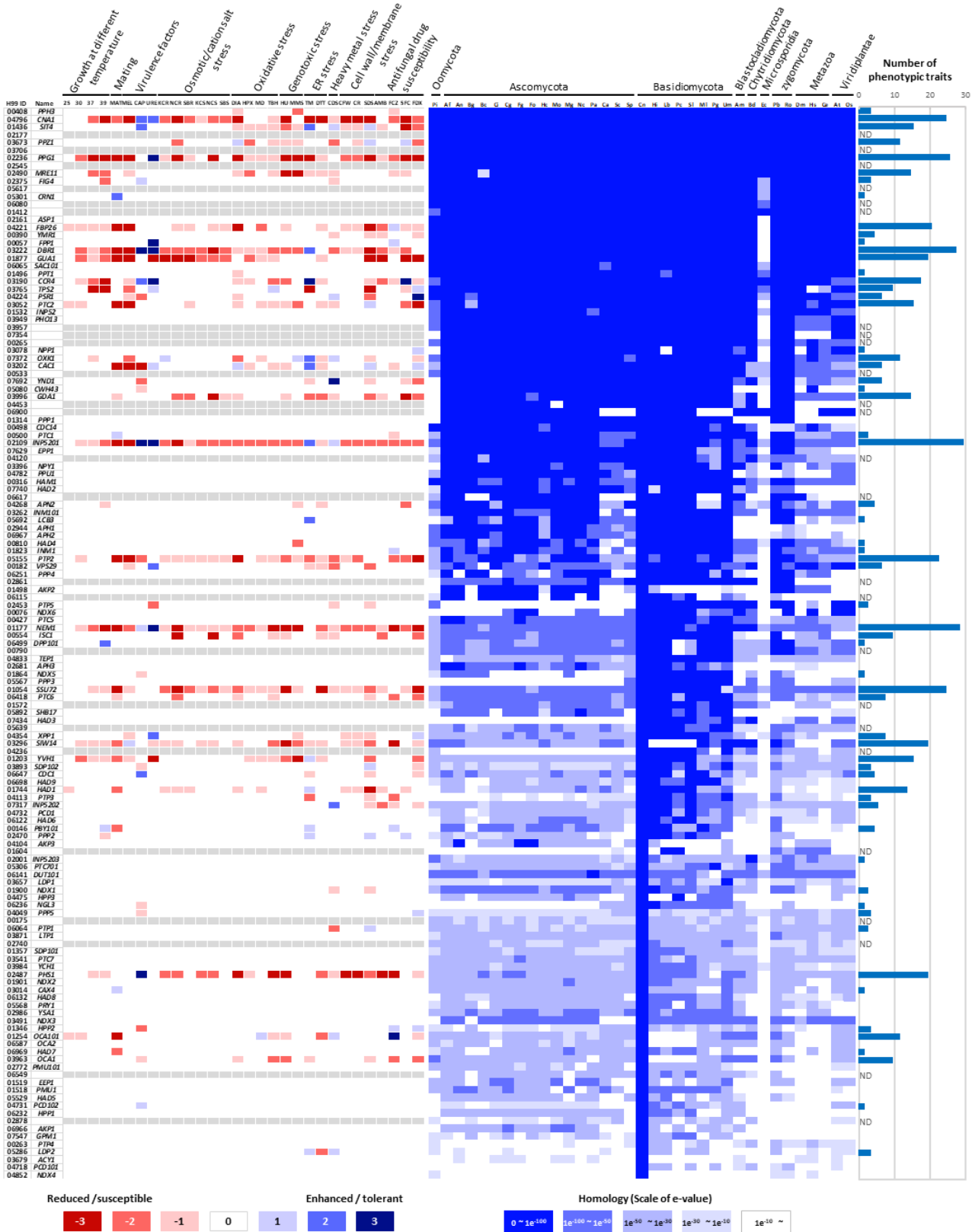


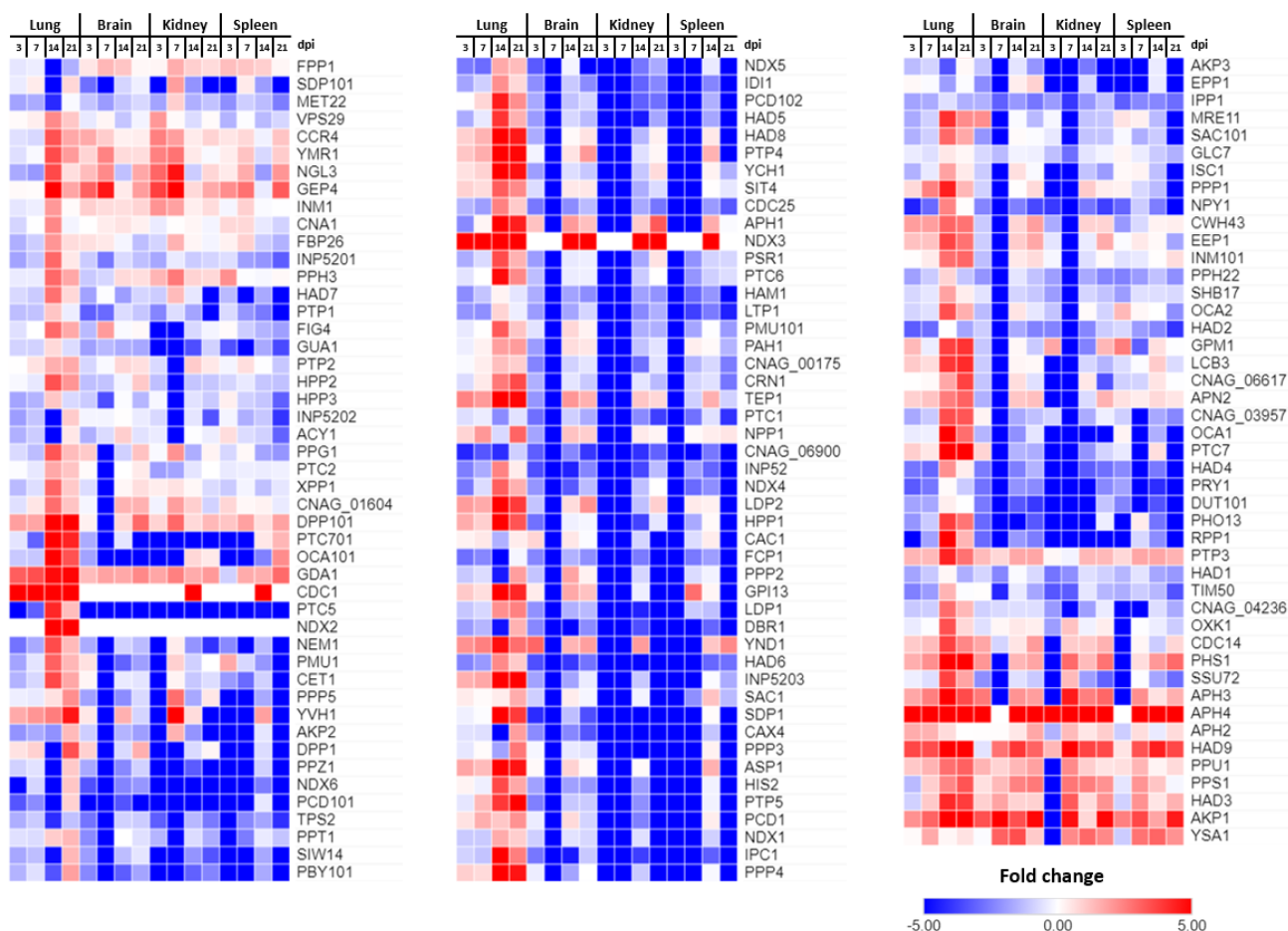
Supplementary Information

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

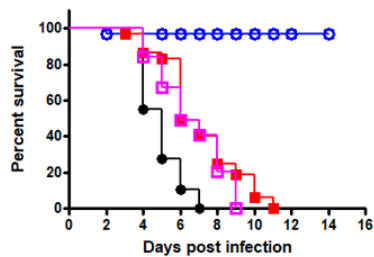
Jin *et al.*



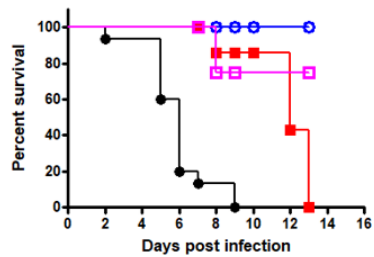
Supplementary Figure 2. Phylogenetic relationship and phenotypic traits of phosphatases in *C. neoformans*. BLAST matrix comparative search for each phosphatase was performed using the Comparative Fungal Genomics Platform (<http://cfgp.riceblast.snu.ac.kr>). In vitro phenotypic traits were examined under 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. 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, fludioxonil; TM, tunicamycin; DTT, dithiothreitol; CDS, cadmium sulphate; SDS, sodium dodecyl sulphate; 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.



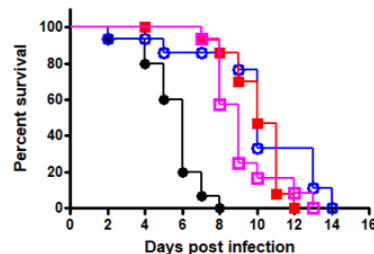
Supplementary Figure 3. *In vivo* gene expression profiling of phosphatases in *C. neoformans*. The *in vivo* gene expression level of 139 phosphatases was monitored using nCounter (NanoString) with RNA samples obtained from wild-type *C. neoformans* strain (H99S)-infected mice organs (lungs, brain, spleen, and kidneys) and gene-specific probes listed in Supplementary Data 7. Three mice were sacrificed 3, 7, 14, or 21 days post-infection (dpi). Infected organs were recovered and lyophilised. Dried organs were homogenised, and total RNA was extracted by using a commercial RNA extraction kit. Hierarchical phenotypic clustering of phosphatases was performed with one minus Pearson correlation using Morpheus (<https://software.broadinstitute.org/morpheus>).



