Systematic functional profiling of transcription factor networks in *Cryptococcus neoformans*

Kwang-Woo Jung1,*, Dong-Hoon Yang1,*, Shinae Maeng1, Kyung-Tae Lee1, Yee-Seul So1, Joohyeon Hong2, Jaeyoung Choi3, Hyo-Jeong Byun1, Hyelim Kim1, Soohyun Bang1, Min-Hee Song1, Jang-Won Lee1, Min Su Kim1, Seo-Young Kim1, Je-Hyun Ji1, Goun Park1, Hyojeong Kwon1, Suyeon Cha1, Gena Lee Meyers1, Li Li Wang1, Jooyoung Jang1, Guilhem Janbon4, Gloria Adedoyin5, Taeyup Kim5, Anna K. Averette5, Joseph Heitman5, Eunji Cheong2, Yong-Hwan Lee3, Yin-Won Lee3 & Yong-Sun Bahn1

*Cryptococcus neoformans* causes life-threatening meningoencephalitis in humans, but its overall biological and pathogenic regulatory circuits remain elusive, particularly due to the presence of an evolutionarily divergent set of transcription factors (TFs). Here, we report the construction of a high-quality library of 322 signature-tagged gene-deletion strains for 155 putative TF genes previously predicted using the DNA-binding domain TF database, and examine their *in vitro* and *in vivo* phenotypic traits under 32 distinct growth conditions. At least one phenotypic trait is exhibited by 145 out of 155 TF mutants (93%) and ~85% of them (132/155) are functionally characterized for the first time in this study. The genotypic and phenotypic data for each TF are available in the *C. neoformans* TF phenome database (http://tf.cryptococcus.org). In conclusion, our phenome-based functional analysis of the *C. neoformans* TF mutant library provides key insights into transcriptional networks of basidiomycetous fungi and human fungal pathogens.
Cryptococcus neoformans is a basidiomycete fungal pathogen that causes meningocencephalitis—mainly in immunocompromised populations—and is responsible for more than 600,000 deaths annually worldwide. However, limited therapeutic options are available for treating cryptococcosis, and a complete understanding of diverse biological aspects of Cryptococcus is urgently required for developing novel therapeutic targets and methods. To this end, the signalling cascades governing the general biology and pathogenicity of C. neoformans have been extensively studied over the past decades. This has allowed us to understand several key metabolic characteristics of C. neoformans through homologous recombination methods for 155 putative transcription factor mutant collection.

To understand C. neoformans TF networks on a global scale, we constructed a high-quality gene-deletion collection through homologous recombination methods for 155 putative C. neoformans TFs previously predicted to contain DNA-binding domains (DBDs). The TF mutant strains are analysed for 30 distinct in vitro phenotypic traits, which cover growth, differentiation, stress responses, antifungal resistance and virulence-factor production. Moreover, we also performed a large-scale virulence test using an insect host model and signature-tagged mutagenesis (STM) scoring in a murine host model. This comprehensive phenotypic data set (phenome) of the TFs, which can be accessed online through the Cryptococcus Transcription Factor Phenome Database (http://tf.cryptococcus.org/) provides a unique opportunity to understand general biological features of C. neoformans and identifies novel putative pathways that could be targeted for the treatment of cryptococcosis. This TF mutant collection and its phenome data are a valuable resource for those studying Cryptococcus and the general fungal research community.

**Results**

**Cryptococcus transcription factor mutant collection.** We first selected putative TFs using the published DBD TF prediction database (http://www.transcriptionfactor.org/). The C. neoformans H99 strain, a serotype A platform strain, contains 188 TFs (148 predicted from Pfam and 96 from SUPERFAMILY). Because these TFs were predicted based on the first version of the annotated H99 genome database, we updated this database with reference to the most recent version of the annotated H99 genome database, which resulted in a final prediction of 178 TFs (Supplementary Data 1). Orthologue mapping based on the BLAST e-value matrix demonstrated that C. neoformans contains several evolutionarily distinct groups of TFs (Supplementary Data 3). The Cryptococcus DBD TFs were classified based on their DBDs (Fig. 1a). Nearly 44% of these TFs (78) contain a fungal Zn2-Cys6 DBD, and among these, 40 also harbour a fungal-specific TF domain. Several TFs contain more than two TF domains (Supplementary Data 1).

To analyse the functions of the TFs, we deleted 155 putative TF genes out of 178 using homologous recombination. To perform a large-scale virulence test, dominant nourseothricin-resistance markers (NATs) containing a series of signature tags of distinct oligonucleotide sequences were employed (Supplementary Data 2). The genotypes of all TF mutants were confirmed by performing Southern blot analysis to verify both the gene deletion and the absence of any ectopic integration of each gene-disruption cassette. To accurately validate the phenotype and exclude unlinked mutational effects, we generated more than two independent TF mutants for all 155 TFs, including 4 TFs (HXL1, ATFI, MBSI and SKN7) that we previously reported10–13, and thus obtained a total of 322 strains. For parallel in vitro and in vivo phenotypic analysis, we deleted 53 TF genes, which were previously deleted in the CM018 strain (a less virulent H99 strain)10, and derived more than two independent mutants. Certain known TFs, including RIM101, ADA2, CUF1, SX11, SP-1/CZ1, NRG1, STE12, BW2C, SRE1, ZNF2 and HAP1/HAP2, were also independently deleted here to accurately compare phenotypes. When two independent TF mutants showed inconsistent phenotypes because of inter-isolate inconsistency, additional TF mutants were generated to exclude outlier mutants. We found that about 8% of gene knockouts (13 TFs) exhibited inconsistent phenotypes, potentially attributable to undetectable mutational artefacts or unexpected alterations in the genome (Supplementary Data 4). This level is highly similar to that reported in a similar study on the ascomycete fungal pathogen Candida albicans11. For the remaining 23 TFs, we could not generate TF mutants. Among these TFs, 6 (ESA1, CEF1, CDC39, RSC8, HSF1 and PZF1) are orthologous to yeast TFs that are essential for growth of Saccharomyces cerevisiae. The remaining 17 TF genes could not be deleted even after repeated attempts at gene disruption and thus they are presumably to be critical or essential for growth in C. neoformans. However, CIR1, MIG1, CNAG_06252 and CNAG_04798 have been successfully deleted previously10,12,13, suggesting that these TFs could be deleted through additional efforts in the future. In summary, we successfully constructed a C. neoformans TF mutant collection that covers 155 TFs and 322 TF mutant strains in total (Fig. 1b).

