Biol. Macromol. 127, 66–75 (2019).

84. Beck, T. & Hall, M. N. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402, 689–692 (1999).

85. Jacinto, E., Guo, B., Arndt, K. T., Schmelzle, T. & Hall, M. N. The TORC1 programme promotes cell growth at the expense of cell division. Nature 497, 495–499 (2013).

86. Mukhopadhyay, A. et al. Direct interaction between the inhibitor 2 and the ethanolamine phosphate of the yeast. I. Detection of mutants. Proc. Natl Acad. Sci. USA 90, 281–285 (1993).

87. O’Meara, T. R. et al. The Cryptococcus neoformans Rim101 transcription factor directly regulates genes required for adaptation to the host. Mol. Cell. Biol. 34, 673–684 (2014).

88. Liu, J. et al. Capsule synthesis by Cryptococcus neoformans. Trans. N. Y. Acad. Sci. 20, 623–648 (1958).

89. Thak, E. J., Kim, J., Lee, D. O., Kim, J. Y. & Kang, H. Structural analysis of N/O glycans assembled on proteins in yeasts. J. Microbiol. 56, 11–23 (2018).

90. Littman, M. L. Capsule synthesis by Cryptococcus neoformans. mBio 10, e02082-18 (2019).

91. Wang, H., Gu, C., Rolfes, R. J., Jessen, H. J. & Shears, S. B. Structural and functional analyses of trehalose-6-phosphate phosphatase from Cryptococcus neoformans (CM1595). Biochem. Biophys. Res. Commun. 413, 248–253 (2013).

92. Chung, V. et al. Safety, tolerability, and preliminary activity of LB-100, an antifungal target.

93. Vazquez, H. M., Vionnet, C., Roubaty, C. & Conzelmann, A. Cdc1 removes the phosphate at O-1 of the first monophosphate of GPI anchors and thus facilitates the integration of GPI proteins into the yeast cell wall. Mol. Cell. Biol. 25, 3375–3388 (2014).

94. Perfect, J. R., Tenor, J. L., Miao, Y. & Brennan, R. G. Trehalose pathway as an antifungal target.

95. O’Meara, T. R. et al. The Cryptococcus neoformans Rim101 transcription factor directly regulates genes required for adaptation to the host. Mol. Cell. Biol. 34, 673–684 (2014).

96. Jain, J., Singh, S., Tandon, S. & Tandon, C. Role of DHH superfamily proteins in nucleic acids metabolism and stress tolerance in prokaryotes and eukaryotes. Int. J. Biol. Macromol. 127, 66–75 (2019).

97. Beck, T. & Hall, M. N. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402, 689–692 (1999).

98. Jacinto, E., Guo, B., Arndt, K. T., Schmelzle, T. & Hall, M. N. The TORC1 programme promotes cell growth at the expense of cell division. Nature 497, 495–499 (2013).

99. O’Meara, T. R. et al. The Cryptococcus neoformans Rim101 transcription factor directly regulates genes required for adaptation to the host. Mol. Cell. Biol. 34, 673–684 (2014).

100. Liu, J. et al. Capsule synthesis by Cryptococcus neoformans. mBio 10, e02082-18 (2019).

101. Littman, M. L. Capsule synthesis by Cryptococcus neoformans. mBio 10, e02082-18 (2019).

102. Liu, J. et al. Capsule synthesis by Cryptococcus neoformans. mBio 10, e02082-18 (2019).

103. Jung, K. W., Kim, S. Y., Okagaki, I. H., Nielsen, K. & Bahn, Y. S. Ste50 adaptor protein governs sexual differentiation of Cryptococcus neoformans via the pheromone-response MAPK signaling pathway. Fungal Genet. Biol. 48, 154–165 (2011).

104. Cheon, S. A. et al. Unique evolution of the UPR pathway with a novel bZIP transcription factor, Hul1, for controlling pathogenicity of Cryptococcus neoformans. PLoS Pathog. 7, e1002177 (2011).

105. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402–408 (2001).

106. Vu, K., Weksler, B., Romero, I., Couraud, P. O. & Gelli, A. Immortalized human brain endothelial cell line hCMEC/D3 as a model of the blood-brain barrier facilitates in vitro studies of central nervous system infection by Cryptococcus neoformans. Eukaryot. Cell 8, 1803–1807 (2009).

107. Santiago-Tirado, F. H., Onken, M. D., Cooper, J. A., Klein, R. S. & Doering, T. L. Trojan horse transit contributes to blood-brain barrier crossing of a eukaryotic pathogen. mBio 8, e02183–e02116 (2017).

108. Choi, J. et al. CFGP 2.0: a versatile web-based platform for supporting comparative and evolutionary genomics of fungi and Oomycetes. Nucleic Acids Res. 41, D714–D719 (2013).

109. Inglis, D. O. et al. Literature-based gene curation and proposed genetic nomenclature for Cryptococcus. Eukaryot. Cell 13, 878–883 (2014).

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Y.-S.B. conceived the project. J.-H.J., K.-T.L., J.H., D.L., E.-H.J., J.-Y.K., Y.L., S.-H.L., Y.-S.S., K.-W.J., D.-G.L., E.J., M.L., Y.-B.J., Y.C., M.H.L., J.-S.K., S.-R.Y., T.-J.C., T.-W.L., H.C., S.-W.K., K.J.S., L.J., E.T., J.C., and A.F.A. performed experiments and analysed the data. Y.-H.L., J.H., H.A.K., E.C., and Y.-S.B. supervised the experimental analysis. J.-H.J., K.-T.L., Y.-H.L., J.H., H.A.K., E.C., and Y.-S.B. wrote the manuscript. All authors have reviewed and approved this manuscript.

Competing interests
The authors declare no competing interests.

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