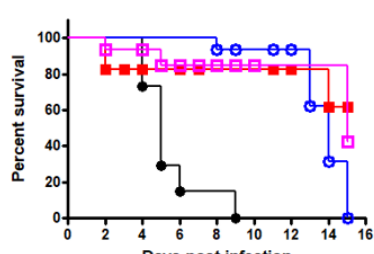
H99
 PBS
 YSB42
 YSB5650
 $P < 0.0001$
 $P = 0.0003$
 (CNAG_03202:CAC1)



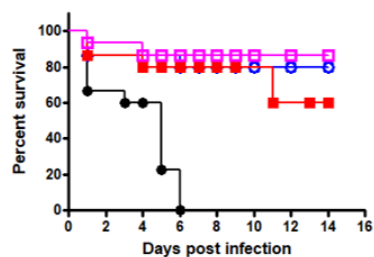
H99
 PBS
 YSB4373
 YSB4433
 $P < 0.0001$
 (CNAG_03765:TPS2)



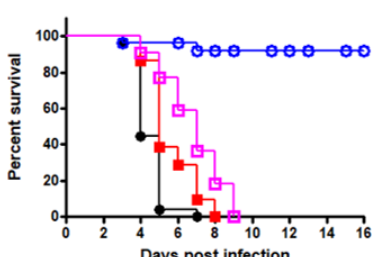
H99
 PBS
 YSB275
 YSB277
 $P < 0.0001$
 (CNAG_05155:PTP2)



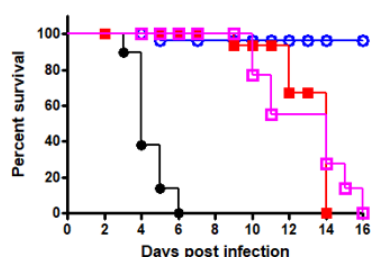
H99
 PBS
 YSB4067
 YSB4068
 $P = 0.0013$
 $P = 0.0002$
 (CNAG_04796:CNA1)



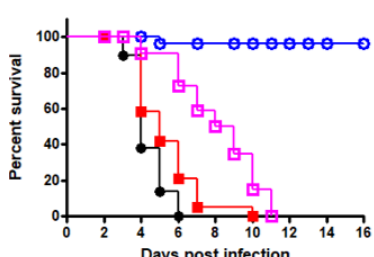
H99
 PBS
 YSB4963
 YSB4964
 $P = 0.0003$
 $P < 0.0001$
 (CNAG_03222:DBR1)



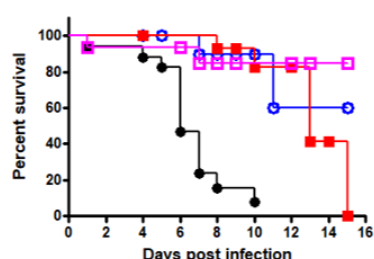
H99
 PBS
 YSB4665
 YSB4666
 $P = 0.0004$
 $P < 0.0001$
 (CNAG_01744:HAD1)



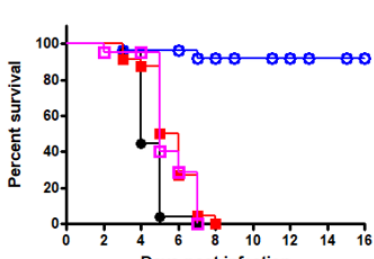
H99
 PBS
 YSB4609
 YSB4929
 $P < 0.0001$
 (CNAG_02109:INP5201)



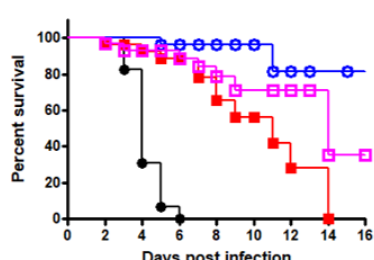
H99
 PBS
 YSB6161
 YSB6163
 $P = 0.0052$
 $P < 0.0001$
 (CNAG_01877:GUA1)



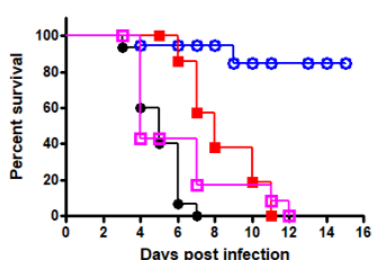
H99
 PBS
 YSB4242
 YSB4243
 $P < 0.0001$
 $P = 0.0003$
 (CNAG_01054:SSU72)



H99
 PBS
 YSB4771
 YSB4772
 $P = 0.0002$
 $P = 0.0002$
 (CNAG_01177:NEM1)

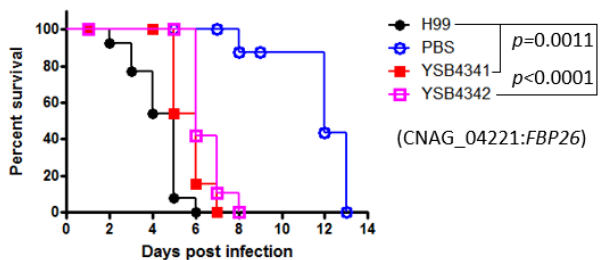
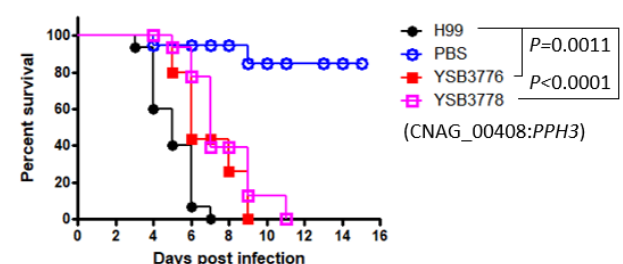
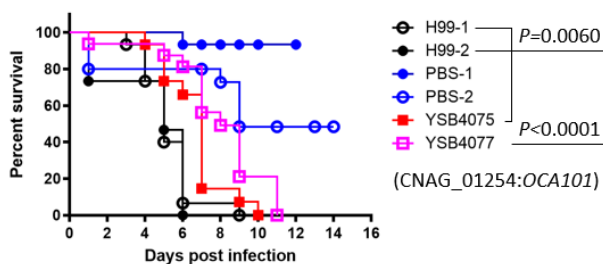
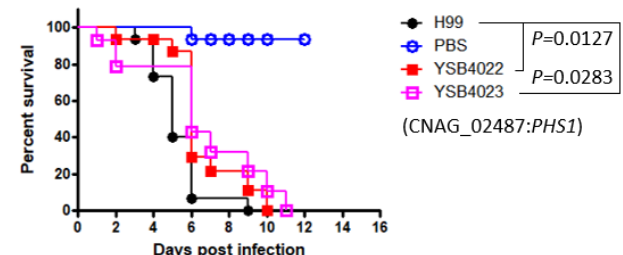
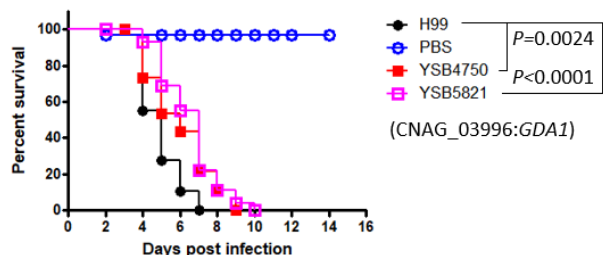
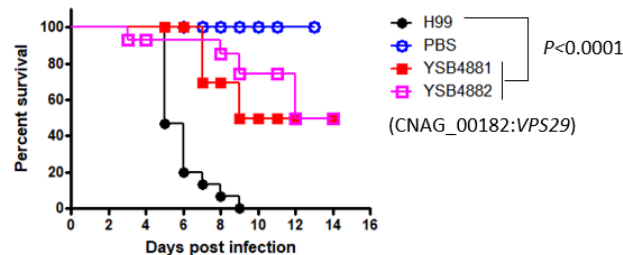
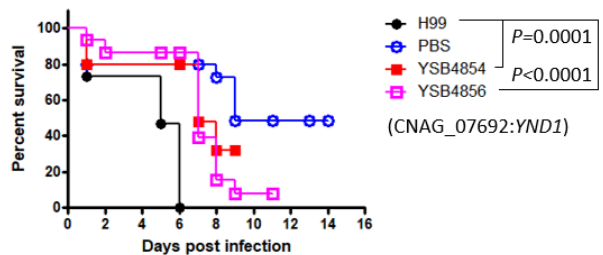
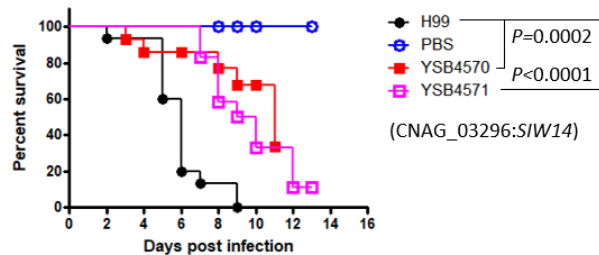
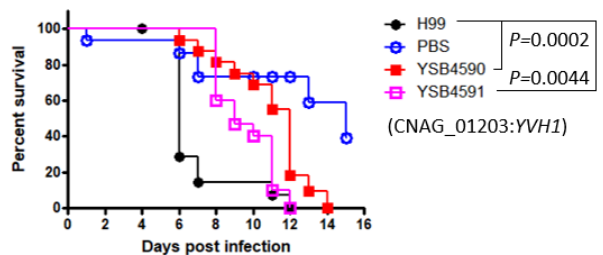
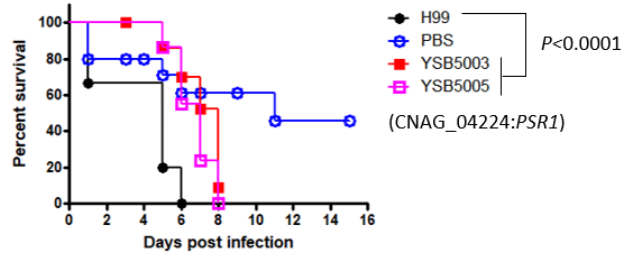
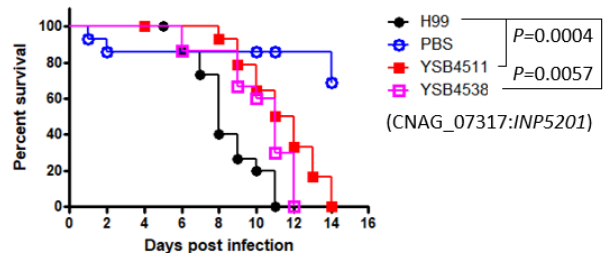


H99
 PBS
 YSB5772
 YSB5940
 $P < 0.0001$
 (CNAG_02236:PPG1)