Out of the 155 TFs whose mutants were constructed, 57 TF genes possess names designated in published studies or reserved by other researchers through registration in FungiDB (www.fungi-db.org). For the remaining 98 TFs, we provided gene names by following the systematic genetic nomenclature flowchart in C. neoformans recently reported by Inglis et al.14 (Supplementary Data 1).

**Phenotypic profiling of the Cryptococcus TF mutant library.** For the 322 TF mutants constructed, we performed a series of phenotypic analyses for the following phenotypic classes: growth, differentiation and morphology, stress responses, antifungal drug resistance, virulence-factor production and in vivo virulence (Fig. 1b). This overall phenome data set generated for the TF mutant collection is illustrated together with a colour scale in the phenome heat map in Fig. 2 and Supplementary Data 4. Data for transcript levels of each TF measured by RNA sequencing analyses under six distinct growth conditions were obtained from a recent H99 genome analysis report8 and also demonstrated as a heat map (Fig. 2; Supplementary Data 4). The phenotypic analysis revealed that about 93% of the TF mutants (145/155) exhibited at least one discernable phenotype, suggesting a high functional coverage of this TF mutant collection. Almost 85% of the TFs
(132/155) have not been functionally characterized before in *C. neoformans*. All of these phenome data are publicly available in the *Cryptococcus neoformans* TF database (http://tf.cryptococcus.org).

**Figure 1 | Overview of Cryptococcus neoformans transcription factors and strategies for their systematic deletion and phenome-based analysis.** (a) Pie chart showing the class and distribution of *C. neoformans* TFs. Each TF was classified based on the DBDs predicted using Superfamily (http://www.supfam.org/SUPERFAMILY/) and Pfam (http://pfam.xfam.org/) databases or Cryptococcus genome database (http://www.broadinstitute.org). Certain TFs contain multiple DBDs (Supplementary Data 1). (b) Flowchart of the construction of the *C. neoformans* TF mutant library and *in vitro* and *in vivo* phenome-based analyses.
to high (37–39 °C). Deletion of some TFs (BZP2, CUF1, LIV4, GAT5, FZC6 and NRG1) resulted in temperature-independent growth defects (Supplementary Fig. 1). The growth defect of the cuf1Δ mutant was due to its inability to uptake copper, because external addition of CuSO4 restored its wild-type (WT) growth (Supplementary Fig. 2). Deletion of the following group of TFs caused growth defects only at high temperature (37–39 °C): HXL1, CRZ1, ATF1, ADA2, HAP1, ARO80, USV101, FZC31, FZC30, FZC1, MIZ1, APN2, GAT6, MBS2, SRE1 and ERT1 (Supplementary Fig. 1). Among these, only HXL1, which is a TF downstream of the Ire1 kinase in the UPR pathway, exhibited a severe growth defect at host physiological temperature. The hob1Δ and hlh3Δ mutants showed WT growth at 30 °C but exhibited growth defects at 25 °C or high temperature. By contrast, deletion of MLN1, MCM1 and FZC46 promoted the growth of C. neoformans at 39 °C. Collectively, these results suggest that multiple TFs (total 27 TFs) control—both positively and negatively—the growth and thermostolerance of C. neoformans.

In a natural environment, C. neoformans exists mainly in the yeast form but undergoes either bisexual differentiation with cells of the opposite mating type or unisexual differentiation with cells of the same mating type to produce filamentous forms and generate infectious basidiospores. These developmental processes contribute to the generation of the genetic diversity of the pathogen. Our systematic analysis revealed that 37 TFs were involved in mating (Fig. 3; Supplementary Fig. 3). Among the novel mating-regulating TFs discovered in this study, deletion of BZP2, USV101, FZC1 and ZAP104 severely reduced mating, even in unilateral mating, whereas deletion of HLH1, HAP2 and GAT1 highly enhanced mating efficiency (Fig. 3a). To determine the mating steps in which these TFs are involved, we measured the efficiency of cell fusion and pheromone production, which precede the filamentation step. The bzp2Δ, usv101Δ, fzc1Δ and zap104A mutants lacked the ability to engage in cell fusion with the MATa control strain (Fig. 3b) and also failed to induce pheromone gene (MFx1) expression upon mating (Fig. 3c), suggesting that Bzp2, Usv101, Fzc1 and Zap104 promote pheromone gene expression, which results in a subsequent increase in cell fusion. Conversely, in the hlh1Δ, haph2Δ and gat1Δ mutants, cell-fusion efficiency was increased two- to threefold (Fig. 3b) and pheromone gene expression was highly enhanced (Fig. 3c), suggesting that Hlh1, Hap2 and Gat1 are negative regulators of pheromone gene expression. By contrast, SKN7, whose deletion promoted mating (Fig. 3a), was dispensable for both pheromone gene expression and cell fusion (Fig. 3b,c), indicating that it is likely involved in a later stage of mating. In summary, numerous TFs are involved in the different mating steps of C. neoformans, demonstrating that multiple signalling cascades modulate its development.

TFs modulating virulence-factor production in C. neoformans.

To support survival and proliferation within the host, C. neoformans is armed with several virulence factors, which include capsule and melanin. Capsule is a glucuronoxylomannan-or galactoxylomannan-based polysaccharide that protects cells from being phagocytosed by host phagocytic cells. Melanin, a black-brown pigment made of polyphenol complexes, confers both antiphagocytic and antioxidative activity to cells.

Our systematic analysis identified 49 TFs (20 positive and 29 negative regulators) involved in capsule production (Fig. 4a; Supplementary Fig. 4). In addition to previously reported capsule-regulating TFs, such as Ada2 (ref. 18), Gat201 (ref. 10), Atf1 (ref. 8) and Mbs1 (ref. 7), we identified several novel capsule-regulating TFs in this study. The zap104Δ, yap1Δ and rds2Δ mutants also exhibited severely reduced capsule production, on par with the extent observed in the gat201Δ and ada2Δ mutants (Fig. 4a). By contrast, deletion of HOB7, CLR3 and FZC51 greatly enhanced capsule production (Fig. 4a).

Our analysis also uncovered 27 TFs (11 positive regulators and 16 negative regulators) involved in melanin production (Fig. 4b; Supplementary Fig. 5). A few of these TF, including Cuf1, Ste12, Mbs1, Skn7 and Atf1, have been reported previously. In addition, we found that the fzc8Δ, hob1Δ and bmp4Δ mutants exhibited greatly reduced melanin production; the reduction was similar to that of the cuf1Δ mutant. Our results show that Hob1 and Fzc8 promote the expression of LAC1, which is the major laccase involved in melanin synthesis, under glucose-starvation conditions, whereas Bmp4 and Cuf1 are not directly involved in LAC1 expression (Fig. 4c). By contrast, the deletion of HLH1, HLH2, YAP1 and FZC1 greatly enhanced melanin production, although only Hlh1 negatively regulated LAC1 expression (Fig. 4c).