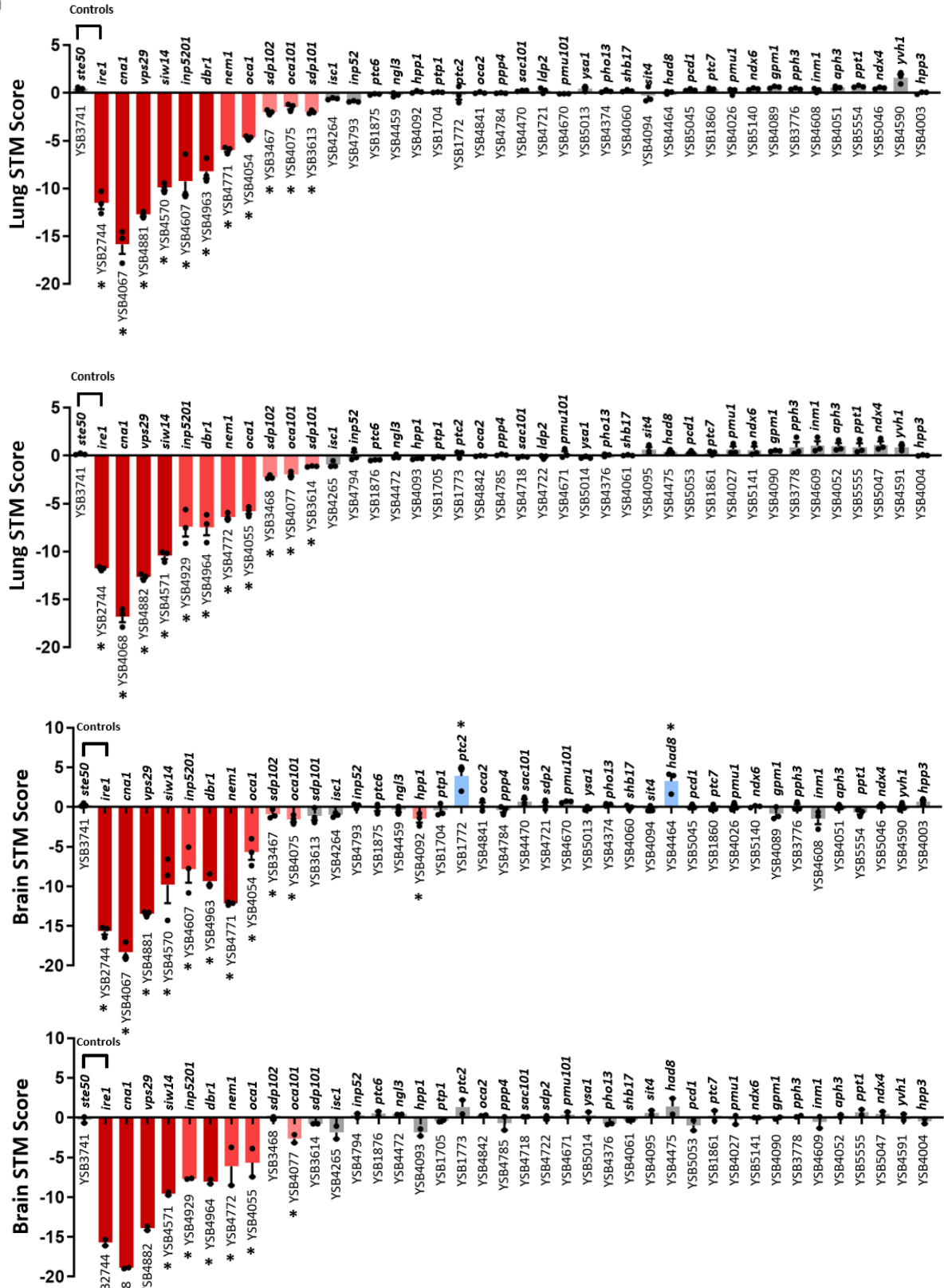
H99
 PBS
 YSB4094
 YSB4095
 $P < 0.0001$
 $P = 0.1413$
 (CNAG_01436:SIT4)

Continued



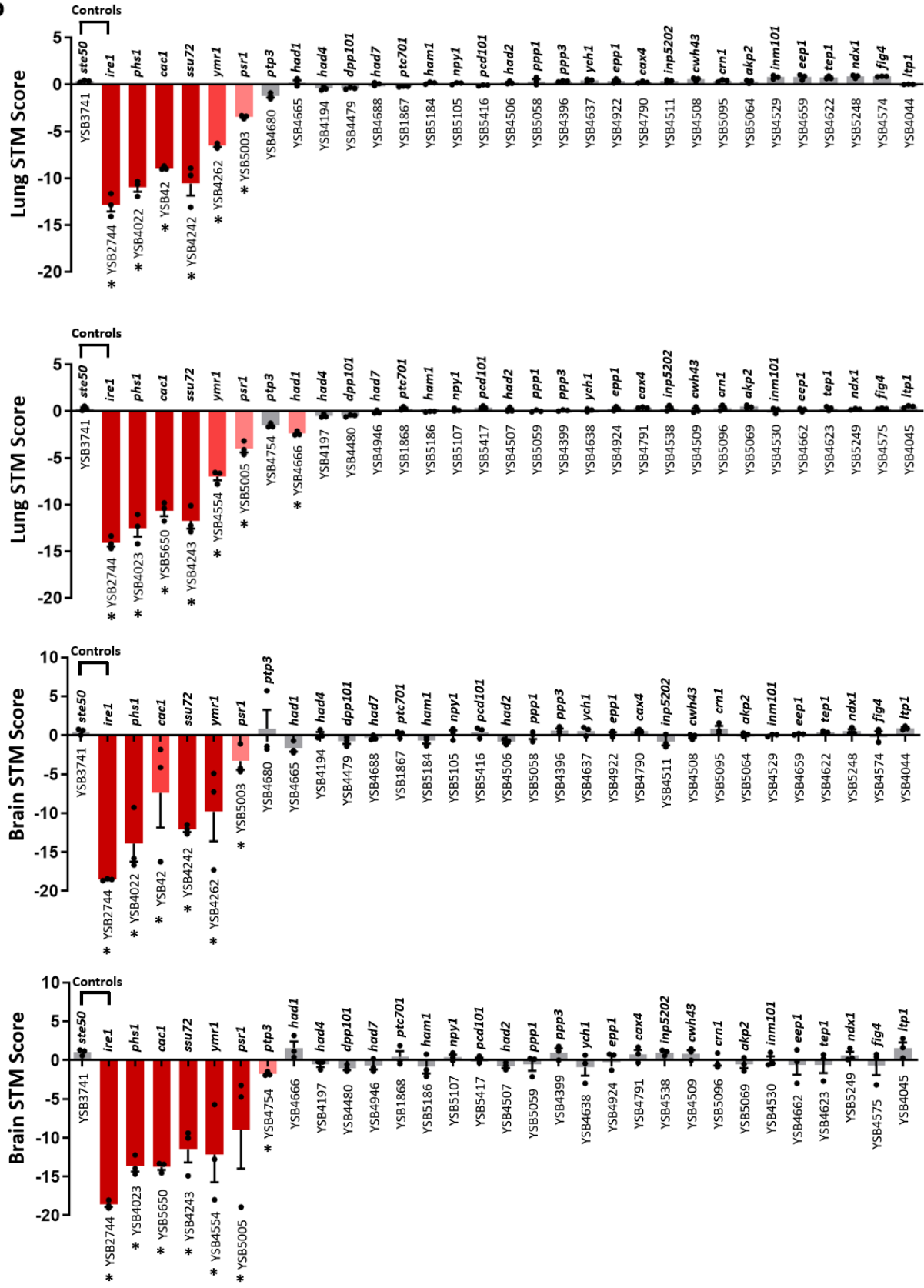
Supplementary Figure 4. Identification of virulence-regulating phosphatases in *C. neoformans* in an insect-killing assay. Each phosphatase mutant was cultured in YPD medium at 30°C for 16 h, pelleted, and washed three times with PBS. Four thousand phosphatase mutant cells were injected into *Galleria mellonella* larvae (at least 15 larvae per group). PBS-only was used as a non-infective negative control, and the H99S strain was used as a positive control. Infected larvae were placed in Petri dishes, incubated in a humidified container at 37°C, and monitored daily. *P* values were calculated using the log-rank (Mantel-Cox) test to measure statistical differences between the wild-type (WT) strain (H99S) and each phosphatase mutant strain. Two independently constructed mutants for each phosphatase gene were monitored.