Another crucial virulence factor is urease, a nickel-dependent protein complex (Ure1, Ure4, Ure6 and Ure7) that converts urea into ammonia, which serves as a nitrogen source. Urease is necessary for sequestration of the pathogen in brain microcapillary beds and crossing the blood–brain barrier through the disruption of tight junctions. We determined that 19 TFs are involved in either positively or negatively regulating urease production (Supplementary Fig. 6). In summary, our systematic analysis identified a plethora of novel TFs involved in the production of virulence factors in C. neoformans.

TFs modulating antifungal drug and stress responses. For the treatment of cryptococcosis, amphotericin B (AmpB) with or without fluconitne (5-FC) and fluconazole (FCZ) are widely utilized. However, in addition to the toxic side effects of such drugs, the emergence of antifungal drug-resistant Cryptococcus strains has caused serious clinical problems. To identify any TFs involved in antifungal drug resistance, we monitored the alteration of antifungal drug susceptibility among the C. neoformans TF mutant strains. Numerous TFs were found to be involved in antifungal drug resistance (Supplementary Table 1), implying that Cryptococcus can potentially adapt to current antifungal drugs in versatile manners. In response to FCZ, mutants of 55/155 TFs (35.5%) exhibited either increased susceptibility (35 TFs) or resistance (20 TFs), suggesting that azole resistance could readily occur through the modulation of diverse TFs. However, in response to AmpB, mutants of 55 genes exhibited differential susceptibility, with most (47 TFs) showing increased susceptibility and only 8 TF mutants exhibiting increased resistance (Supplementary Table 1). These data support the clinical observation that compared with azole-resistant C. neoformans mutants.
resistance, polyene resistance is rarely observed. Furthermore, supporting the observation that the 5-FC readily elicits the development of drug-resistant strains, our results showed that 27 TFs differentially regulate flucytosine resistance (Supplementary Table 1).

We noted that the deletion of some TFs regulated azole and polyene susceptibility in an opposite manner (Fig. 5a; Supplementary Table 1), possibly because these might directly control ERG11 expression and sterol biosynthesis and affect polyene-binding capacity. Two of these TFs were previously reported to be Erg11 regulators. Sre1, a key sterol regulatory TF, negatively regulates basal ERG11 expression and therefore its deletion increases azole resistance but decreases polyene resistance in C. neoformans. To further test whether other TFs are also involved in ERG11 regulation, we measured ERG11 expression levels in these TF mutants under both sterol-replete and -depleted conditions (Fig. 5b). As expected, basal and induced ERG11 levels were substantially lower in the sre1Δ mutant than in the WT strain. Notably, deleting HOB1 markedly increased the basal expression levels of ERG11. To determine whether Hob1 is involved in the regulation of other ERG genes, we monitored the expression of ERG2, ERG3, ERG5 and ERG25 in the WT, hob1Δ and sre1Δ strains under sterol-replete and -depleted conditions. The expression of all of these ERG genes was induced in response to sterol depletion through FCZ treatment in the WT strain, but not in the sre1Δ strain (Fig. 5c). Deletion of HOBI markedly induced the basal expression of ERG2 (Fig. 5c). By contrast, under sterol depletion, the induction of ERG2, ERG3, ERG5, ERG11 and ERG25 was decreased in the hob1Δ mutant (Fig. 5c). The tight regulation of ERG expression appeared to be mostly absent in the hob1Δ mutant, indicating that Hob1 is a key regulator of ergosterol gene expression.

Notably, the TFs involved in sterol biosynthesis also appeared to be involved in environmental stress responses and adaptation, which are critical for the survival and proliferation of C. neoformans within the host because the pathogen encounters drastic environmental changes during infection. Reflecting diverse types of external stresses, 145 TFs were identified to be involved in sensing and responding to at least one type of stress (Fig. 2; Supplementary Fig. 7). Among these TFs, the two sterol regulators, Sre1 and Hob1, appeared to be general stress-responsive TFs that govern multiple stress responses and adaptations. Strikingly, the deletion of HOBI or SREI substantially reduced resistance to osmotic/salt, oxidizing/reducing, genotoxic, endoplasmic reticulum (ER) and cell wall/membrane stresses (Fig. 5d). The hob1Δ and sre1Δ mutants exhibited similar stress resistance/susceptibility patterns under
most of the tested environmental stresses, and this is in stark contrast to their opposite resistance patterns towards FCZ and AmpB; this result strongly suggested that sterol homeostasis is critical for controlling stress response and adaptation in *C. neoformans* (Fig. 5e).

**TFs affecting infectivity and virulence of *C. neoformans***

Identification of TFs required for the pathogenicity of *C. neoformans* is critical for future development of novel antifungal drugs and therapeutic methods. Here, we employed two large-scale assays: (1) a virulence assay conducted in the invertebrate insect larval model system *Galleria mellonella*; and (2) a STM-based infectivity assay conducted in a murine inhalation model. These assays using one insect host and one mammalian host model have been widely adopted for large-scale virulence assays: (1) a virulence assay conducted in the invertebrate insect larval model system *Galleria mellonella*, and (2) a STM-based infectivity assay conducted in a murine inhalation model. These assays using one insect host and one mammalian host model have been widely adopted for large-scale virulence assays in other fungi as well as *C. neoformans*.10

Using the STM-based murine infectivity assay, we identified 40 virulence genes (Fig. 6b; Supplementary Fig. 9; Supplementary Table 2). The STM score for each mutant was calculated based on the quantitative PCR score = Log2(output/input) in the lung from the sacrificed mice (average score from three mice). Among all the sets studied, the *ire1*Δ mutant, which is a non-virulent control strain, exhibited a highly reduced STM score (−7.05 ± 1.49), whereas the *ste50Δ* mutant, a virulent control strain, showed an STM score of 0.52 ± 1.07. Further supporting the quality of the STM assay, 11/40 TFs identified here were previously reported to be involved in virulence9,10,30. The *gat201Δ* and *pdr802Δ* mutants exhibited drastically reduced STM scores (−11.125 and −7.212, respectively) as reported10. Similarly, the STM scores of the *zap104*Δ and *liv1*Δ mutants were also decreased (−5.528 and −3.875, respectively)10. Furthermore, the *hxl1Δ*, *nrg1*Δ and *bw2c*Δ mutants also showed highly reduced STM scores. The 11/40 TFs identified by the STM analysis (*GAT201*, *PDR802*, *HXL1*, *BWC2*, *NRG1*, *FZC1*, *FZC50* and *FZC31*) significantly reduced the virulence of *C. neoformans*.

Using the STM-based murine infectivity assay, we confirmed that 17 TF genes are involved in the virulence of *C. neoformans* (Fig. 6a; Supplementary Fig. 8; Supplementary Table 2). The mutants identified include nine TF mutants (*hxl1Δ, ada2Δ, sre1Δ, nrg1Δ, bw2c2Δ, crz1Δ, pdr802Δ, gat201Δ* and *gat204Δ*) that were previously reported to show reduced virulence in a murine model of systemic cryptococcosis9,10,18,27–32. This further indicated a strong correlation between the insect and murine models in terms of the pathogenicity of *C. neoformans*. Besides the deletion of these known TFs, deletion of *HOB1, BZP2, USV101, YAP1, FZC51, FZC1*, *FZC31, FZC50* and *FZC31* significantly reduced the virulence of *C. neoformans*.
The virulence assay data from the insect model were statistically significantly correlated with the STN-based infectivity data from the murine model based on the Pearson correlation coefficient (PCC) analysis (Fig. 7). Among the novel virulence-related TFs that were screened using only the STM-based murine model, the fcz1Δ and ddt1Δ mutants exhibited highly reduced STM scores (−4.328 and −4.832, respectively). Our phenotype database revealed that the fcz1Δ mutant exhibited increased susceptibility to osmotic, oxidative and cell membrane stresses, which might collectively affect virulence. By contrast, the only notable phenotype observed in the case of the ddt1Δ mutant was a weak diithiothreitol (DTT) sensitivity, which is not likely responsible for the marked decrease in the survival of the mutant in the lung because several other DTT-sensitive TF mutants were as virulent as the WT strain. Therefore, Ddt1 is likely to modulate one or more virulence factors of C. neoformans that were not addressed in this study.

To further understand the correlation among phenotypic traits and virulence, we measured the degree of linear dependence by calculating all PCCs between two possible phenotypic combinations tested in this study; these correlations are illustrated in a combined network (Fig. 7). The correlation network revealed that the virulence of C. neoformans is highly correlated to growth at distinct temperatures and osmotic and cell wall/membrane-stress responses and moderately related to oxidative, genotoxic and ER stress responses. Notably, these stress phenotypes were also highly inter-correlated (Fig. 7), suggesting that several core stress signalling networks might exist, including
the known Hog1, Pkc1/Mpk1 and UPR pathways. By contrast, mating and resistance to antifungal drugs, except to resistance to AmpB, were not significantly related to the virulence of *C. neoformans*. The production of virulence factors did not appear to be correlated to *in vivo* virulence, and this is likely because increased virulence-factor production often did not result in increased virulence. Supporting this, reduced capsule production was found to be highly correlated to reduced virulence by PCC analysis (*P* < 0.05).

**Discussion**

In this study, we constructed 322 gene-deletion strains representing 155 TFs to systematically analyse their *in vitro* and *in vivo* phenotypic traits in *C. neoformans*. Although large-scale TF phenome data are available for *S. cerevisiae*, *Schizosaccharomyces pombe*, *C. albicans*, *Candida glabrata*, *Fusarium graminearum* and *Neurospora crassa*[^11][^33][^34][^35][^36], all of these are ascomycetes that phylogenetically diverged from the basidiomycetes at least 500 million years ago[^37], and only a limited number of *C. neoformans* TF genes appear to have phylogenetically relevant orthologues in the ascomycete model fungi. As we excluded general TFs and sequence-nonspecific DNA-binding proteins, this is likely not a complete set of TFs but represents a majority of the sequence-specific DNA-binding TFs present in the *C. neoformans* genome, and as such the data presented here provide comprehensive insights into transcriptional networks in diverse basidiomycetous fungi and human fungal pathogens.

Our *Cryptococcus* TF mutant library and its phenome database have an exceptionally high functional coverage (93%) compared with those performed in other pathogenic ascomycetous fungal species. Homann *et al.*[^11] constructed 166 TF mutants in *C. albicans* and tested their phenotypic traits under 50 different growth conditions and found that over the 50% of them exhibit at

---

[^11]: 11[^11]
[^33]: 33[^33]
[^34]: 34[^34]
[^35]: 35[^35]
[^36]: 36[^36]
[^37]: 37[^37]
least one moderate phenotype. Son et al.\textsuperscript{36} constructed 657 TF mutants in \textit{F. graminearum} and analysed their phenotypic traits under 17 different growth conditions. Interestingly, however, only 26% of them displayed discernible phenotypes\textsuperscript{36}. Schwarzmuller et al.\textsuperscript{35} constructed 619 deletion mutants containing 177 TFs in \textit{C. glabrata} and found that about 32% of them exhibited at least one discernible phenotypic trait. Therefore, our high functional coverage TF phenome analysis not only led to the discovery of novel phenotypic traits of previously characterized TFs, but also identified several novel TFs involved in diverse biological aspects of \textit{C. neoformans}.

Out of \textit{C. neoformans}'s 178 TFs, only a limited number of orthologues are present in the model yeasts and fungi: 53 in \textit{S. pombe}, 68 in \textit{N. crassa}, 82 in \textit{F. graminearum}, 64 in \textit{C. albicans}, 51 in \textit{S. cerevisiae} and 53 genes in \textit{C. glabrata} (Supplementary Data 5). Among them, 22 TFs (Esa1, Top3, Cef1, Rsc8, Pzf1, Mig1, Run1, Jjj1, Ada2, Mbs1, Fab1, Hel2, Gat1, Miz1, Skn7, Mcm1, Liv4, Crz1, Usv101, Cuf1, Mbf1 and Rlm1) are conserved and considered to be core fungal TFs. Supporting their core functions, six of them (Esa1, Top3, Cef1, Rsc8, Pzf1 and Mig1) are essential in \textit{S. cerevisiae} and four of them (Esa1, Cef1, Rsc8 and Pzf1) also appeared to be essential in \textit{C. neoformans}. In this study, we constructed mutants for 16 out of the 22 core TFs and discovered that their phenotypes cover a broad range of biological processes. Strikingly, the finding that nine of them (Jjj1, Ada2, Mbs1, Gat1, Skn7, Mcm1, Usv101, Crz1 and Cuf1) are involved in virulence-factor production (capsule, melanin and urease) and virulence suggests that \textit{C. neoformans} evolved several core TF networks into pathogenicity-related signalling networks.