a



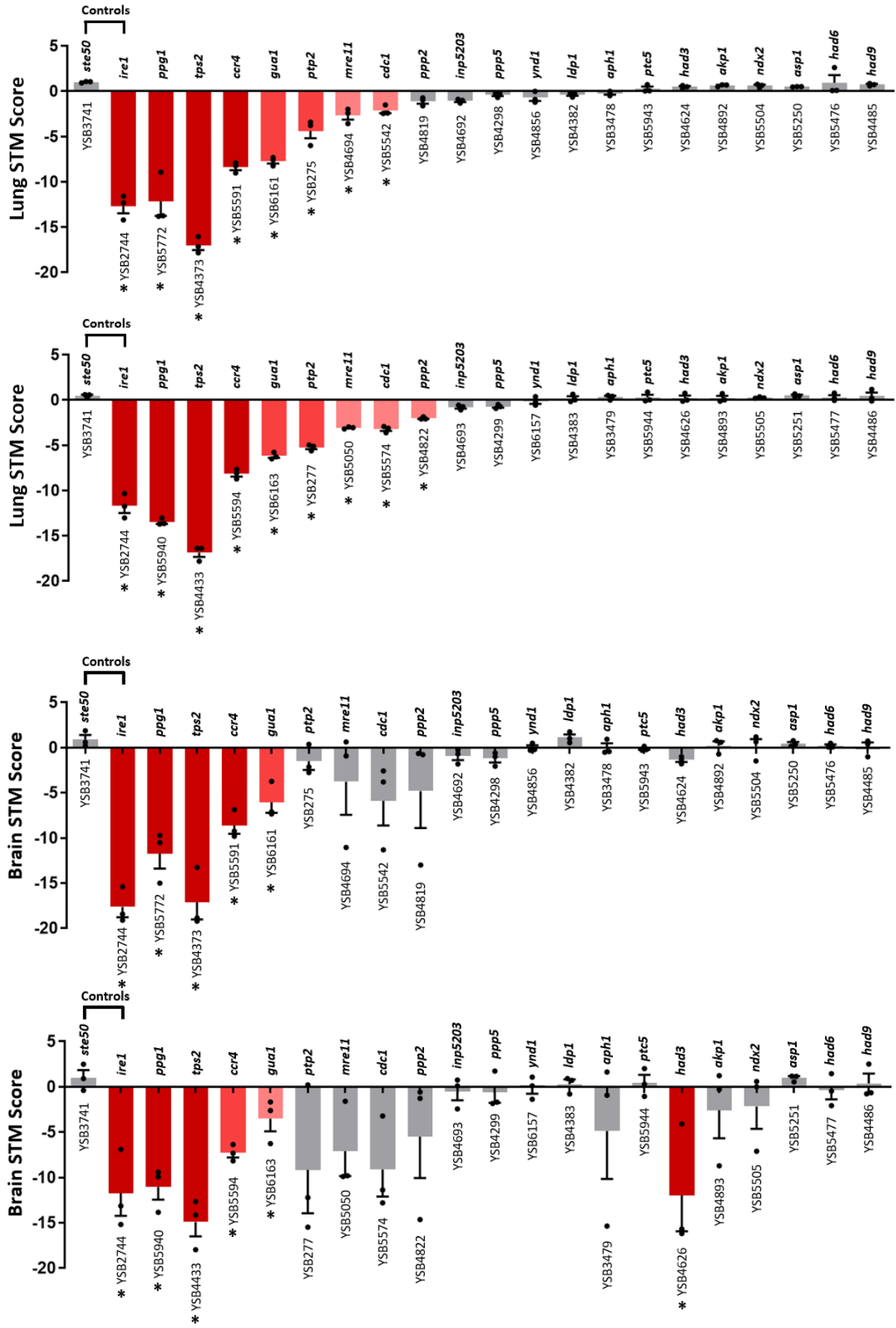
Continued

b

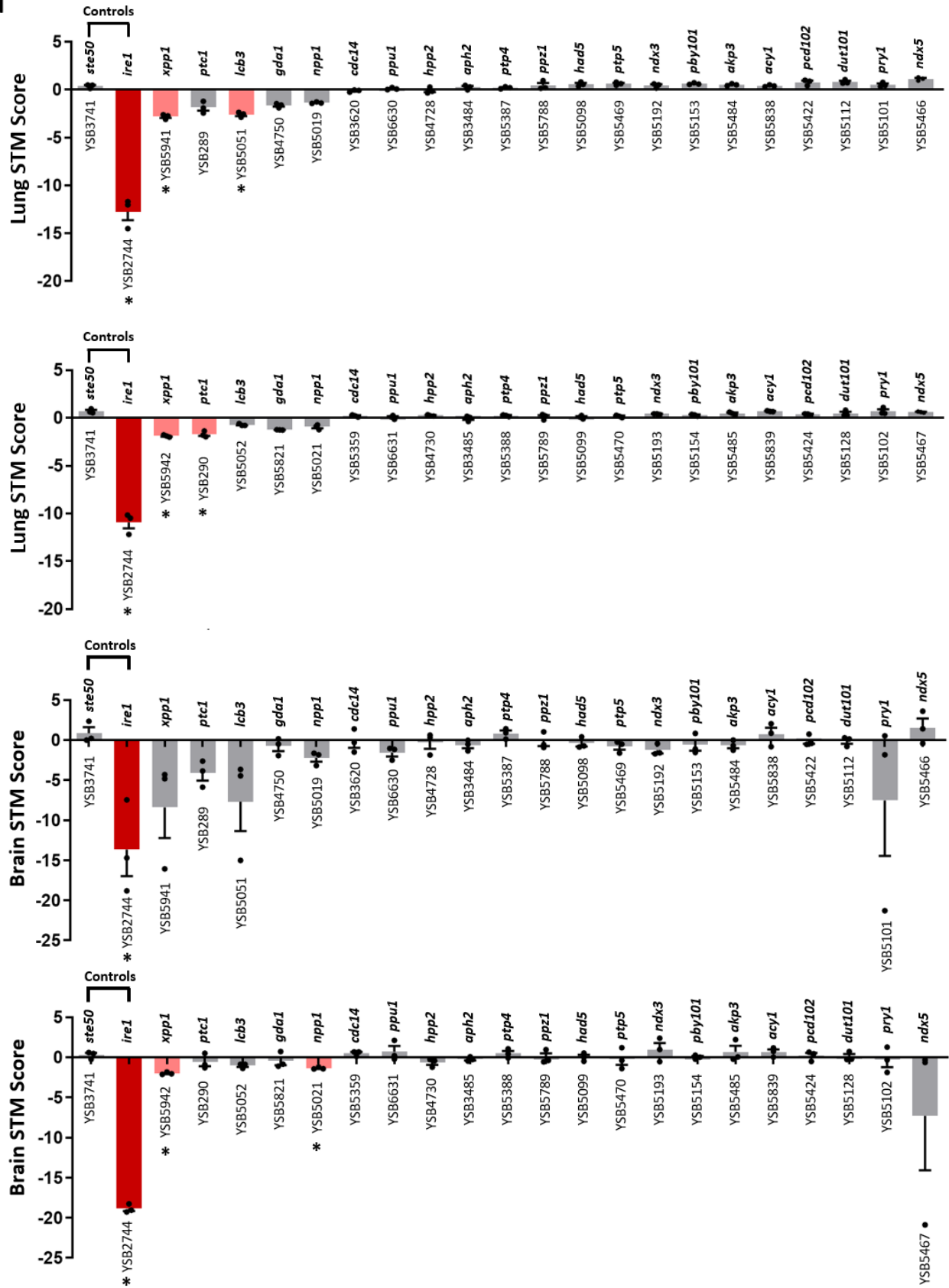


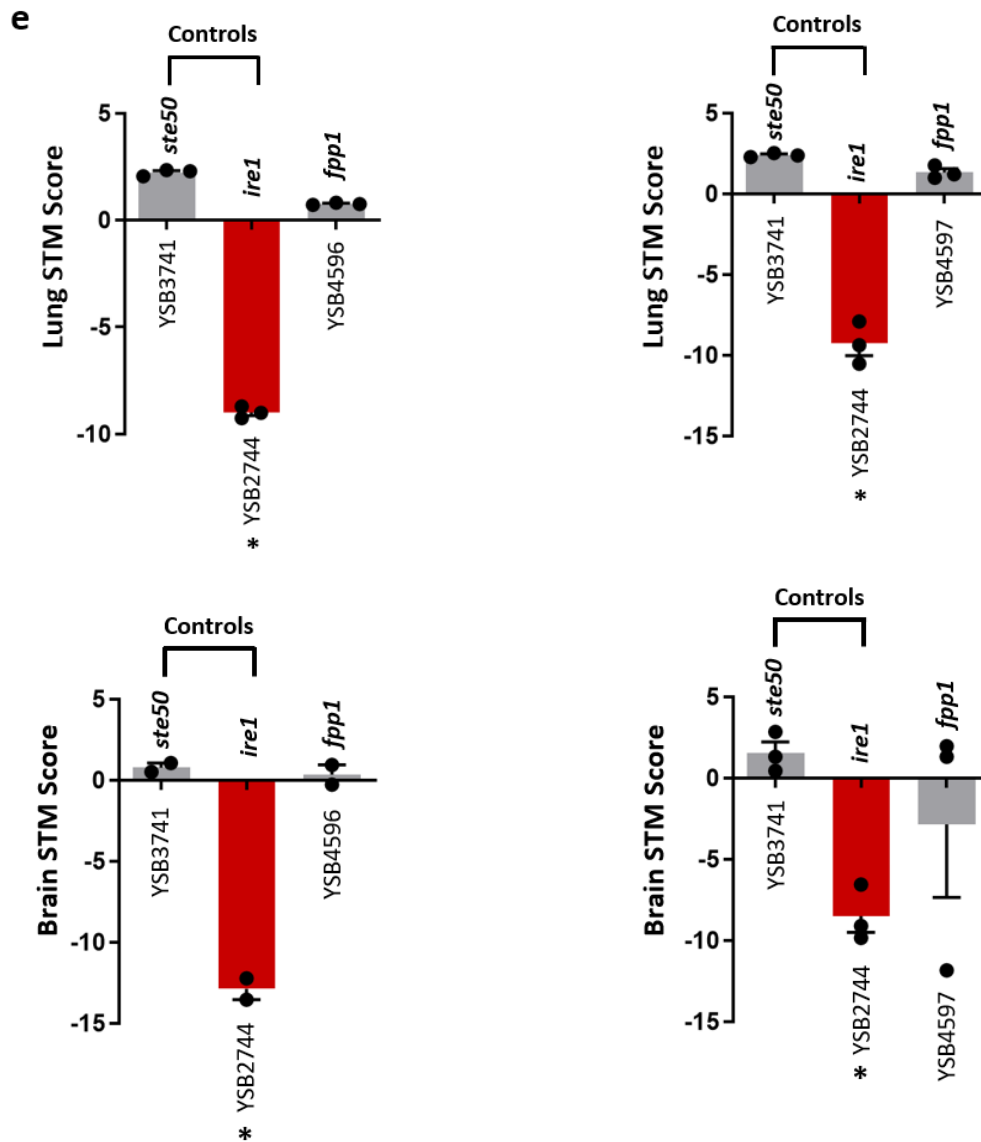
Continued

C

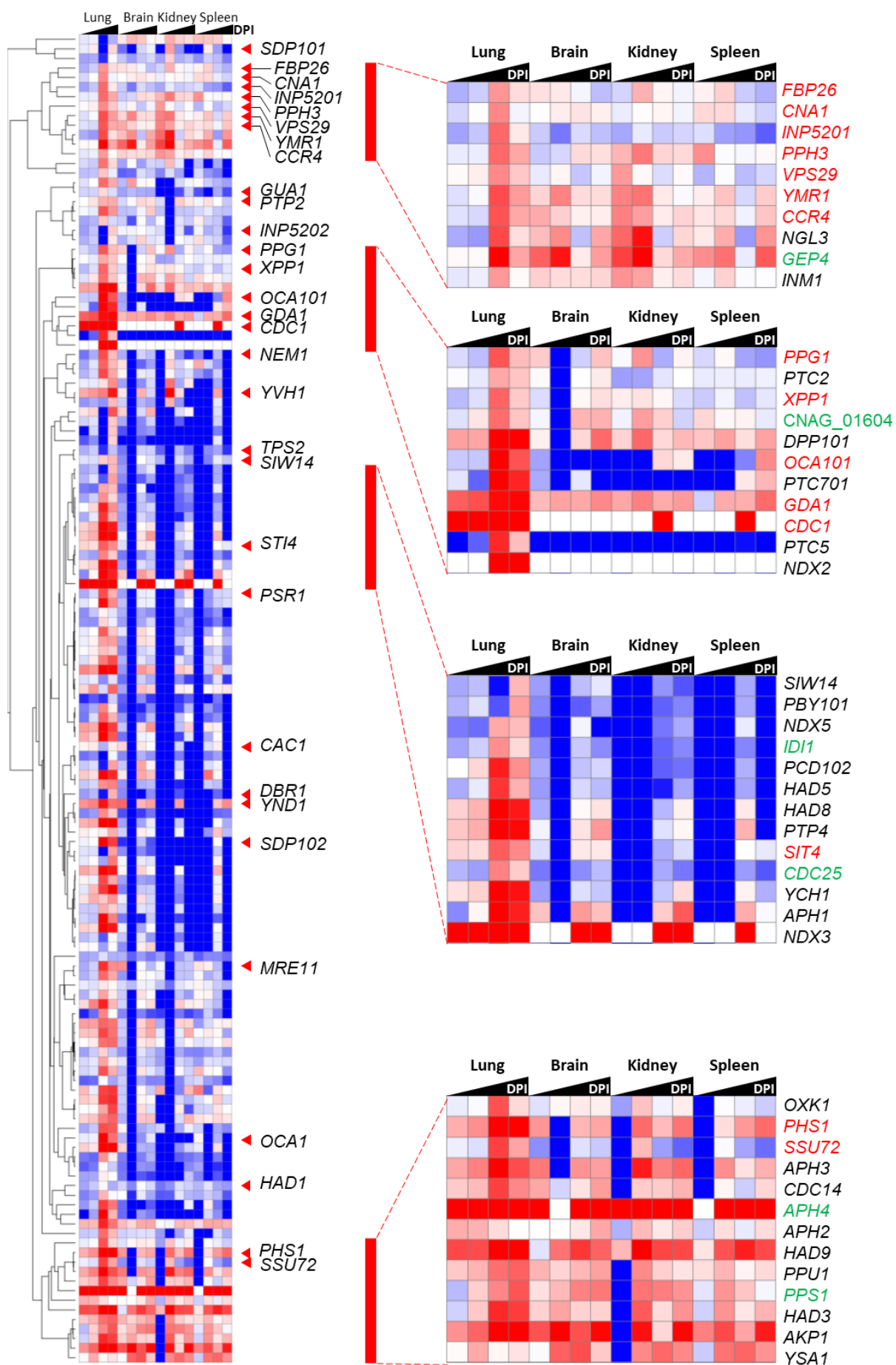


Continued

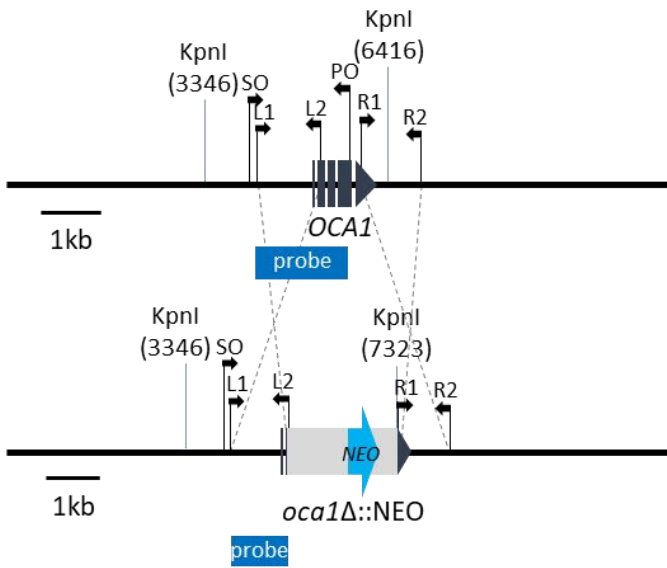
a*Continued*



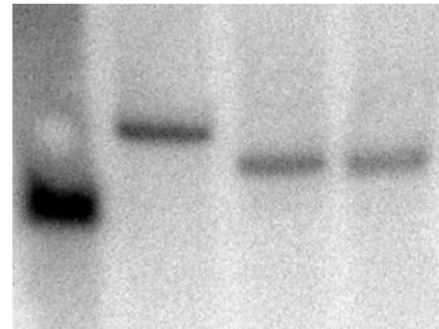
Supplementary Figure 5. Identification of infectivity-regulating phosphatases in *C. neoformans* in a signature-tagged mutagenesis (STM)-based murine infectivity assay. Lung and brain STM scores were calculated by quantitative PCR using a common primer and the signature tag-specific primers listed in Supplementary Data 4. The *ste50* Δ and *ire* Δ mutants were used as virulent positive control and avirulent negative control strains, respectively, as previously reported^{3,4}. **(a-e)** Phosphatase mutants were divided into five sets. Each panel indicates a separate pooled set of mutants. Each set included two independent strains of each phosphatase mutant. The statistically significant was calculated by one-way ANOVA analysis with Bonferroni's multiple comparison test. Data are presented as mean values $\pm$ standard error of mean (SEM) (*, $P < 0.05$) and presented with different colour codes: pink (< -2), reddish (< -4), red (< -8), and light blue (> 2).