Common phenotypic traits examined by this and other studies in the systematic analyses of fungal TF networks include development and differentiation. For 103 TFs studied in \textit{N. crassa}, 88 of them have orthologues in the \textit{F. graminearum} genome\textsuperscript{33,36}. Among them, only nine mutants exhibit similar phenotypes in differentiation processes of both fungi. By contrast, only 13 out of 103 \textit{N. crassa} TFs have recognizable orthologues in the \textit{C. neoformans} genome. Interestingly, however, nine of them are involved in differentiation of \textit{Neurospora} and six of them (STE12, ATF1, BWC2, GAT201, HOB7 and SRE1) were shown to play a role in sexual differentiation in \textit{C. neoformans}. Furthermore, 27 of 82 \textit{F. graminearum} TFs, which are highly homologous to \textit{Cryptococcus} TFs, are involved in sexual development of the plant fungal pathogen and 10 of them (Hob7, Hcm1, Znf2, Gat1, Fzc26, Zap104, Ada2, Rum1, Mcm1 and Usv101) also exhibited altered mating response in \textit{C. neoformans}. Therefore, among many phenotypic traits, some genes involved in sexual differentiation appeared to be functionally well conserved among fungi, although each fungus also contains a unique set of TFs to govern its own differentiation processes.

In fact, the first large-scale functional analysis of \textit{C. neoformans} genes has been previously performed by Liu et al.\textsuperscript{10}, who constructed deletion mutants for 1,201 genes, including 58 TFs, and analysed three virulence-related \textit{in vitro} phenotypic traits (capsule, melanin and growth at 37 °C) and \textit{in vivo} pulmonary infectivity. However, their mutant collection consists of only one PCR-confirmed mutant strain for each gene in the background of the CMO18 strain, which is virulence-attenuated, nearly sterile H99 strain\textsuperscript{4,10}. By contrast, our TF mutant collection consists of two independent Southern blot analysis-confirmed mutants for all 155 TFs in the background of the H99S strain that retains full virulence and fertility\textsuperscript{6}. For parallel phenotypic comparison, we reconstructed 53 out of their CMO18 58 TF mutants in the H99S strain background. Due to differences in strain background and experimental conditions, we found only partial overlap between the two \textit{in vitro} phenotypic analyses. Melanin-regulating TFs identified by this study were not discovered by Liu et al. because the CMO18 strain is defective in capsule production and they used different melanin-inducing media. As the two studies also used two different capsule-inducing media, only one TF, Gat201, was commonly found to be highly defective in capsule production by these two studies. Our study identified numerous novel TFs orchestrating production of capsule and melanin. Particularly, we found that Hob1, Fzc8 and Hlh1 are involved in regulation of \textit{LAC1} expression. By contrast, we found a significant overlap in terms of the infectivity phenotype between the two studies.

In this study, we identified novel regulators of sterol biosynthesis in \textit{C. neoformans} in addition to a known sterol regulator, Sre1. In particular, the homeobox protein Hob1 appears to function as a key regulator of sterol biosynthesis by affecting multiple ergosterol biosynthesis genes. Our data suggest that Hob1 must effectively repress sterol biosynthesis genes under sterol-replete conditions. Without this control, cells might not be able to adapt to environmental changes, as indicated by our finding that the \textit{hob1}A mutants exhibited patterns of extreme stress sensitivity to multiple stresses that were similar to the sensitivity patterns exhibited by the \textit{sre1}A mutant. In \textit{S. cerevisiae}, Mot3 (a C2-H2 zinc-finger TF) and Rox1 (an HMG domain TF), which are not homologous to Hob1, play similar repressive roles in sterol biosynthesis in a HOG pathway-dependent manner\textsuperscript{38}, suggesting that sterol biosynthesis in \textit{C. neoformans} appears to be governed by a mechanism that is considerably distinct from that in \textit{S. cerevisiae}.
Another key finding of this study with clinical and pharmaceutical implications is the discovery of an unprecedented number of TFs involved in virulence of *C. neoformans*, including several structurally and functionally unique TFs. Although TFs are considered to be less optimal drug targets in general, it is not impossible to develop TF-specific drugs as witnessed in the development of a STAT4 inhibitor, Lisofoylline, for treating diabetes and STAT3 inhibitors for cancer treatment. In the small-molecule-mediated inhibition of FOXM1 transcriptional programme. Here, we determined that 45 genes (32 novel and 13 known TFs) are involved in pathogenicity of *C. neoformans* based on the insect-based virulence assay and STBM-based murine infectivity assay, which are two complementary assays for large-scale screening of potential virulence factors.

Particularly, the virulence-related TFs confirmed using both approaches warrant particular attention. Besides recognized TFs, these include novel TFs, such as UsV101, Hob1, Fzc1, Fzc31, and Zic2. Virulence defects observed in the *hob1Δ* and *usv101Δ* mutants might result from increased thermosensitivity and severe defects in melanin production and multiple stress responses. By contrast, obvious virulence-related phenotypic traits were not detected in the *fzc2Δ, fzc1Δ* and *fzc31Δ* mutants. The *fzc1Δ* mutant in particular exhibited highly enhanced capsule and melanin production. Therefore, these TFs might function in the production of other virulence factor(s), which could include capsule-independent antiphagocytic factors, phospholipases, inositol uptake and metabolic systems and giant/titan cell formation.

Therefore, an expanded phenotypic profiling of the TF mutant library will further reveal the molecular functions of each virulence-related TF in the pathogenicity of *C. neoformans*.

When virulence assay data in human pathogenic fungi from this and others studies were compared, we found several evolutionarily conserved virulence-regulating TFs, which could be exploited as broad-spectrum antifungal drug targets. When two large-scale virulence analyses of *C. neoformans* and *A. fumigatus* TF mutants were compared, the five TFs (Nrg1, Rim101, Crz1, UsV101 and Zap104) appear to be commonly involved in virulence of the two fungal pathogens. Crz1 is also known to be required for the virulence of *A. fumigatus* and *G. glabrata*. Comparison of our virulence–phenotype correlation network showed that in vivo virulence of *C. neoformans* is highly correlated to stress responses, but not to mating and differentiation. By contrast, morphological switching and differentiation processes appear to be highly correlated with virulence of other fungal pathogens. In *F. graminearum*, sexual development is highly correlated to virulence. In *C. albicans*, Noble et al. identified 115 infectivity-attenuated mutants, about 40% of which exhibits altered morphological switching and proliferation.

Sexual differentiation was not connected to virulence of *C. neoformans*, other types of morphological changes could be related to virulence. The production of titan cells, which is the only known morphological switching event occurring during host infection, is required for full virulence of *C. neoformans*. As our study has not monitored the role of each TF in titan cell formation systematically, it is possible that TF networks governing morphogenesis could be critical for virulence. Recently, two TFs, Rim101 and Mbs1 were found to be involved in titan cell formation. Future screening of titan cell-regulating TFs could significantly correlate morphogenesis to virulence in *C. neoformans*.

In conclusion, the current systematic functional profiling of transcriptional networks will enhance our ability to comprehensively understand the complex signalling networks that govern the general biology and pathogenicity of *C. neoformans*.

Methods

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

Construction of the *C. neoformans* TF mutant library. TF mutant strains were constructed in the *C. neoformans* serotype A H99 strain background. Gene-disruption cassette constructions containing the nourseothricin-resistance marker (NAT) and signature-tagged sequences were generated using overlap PCR or NAT-split marker/ double-joint PCR strategies. Briefly, for the overlap PCR method, the 5’- and 3’-flanking regions of the TF genes were amplified by using primers L1 and L2 and primers R1 and R2, respectively, together with *H99* genomic DNA in the first round of PCR. Primers M13F (M13 forward extended) and M13R (M13 reverse extended) were used for amplifying the dominant selectable marker (NAT) containing signature-tagged sequences. In the second round of PCR, the TF gene-disruption cassettes were generated by means of overlap PCR performed using primers L1 and R2 and the first-round PCR products as templates. In the double-joint PCR method, the 5’- and 3’-flanking regions of the TF genes were amplified using, respectively, the *Bio-Rad* (Bio-Rad) and into the *H99* genomic DNA in the first round of PCR. The 5’ and 3’-regions of NAT-split markers were amplified using primers M13F and NSL and primers M13R and NSR, respectively, together with pNATSTM, which harboured unique signature-tagged sequences. The amplified gene-disruption cassettes were combined with 600 μg of gold microcarrier beads and were introduced into the *H99* genome by electroporative transformation apparatus. Stable nourseothricin-resistant transformants were initially screened by means of diagnostic PCR. The accuracy of the genotypes of the positive transformants was validated by means of Southern blot analysis.

*Cryptococcus* genomic DNA was extracted using the CTAB (cetyl trimethyl ammonium bromide) method. Isolated genomic DNA from each TF mutant was digested with the indicated restriction enzyme. The digested genomic DNAs were separated by 1% agarose gel electrophoresis. The agarose gel was transferred into the denatured buffer containing 0.5 M NaOH and 1.5 M NaCl for 45 min. Next, the agarose gel was transferred into the neutralization buffer containing 1.5 M NaCl and 1 M Tris adjusted with pH 8 for 45 min. The digested genomic DNA were transferred to the nylon membrane using 10 × SSC buffer and fixed by 1,200 mJ −1 ultraviolet exposure. The membrane was hybridized with a gene-specific and radioactively labelled probe using modified church hybridization method. Isolated genomic DNA from each TF mutant was digested with the indicated restriction enzyme and the number of colonies on each plate was determined.

C. *neoformans* H99S strain background. Gene-disruption cassette constructions containing the nourseothricin-resistance marker (NAT) and signature-tagged sequences were generated using overlap PCR or NAT-split marker/double-joint PCR strategies. Briefly, for the overlap PCR method, the 5’- and 3’-flanking regions of the TF genes were amplified by using primers L1 and L2 and primers R1 and R2, respectively, together with *H99* genomic DNA in the first round of PCR. Primers M13F (M13 forward extended) and M13R (M13 reverse extended) were used for amplifying the dominant selectable marker (NAT) containing signature-tagged sequences. In the second round of PCR, the TF gene-disruption cassettes were generated by means of overlap PCR performed using primers L1 and R2 and the first-round PCR products as templates. In the double-joint PCR method, the 5’- and 3’-flanking regions of the TF genes were amplified using, respectively, the *Bio-Rad* (Bio-Rad) and into the *H99* genomic DNA in the first round of PCR. The 5’ and 3’-regions of NAT-split markers were amplified using primers M13F and NSL and primers M13R and NSR, respectively, together with pNATSTM, which harboured unique signature-tagged sequences. The amplified gene-disruption cassettes were combined with 600 μg of gold microcarrier beads and were introduced into the *H99* genome by electroporative transformation apparatus. Stable nourseothricin-resistant transformants were initially screened by means of diagnostic PCR. The accuracy of the genotypes of the positive transformants was validated by means of Southern blot analysis.

*Cryptococcus* genomic DNA was extracted using the CTAB (cetyl trimethyl ammonium bromide) method. Isolated genomic DNA from each TF mutant was digested with the indicated restriction enzyme and the number of colonies on each plate was determined.

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

Another key finding of this study with clinical and pharmaceutical implications is the discovery of an unprecedented number of TFs involved in virulence of *C. neoformans*, including several structurally and functionally unique TFs. Although TFs are considered to be less optimal drug targets in general, it is not impossible to develop TF-specific drugs as witnessed in the development of a STAT4 inhibitor, Lisofoylline, for treating diabetes and STAT3 inhibitors for cancer treatment. In the small-molecule-mediated inhibition of FOXM1 transcriptional programme. Here, we determined that 45 genes (32 novel and 13 known TFs) are involved in pathogenicity of *C. neoformans* based on the insect-based virulence assay and STBM-based murine infectivity assay, which are two complementary assays for large-scale screening of potential virulence factors. Particularly, the virulence-related TFs confirmed using both approaches warrant particular attention. Besides recognized TFs, these include novel TFs, such as UsV101, Hob1, Fzc1, Fzc31, and Zic2. Virulence defects observed in the *hob1Δ* and *usv101Δ* mutants might result from increased thermosensitivity and severe defects in melanin production and multiple stress responses. By contrast, obvious virulence-related phenotypic traits were not detected in the *fzc2Δ, fzc1Δ* and *fzc31Δ* mutants. The *fzc1Δ* mutant in particular exhibited highly enhanced capsule and melanin production. Therefore, these TFs might function in the production of other virulence factor(s), which could include capsule-independent antiphagocytic factors, phospholipases, inositol uptake and metabolic systems and giant/titan cell formation. Therefore, an expanded phenotypic profiling of the TF mutant library will further reveal the molecular functions of each virulence-related TF in the pathogenicity of *C. neoformans*.