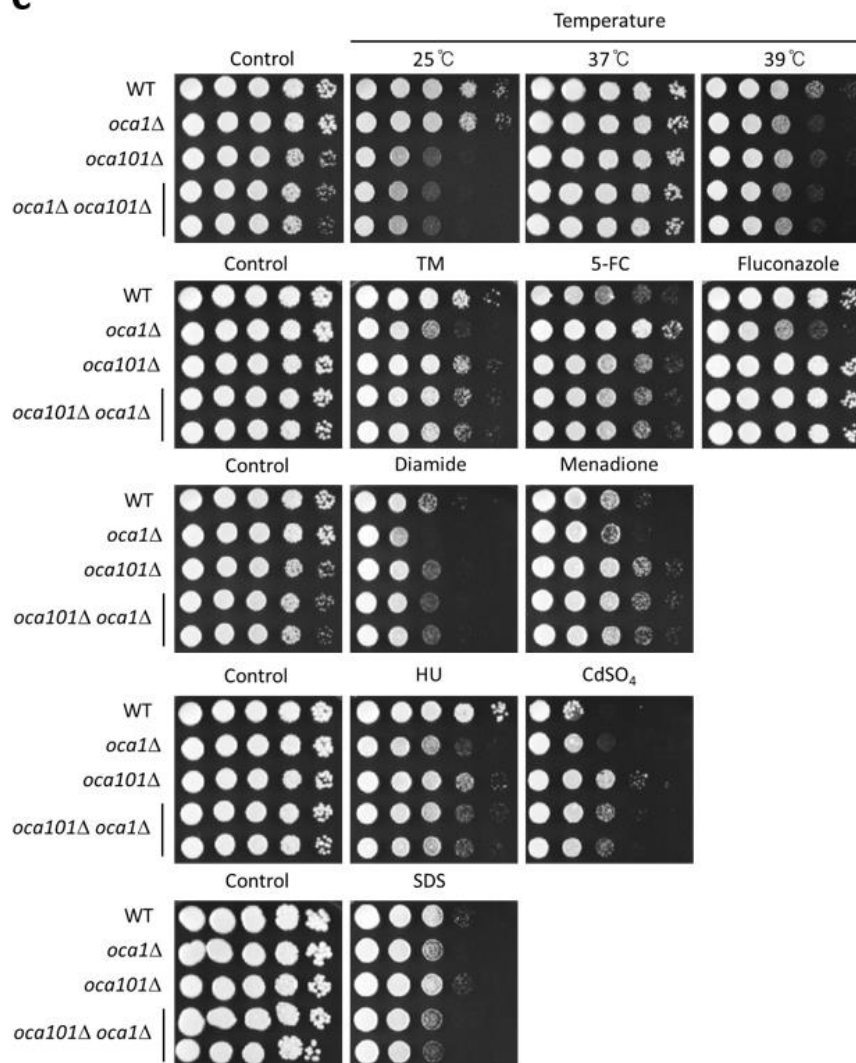
Supplementary Figure 6. Clustering of *in vivo* gene expression data of *Cryptococcus neoformans* phosphatases. *In vivo* gene expression profiles of *C. neoformans* phosphatases were hierarchically clustered using one minus Pearson correlation in Morpheus (<https://software.broadinstitute.org/morpheus>). The original *in vivo* gene expression data obtained from the NanoString nCounter analysis are available in Supplementary Data 8. Of the 139 phosphatases, the pathogenicity-related phosphatases are given in the left panel. The groups highlighted in the right panel include the phosphatase genes that exhibited high *in vivo* expression in the lungs, brain, and/or kidney. Red letters indicate the pathogenicity-related phosphatases and green letters indicate putative essential phosphatases in *C. neoformans*.

a**b**

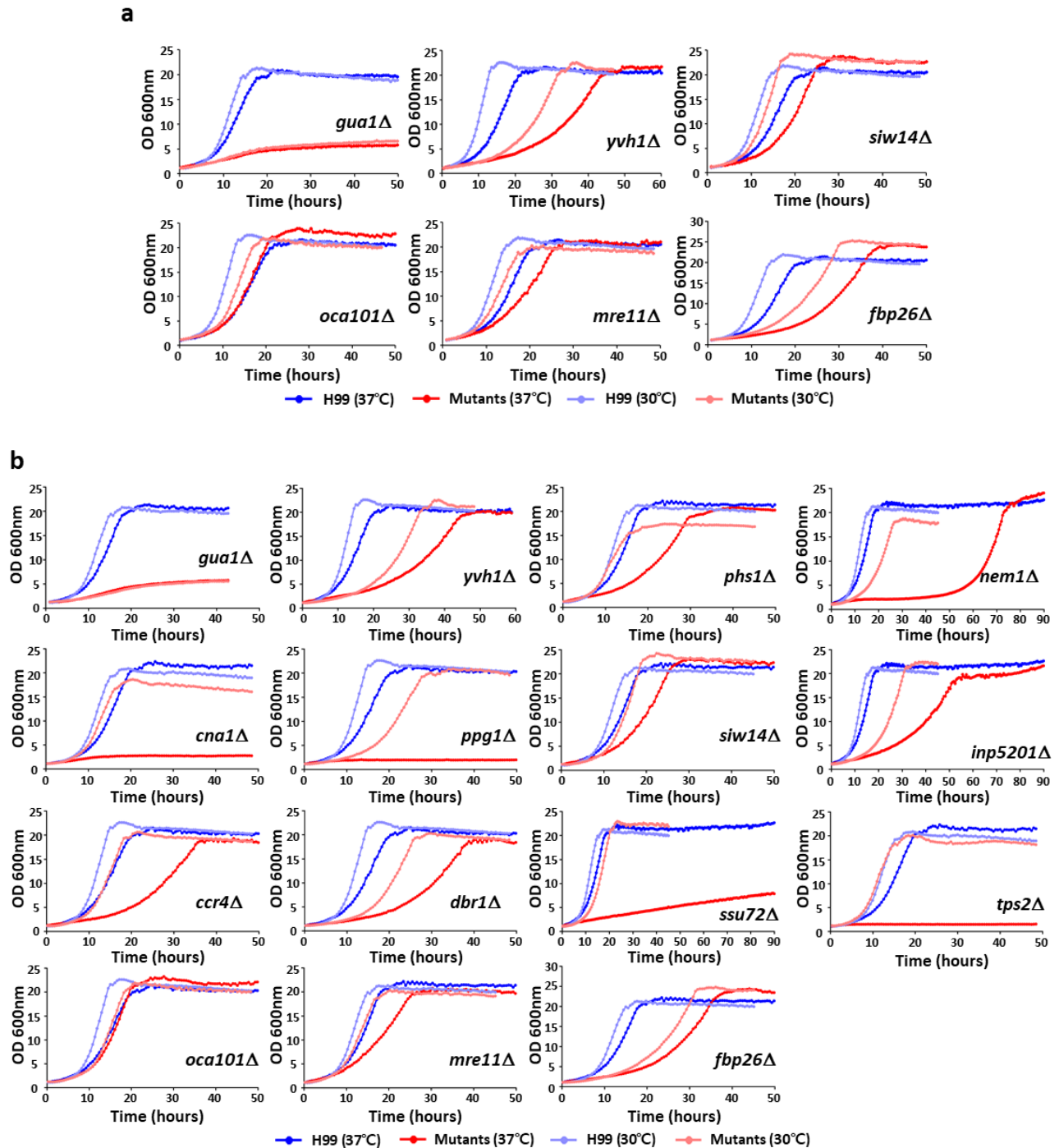
oca101Δ::NAT *oca101Δ::NAT oca1Δ::NEO*
 YSB4075 YSB6634 YSB6635