When virulence assay data in human pathogenic fungi from this and others studies were compared, we found several evolutionarily conserved virulence-regulating TFs, which could be exploited as broad-spectrum antifungal drug targets. When two large-scale virulence analyses of *C. neoformans* and *A. fumigatus* TF mutants were compared, the five TFs (Nrg1, Rim101, Crz1, UsV101 and Zap104) appear to be commonly involved in virulence of the two fungal pathogens. Crz1 is also known to be required for the virulence of *A. fumigatus* and *G. glabrata*. Comparison of our virulence–phenotype correlation network showed that in vivo virulence of *C. neoformans* is highly correlated to stress responses, but not to mating and differentiation. By contrast, morphological switching and differentiation processes appear to be highly correlated with virulence of other fungal pathogens. In *F. graminearum*, sexual development is highly correlated to virulence. In *C. albicans*, Noble et al. identified 115 infectivity-attenuated mutants, about 40% of which exhibits altered morphological switching and proliferation. Although sexual differentiation was not connected to virulence of *C. neoformans*, other types of morphological changes could be related to virulence. The production of titan cells, which is the only known morphological switching event occurring during host infection, is required for full virulence of *C. neoformans*. As our study has not monitored the role of each TF in titan cell formation systematically, it is possible that TF networks governing morphogenesis could be critical for virulence. Recently, two TFs, Rim101 and Mbs1 were found to be involved in titan cell formation. Future screening of titan cell-regulating TFs could significantly correlate morphogenesis to virulence in *C. neoformans*.

In conclusion, the current systematic functional profiling of transcriptional networks will enhance our ability to comprehensively understand the complex signalling networks that govern the general biology and pathogenicity of *C. neoformans*.
For monitoring pheromone gene expression, the MA2A and KN99a strains were mixed with equal concentration of cells (10^8 cells per ml) spread onto the V8 medium and incubated in the dark at room temperature for 12 h. The cells were scraped, pelleted, frozen in liquid nitrogen and lyophilized overnight for total RNA isolation, followed by northern blot analysis with a specific mating pheromone gene (MFA1)-specific probe.

Expression analysis by northern blot and quantitative RT-PCR. Total RNA was isolated from each sample using TriZol reagent. For northern blot analysis, 10 μg of RNA was separated on 1% agarose gel made with diethyl pyrocarbonate-treated water and 1 x MOPS running buffer by electrophoresis. The gel was washed three times with distilled water, transferred to a nylon membrane using 2 x SSC buffer and fixed by 1,200 J m⁻² ultraviolet exposure. The membrane was hybridized with a gene-specific and radioactively labelled probe using modified church hybridization buffer. The membrane was washed with the washing buffer 1 and 2, and then the membrane was exposed to autoradiography film for 1–2 days. To analyse the expression of ERG2, ERG3, ERG5, ERG11 and ERG25, we grew WT, sreΔ and hod1Δ mutants in liquid YPD medium overnight. The overnight culture was then inoculated in 100 ml of fresh YPD medium at 30 °C and grown until the OD600 of the culture reached ~1.0. To prepare the zero-time sample, 50 ml of cell culture was sampled and the remaining culture was treated with FCZ (final concentration: 10 μg ml⁻¹) for 90 min. Total RNA was isolated using TRIzol reagent and complementary DNA was synthesized using M-MuLV reverse transcriptase (Thermo scientific). Northern blot analysis was performed with each ERG gene-specific probe that was amplified with ERG gene-specific primers with the total RNA in cells treated or not treated with FCZ. Primers: B5789 and B5790 for ERG3, B5789 and B5791 for ERG5, B5158 for ERG8 for ERG11; B1718 and B1719 for ERG25. Quantitative real-time PCR was performed with each gene-specific primer using a MyiQ2 Real-Time PCR detection system (Bio-Rad). Primers: B5789 and B6838 for ERG2; B1720 and B6839 for ERG3; B671 and B672 for ERG5; B677 and B678 for ERG11; B2065 and B6840 for ERG25; and B679 and B680 for ACT1.

In vitro virulence-factor production assay. Capsule production was measured in both qualitative and quantitative manners. Cells were grown at 30 °C in liquid YPD medium for 16 h, spotted onto Dulbecco’s Modified Eagle’s (DME) solid medium and incubated at 37 °C for 2 days. Then cells were scraped and washed with PBS. For qualitative measurement, capsules were stained by India ink (Bactidrop; Remel) and visualized using an Olympus BX51 microscope equipped with a Spot insight digital camera (Diagnostic Instrument Inc.). For quantitative measurement, the cells collected from DME solid medium were fixed with 10% formalin and an equal number of cells (2.5 x 10^7 cells per ml) was loaded into a haematocrit capillary tube, which was subsequently placed vertically to allow cells to be packed by gravity for 10 days. The packed cell volume ratio was measured by calculating the ratio of the length of the packed cell volume phase to the length of the total volume phase (cells medium). The relative packed cell volume of each mutant was measured by calculating the ratio of the cell volume of the WT packed cell volume ratio. Triplicate technical experiments with two or more independent strains were performed. Statistical difference in relative packed cell volume was determined by one-way analysis of variance with Bonferroni’s multi-comparison test (Graphpad software). For monitoring capsule production, each strain was cultured at 30 °C in liquid YPD medium for 16 h, spotted on Niger seed agar medium containing 0.1 or 0.3% glucose, incubated at 30 °C for 7–10 days and collected by scraping to isolate output genomic DNA. Both input and output genomic DNAs were extracted using the CTAB method. Quantitative PCR analysis was performed with the various tag-specific primers listed in Supplementary Data 2 using a MyiQ2 Real-Time PCR detection system (Bio-Rad). We used the 2^ΔΔCt method to determine the STM score. To determine the input of genomic DNA with a specific tag, a ΔCt(ΔActtag-input) was calculated by comparing the ΔACT1 with average of Cttag from genomic DNA of pooled TF strains (Cttag – ΔACT1). To determine the output genomic DNA with a specific tag, a ΔCt(ΔActtag-output) was calculated by comparing the ΔACT1 with average of Cttag from genomic DNA from each lung of the sacrificed mouse (Cttag – ΔACT1). The STM score of each TF mutant was determined as the Log2 ΔACT1 (ΔActtag – ΔCt).