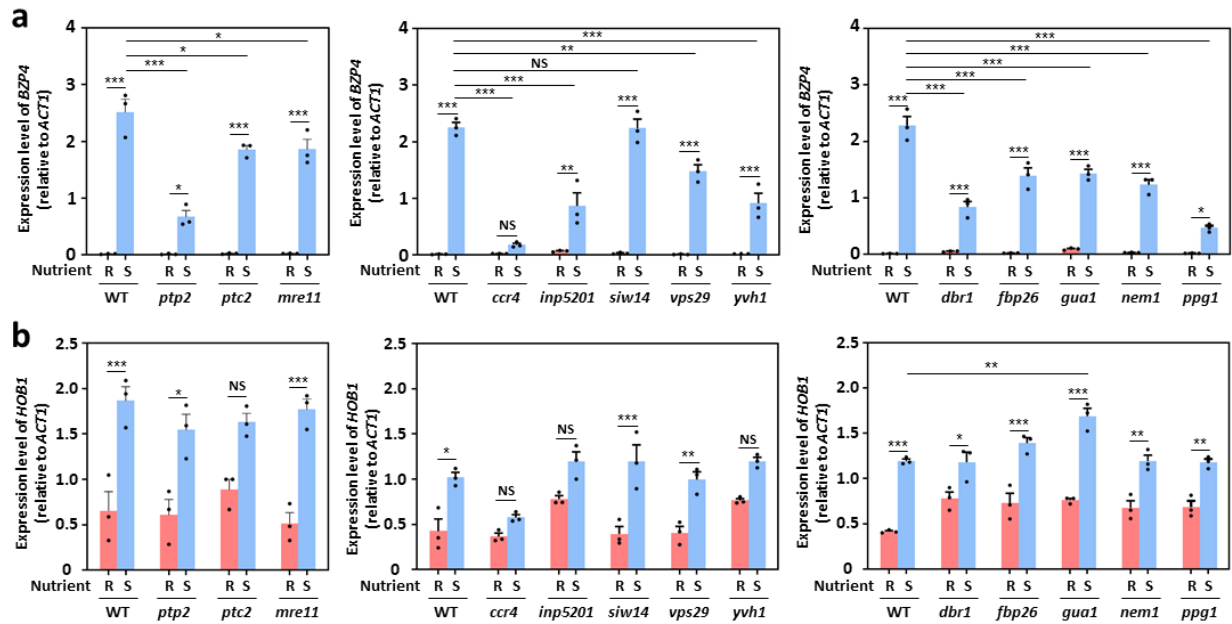
Expected size (KpnI digestion)
 WT : 3070 bp
 Mutant : 3977 bp

c

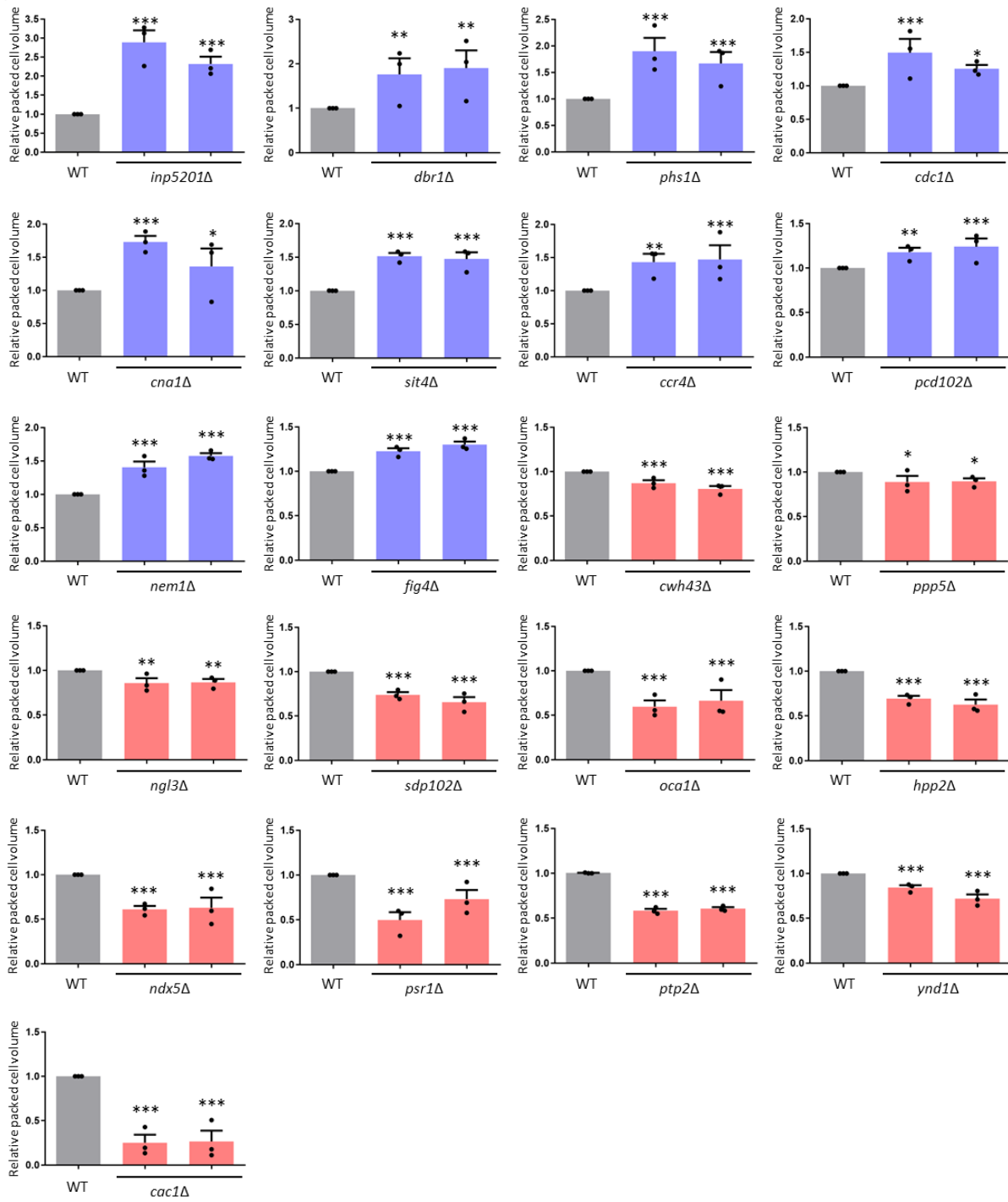
Supplementary Figure 7. *In vitro* phenotypic traits of the *oca1*Δ, *oca101*Δ, and *oca1*Δ *oca101*Δ mutants. (a) Strategy for disruption of the *OCA1* gene in *oca101*Δ mutant strain (YSB4075). (b) Genotype of *oca1*Δ *oca101*Δ mutant strains were confirmed by Southern blot analysis using genomic DNA digested with the restriction enzyme KpnI. The Southern blot analysis was repeated twice and one representative image was shown here. Source data are provided as a Source Data file. (c) Wild-type (WT; H99S), *oca1*Δ, *oca101*Δ, and *oca1*Δ *oca101*Δ mutant strains grown for 16 h at 30°C in liquid YPD medium, serially diluted 10-fold (1 to 10⁴), and spotted on YPD agar media containing the indicated concentrations of the following chemical agents: 0.3 μg per ml tunicamycin (TM), 500 μg per ml flucytosine (5-FC), 13 μg per ml fluconazole (FCZ), 2.5 mM diamide, 0.02 mM menadione, 110 mM hydroxyl urea (HU), 25 μM cadmium sulphate (CdSO₄), or 0.03% sodium dodecyl sulphate (SDS). Cells were incubated at 30°C or indicated temperature and photographed after 2–3 days. This is the representative of more than three biologically independent experiments.

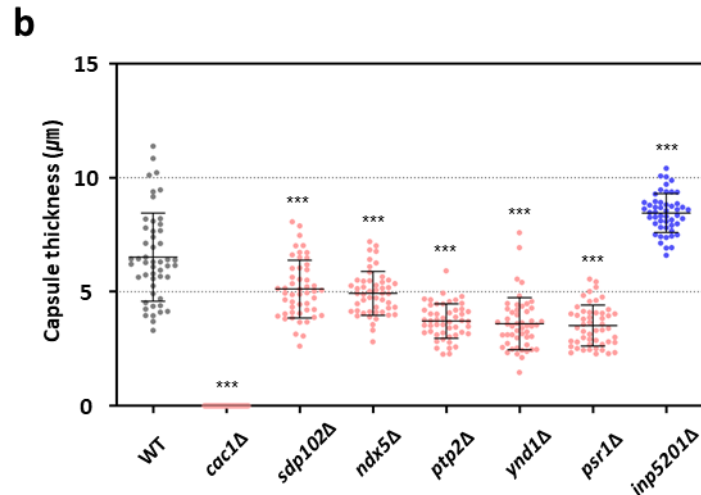


Supplementary Figure 8. Growth curves of phosphatase mutants defective in growth at mammalian body temperature. (a-b) and Figure 4a show the growth rates of the temperature sensitive phosphatase mutants from two biologically independent experiments. *Cryptococcus neoformans* wild-type (WT) strain (H99S) and phosphatase mutants were cultured at 30°C for 16 h and then inoculated into fresh liquid YPD medium ($OD_{600nm} = 0.2$). Cells were then incubated at 30°C or 37°C in a multi-channel bioreactor (Biosan Laboratories, Inc., Warren, MI), and OD_{600nm} was automatically measured for 40 to 90 h.

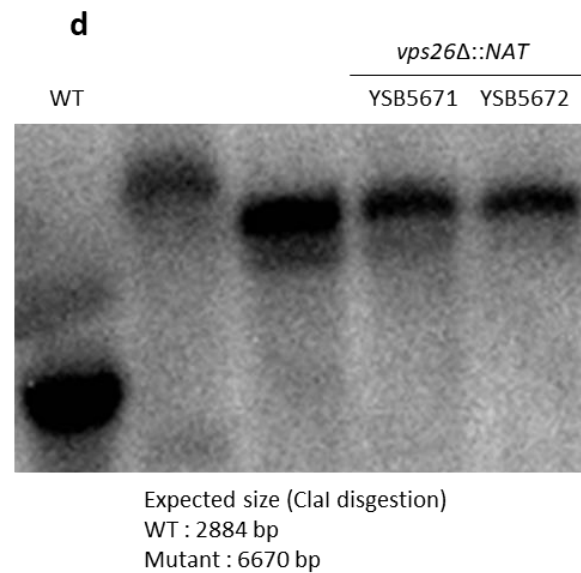
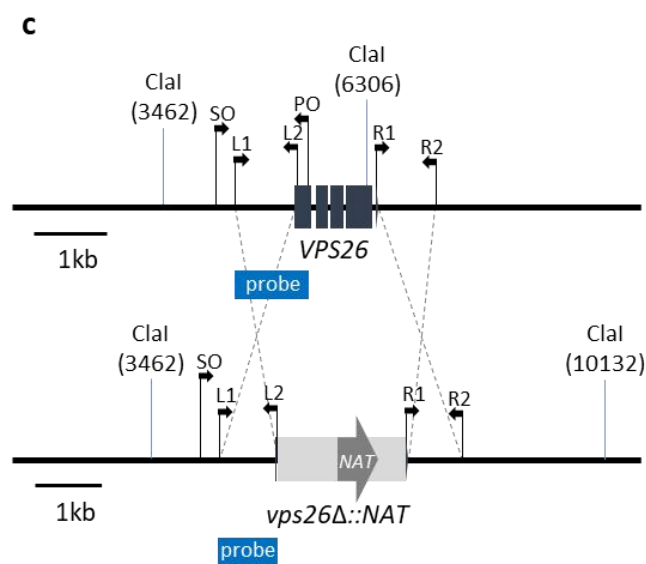
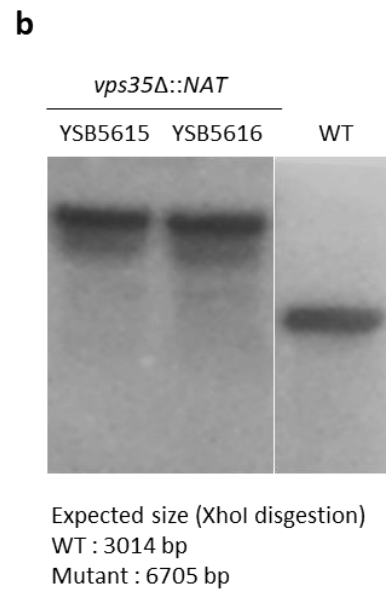
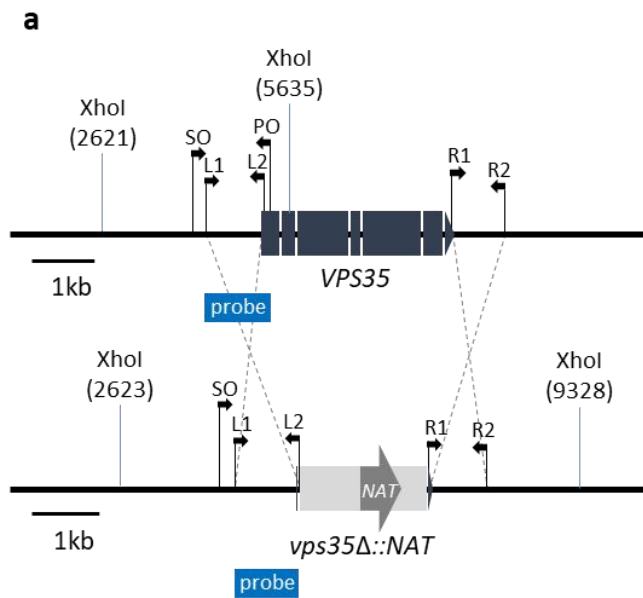


Supplementary Figure 9. Expression levels of known melanin-regulating genes in phosphatase mutants. (a-b) Gene expression (qRT-PCR) of melanin-regulating genes *BZP4*, and *HOB1* in both nutrient-rich (R) and nutrient-starvation (S) conditions. RNA was extracted from three biological replicates with three technical replicates of wild-type and melanin-regulating phosphatase mutants; expression was normalised to *ACT1*. Statistical significance was calculated by one-way ANOVA analysis with Bonferroni's multiple comparison test. Data are presented as mean values $\pm$ standard error of mean (SEM) (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$).

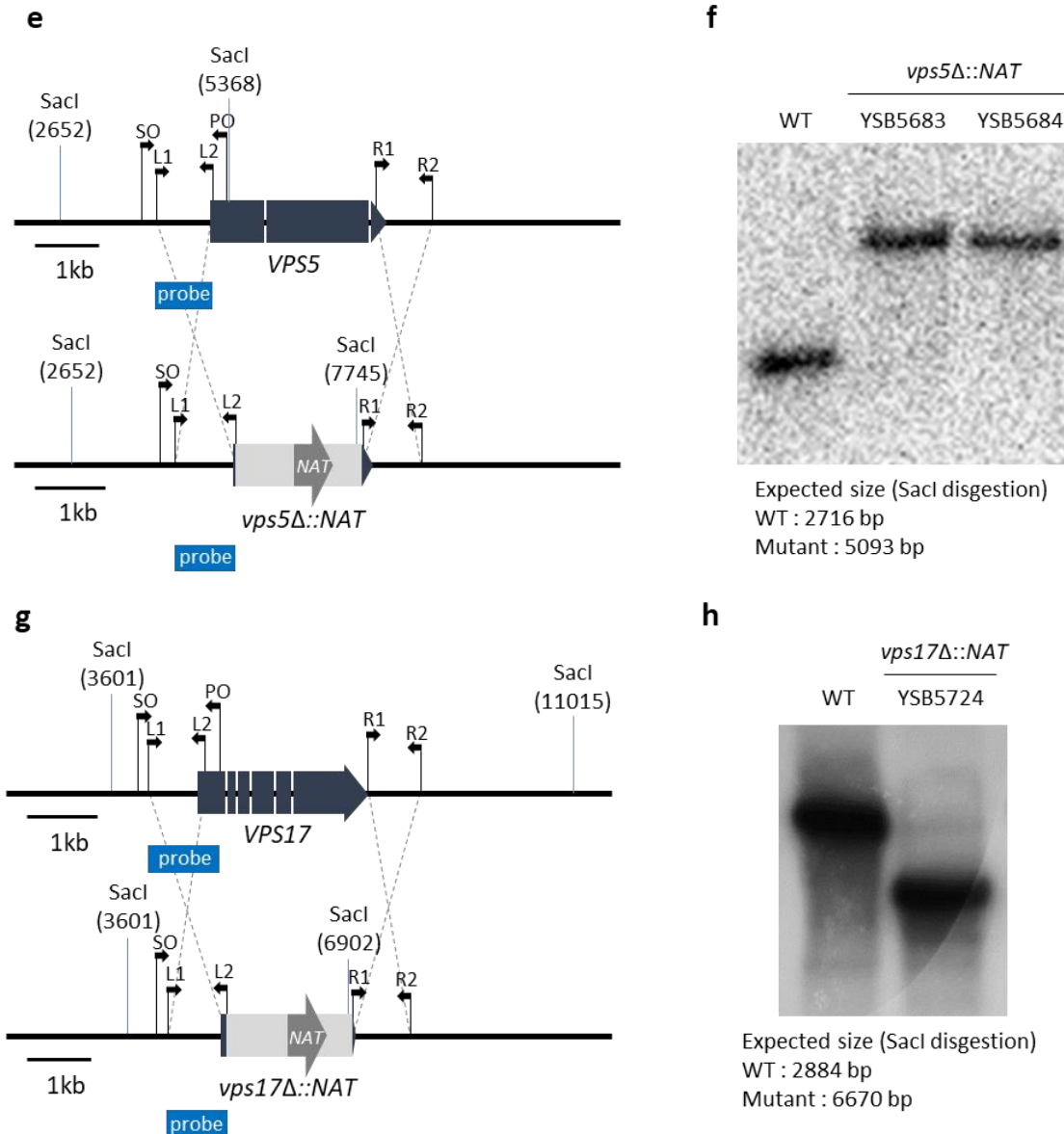
a*Continued*



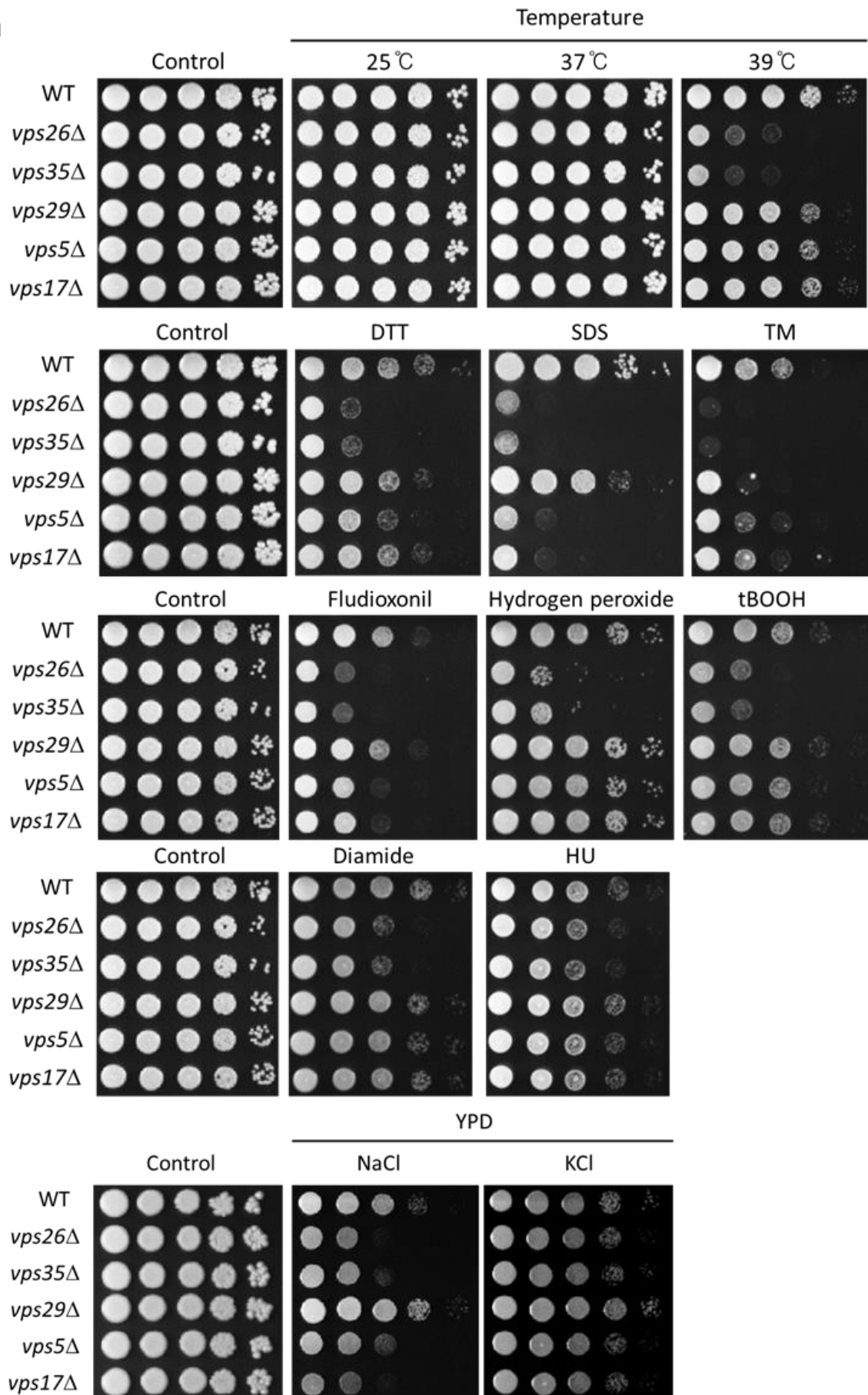
Supplementary Figure 10. Capsule-regulating phosphatases in *C. neoformans*. (a) Each phosphatase mutant was cultured for 16 h at 30°C, spotted onto Dulbecco's Modified Eagle's (DME) agar medium, and incubated at 37°C for 2 days to induce capsule production. Cells were then scraped and fixed by 10% formalin. Fixed cells were adjusted to 3×10^8 cells per ml, and 50 μ l of the cell suspension was injected into microhaematocrit capillary tubes (Kimble Chase, Rockwood, TN). Packed cell volume ratio (packed cell phase / total phase) was measured and the relative packed cell volume of each mutant was calculated by normalizing each ratio with packed cell volume ratio of wild-type (WT) H99S strain. Statistical differences in relative packed cell volume ratios were determined by one-way ANOVA analysis with Bonferroni's multiple comparison test. Data are presented as mean values $\pm$ standard error of mean (SEM) (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$). Each data plot indicates the three biologically independent experiments with three technical replicates. (b) Capsule production assay was performed using capsule-inducing media (Littman's agar medium). To measure capsule thickness (total diameter – cell body diameter), 50 cells were measured from the wild-type (WT) strain and 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 $\pm$ standard deviation (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$). This graph is representative of more than three independent experiments.

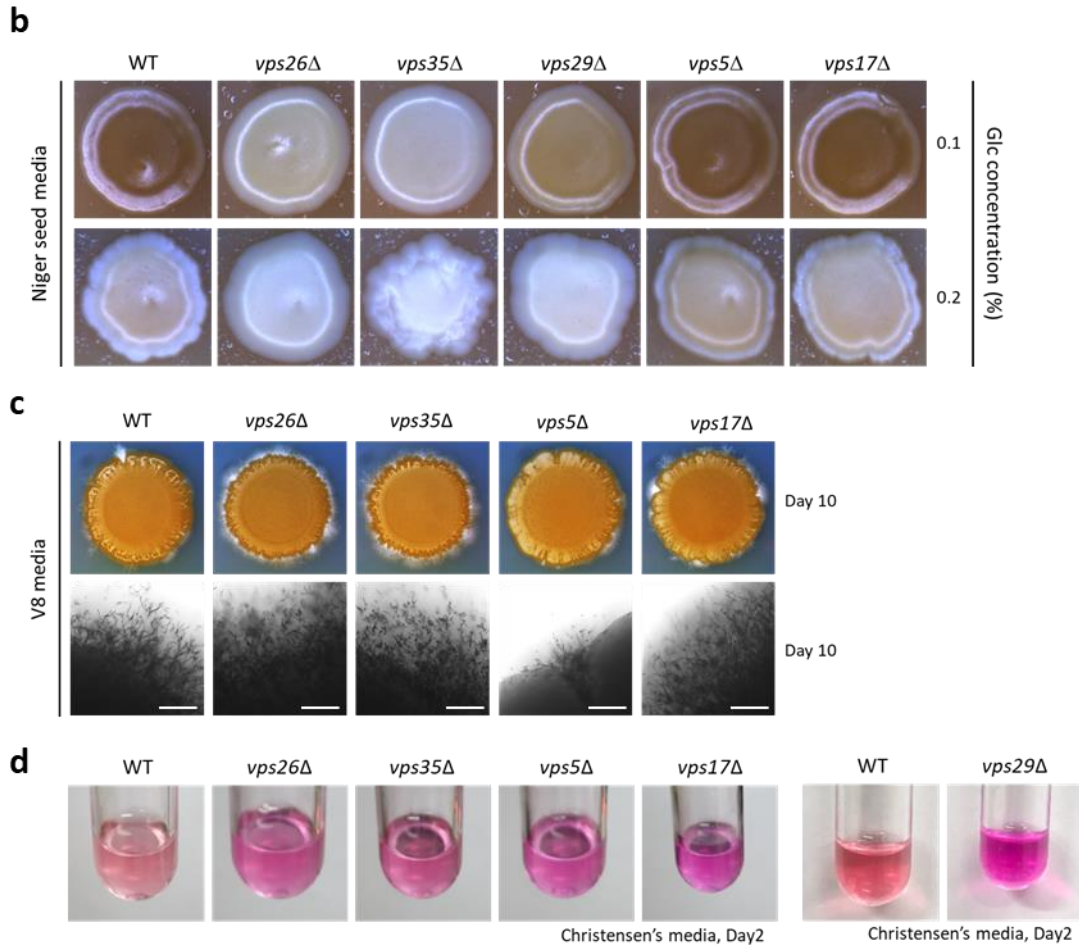


Continued

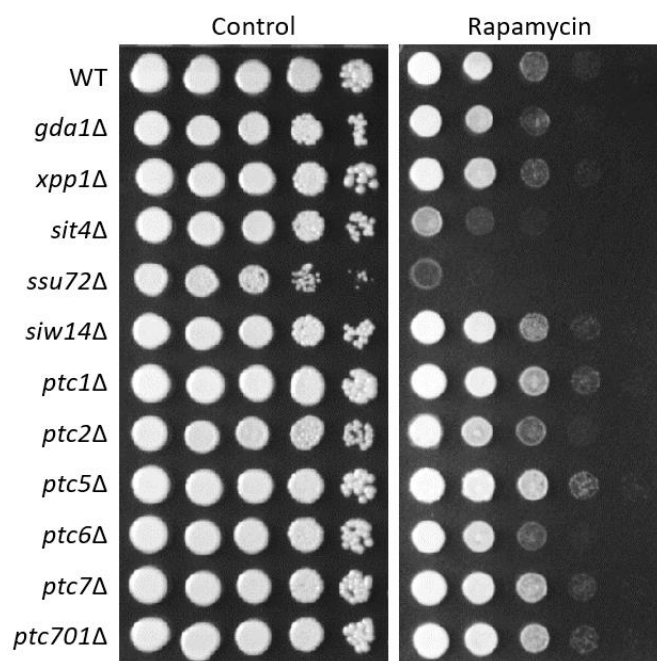


Supplementary Figure 11. Construction of retromer mutant strains. (a) Strategy for disruption of the *VPS35* gene in *C. neoformans* H99S. (b) Genotype of *vps35Δ* mutant strains were confirmed by Southern blot analysis using genomic DNA digested with the restriction enzyme *Xho*I. The Southern blot analysis was repeated twice and one representative image was shown here. (c) Strategy for disruption of the *VPS26* gene in *C. neoformans* H99S. (d) Genotype of *vps26Δ* mutant strains were confirmed by Southern blot analysis using genomic DNA digested with the restriction enzyme *Cla*I. The Southern blot analysis was repeated twice and one representative image was shown here. (e) Strategy for disruption of the *VPS5* gene in *C. neoformans* H99S. (f) Genotype of *vps5Δ* mutant strains were confirmed by Southern blot analysis using genomic DNA digested with the restriction enzyme *Sac*I. The Southern blot analysis was repeated twice and one representative image was shown here. (g) Strategy for disruption of the *VPS17* gene in *C. neoformans* H99S. (h) Genotype of *vps17Δ* mutant strain was confirmed by Southern blot analysis using genomic DNA digested with the restriction enzyme *Sac*I. The Southern blot analysis was repeated twice and one representative image was shown here. Source data are provided as a Source Data file.

a



Supplementary Figure 12. *In vitro* phenotypic traits of retromer complex. (a-d) All data were representative of more than three biologically independent experiments. (a) Wild-type (WT) H99S strain and *vps29Δ*, *vps26Δ*, *vps35Δ*, *vps5Δ*, and *vps17Δ* mutants were grown for 16 h at 30°C in liquid YPD medium, serially diluted 10-fold (1 to 10⁴), and spotted on YPD agar media containing the indicated concentration of the following chemical agents: 0.3 μg per ml tunicamycin (TM), 500 μg per ml flucytosine (5-FC), 13 μg per ml fluconazole (FCZ), 2.5 mM diamide, 0.02 mM menadione, 110 mM hydroxyl urea (HU), 25 μM cadmium sulphate (CdSO₄), 0.03% sodium dodecyl sulphate (SDS), 1 μg per ml fludioxonil, 3.5 mM hydrogen peroxide, 0.7 mM *tert*-butyl hydroperoxide (tBOOH), 1.5 M KCl (1 M KCl in YP medium), or 1.5 M NaCl (1 M NaCl in YP medium). Cells were incubated at 30°C or the indicated temperature and photographed. (b) Retromer mutant strains were grown for 16 h at 30°C in liquid YPD medium and washed with PBS. Each strain was spotted on Niger seed media containing 0.1% or 0.2% glucose, incubated at 37°C, and photographed after 1–4 days. (c) Retromer mutant strains and the *MATa* (KN99) strain were cultured in YPD medium for 16 h at 30°C, washed twice with phosphate-buffered saline (PBS), mixed at equal concentrations (10⁷ cells per ml), spotted on V8 mating media (pH 5), and incubated at room temperature in the dark for 7 to 14 days. Filamentous growth was observed and photographed weekly. Scale bars, 0.25 mm. (d) Retromer mutant strains were cultured at 30°C for 16 h, washed with PBS, and an equal number of cells (10⁶ cells) was inoculated onto liquid Christensen's media in a 10-ml medical tube (SPL Life Sciences, Gyeonggi, Korea). The tubes were incubated at 30°C in a shaking incubator for 1–3 days and photographed daily.



Supplementary Figure 13. Rapamycin susceptibility of *gda1Δ*, *xpp1Δ*, *sit4Δ*, *ssu72Δ*, *siw14Δ*, and PP2C (Type 2C protein phosphatases) mutants. Wild-type (WT) H99S strain and *gda1Δ*, *xpp1Δ*, *sit4Δ*, *ssu72Δ*, *siw14Δ*, and PP2C mutants (*ptc1Δ*, *ptc2Δ*, *ptc5Δ*, *ptc6Δ*, *ptc7Δ*, and *ptc701Δ*) were grown for 16 h at 30°C in liquid YPD medium, serially diluted 10-fold (1 to 10⁴), and spotted on YPD agar media containing 10 ng per ml rapamycin. Cells were incubated at 30°C and photographed after 6 days. This is representative of three biologically independent experiments.

References

- 1 Jung, K. W. *et al.* Systematic functional profiling of transcription factor networks in *Cryptococcus neoformans*. *Nat Commun* **6**, 6757 (2015).
- 2 Lee, K. T. *et al.* Systematic functional analysis of kinases in the fungal pathogen *Cryptococcus neoformans*. *Nat Commun* **7**, 12766 (2016).
- 3 Cheon, S. A. *et al.* Unique evolution of the UPR pathway with a novel bZIP transcription factor, Hx11, for controlling pathogenicity of *Cryptococcus neoformans*. *PLoS Pathog* **7**, e1002177 (2011).
- 4 Jung, K. W., Kim, S. Y., Okagaki, L. 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).