Cryptococcus transcription factor database. The genome and transcriptome data of the C. neoformans strains were processed using the protocol of the standardized genome data warehouse in Comparative Fungal Genomics Platform (CGFP 2.0; http://cgfp.snu.ac.kr)57. The TF family name for each gene was described based on the prediction of Fungal Transcription Factor Database (http://tfid.snu.ac.kr)58. For detailed information of the predicted genes, pre-computed results of eight bioinformatics programs were provided (InterPro scan, Signalp 3.0, PsortII, TargetP, ChloroP, SecretomeP, predictNLS and TLMHM2))59–66. To browse genomics contexts together with key biological features, Seoul National University Genome Browser (SNUGB; http://genomewebbrowser.snu.ac.kr)67 was incorporated for use with the Cryptococcus TF Database (http://tf.cryptococcus.org). In the pages of Browse Scaffolds, Browse Gene Models and 3 gene-family browsers, direct links to the SNUGB module were provided. MySQL 5.0.81 (source code distribution) and PHP 5.2.6 were used for administering the database and developing web interfaces, respectively. Web pages were provided through Apache 2.2.9 web server.

Construction of Pearson’s correlation networks. We calculated PCC scores by using Prism 5.0 (GraphPad Software Inc.) based on the results of phenotypic tests (strongly resistant phenotype: 3; moderately resistant phenotype: 2; weakly resistant phenotype: 1; WT-like phenotype: 0; weakly sensitive phenotype: –1; moderately sensitive phenotype: –2; and strongly sensitive phenotype: –3). Networks were visualized using Cytoscape software 2.9.0 based on the PCC scores.

Statistical analyses. Statistical analyses were performed using Prism 6.0 (GraphPad Software Inc.). Statistical difference was determined using Bonferroni’s multiple-comparison test.

References
1. Park, B. J. et al. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS 23, 525–530 (2009).
2. Perfect, J. R. et al. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of America. Clin. Infect. Dis. 50, 291–320 (2010).
3. Bahn, Y. S. & Jung, K. W. Stress signaling pathways for the pathogenicity of Cryptococcus neoformans. Eukaryot. Cell 11, 53–67 (2012).
4. Janbon, G. et al. Analysis of the genome and transcriptome of Cryptococcus neoformans var. gattiiw2 phenotype reveals RNA expression and transcription leading to virulence attenuation. PLoS Genet. 10, e1004261 (2014).
5. Kummerfeld, S. K. & Teichmann, S. A. DBD: a transcription factor prediction database. Nucleic Acids Res. 34, D74–D81 (2006).
6. Wilson, D., Charoensawan, V., Kummerfeld, S. K. & Teichmann, S. A. DBD–taxonomically broad transcription factor predictions: new content and functionality. Nucleic Acids Res. 36, D88–D92 (2008).
7. Song, M. H. et al. A flucytosine-responsive Mhp1/Swi1-like protein, Mhs1, plays pleiotropic roles in antifungal drug resistance, stress response, and virulence of Cryptococcus neoformans. Eukaryot. Cell 11, 53–67 (2012).

© 2015 Macmillan Publishers Limited. All rights reserved.
8. Kim, M. S. et al. Comparative transcriptome analysis of the CO2 sensing pathway via differential expression of carbonic anhydrase in Cryptococcus neoformans. *Genetics* 185, 1207–1219 (2010).

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

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

11. Homann, O. R., Dea, J., Noble, S. M. & Johnson, A. D. A phenotypic profile of the Candida albicans regulatory nursery. *PLoS Genet.* 5, e1000783 (2009).

12. Jung, W. H., Sham, A., White, R. & Kronstad, J. W. Iron regulation of the major virulence factors in the AIDS-associated pathogen Cryptococcus neoformans. *PLoS Biol.* 4, e10 (2006).

13. Price, M. S. et al. Cryptococcus neoformans requires a functional glycolytic pathway for disease but not persistence in the host. *MBio* 2, e01013–e01111 (2011).

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

15. Lin, X. & Heitman, J. The biology of the *Cryptococcus neoformans* species complex. *Annu. Rev. Microbiol.* 60, 69–105 (2006).

16. Garcia-Rodas, R. & Zaragoza, O. Catch me if you can: phagocytosis and killing avoidance by Cryptococcus neoformans. *FEBS Lett.* 64, 147–161 (2012).

17. McCaffren, D. C. & Casadevall, A. Capsule and melanin synthesis in Cryptococcus neoformans. *Med. Mycol.* 39(Suppl 1), 19–30 (2001).

18. Haynes, B. C. et al. Toward an integrated model of capsule regulation in Cryptococcus neoformans. *PLoS Pathog.* 7, e1002211 (2011).

19. Wicker, B. L., Edman, U. & Edman, J. C. The *Cryptococcus neoformans* STEI2z gene: a putative Saccharomyces cerevisiae STEII homologue that is mating type specific. *Mol. Microbiol.* 26, 951–960 (1997).

20. Bahn, Y. S., Kojima, K., Cox, G. M. & Heitman, J. A unique fungal two-component system regulates stress responses, drug sensitivity, sexual development, and virulence of Cryptococcus neoformans. *Mol. Biol. Cell* 17, 3122–3135 (2006).

21. Jiang, N. et al. A copper-responsive factor gene CUF1 is required for copper induction of laccase in Cryptococcus neoformans. *FEBS Lett.* 296, 84–90 (2009).

22. Pukkila-Worley, R. et al. Transcriptional network of multiple capsule and melanin genes governed by the *Cryptococcus neoformans* cyclic AMP cascade. *Eukaryot. Cell* 10, 190–201 (2005).

23. Singh, A. et al. Factors required for activation of urease as a virulence determinant in *Cryptococcus neoformans*. *Mbio* 4, e00220–e00111 (2013).

24. Olszewski, M. A. et al. Urease expression by *Cryptococcus neoformans* promotes microvascular sequestration, thereby enhancing central nervous system invasion. *Am. J. Pathol.* 164, 1761–1771 (2004).

25. Cowen, L. E. The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype. *Nat. Rev. Microbiol.* 6, 187–198 (2008).

26. Vermes, A., Guchelaar, H. J. & Dankert, J. Fuclytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. *J. Antimicrob. Chemother.* 46, 171–179 (2000).

27. Chang, Y. C., Bien, C. M., Lee, H., Espenshade, P. J. & Kwon-Chung, K. J. Ste50 adaptor protein governs sexual differentiation of *Cryptococcus neoformans* via the CUF1 class and indication. *Annu. Rev. Pharmacol. Toxicol.* 49, 154–165 (2011).

28. Choe, J. et al. Functional characterization of the *Aspergillus fumigatus* CRZ1 homologue, CrzA. *Mol. Microbiol.* 67, 1274–1281 (2009).

29. Miyazaki, T. et al. Roles of calcineurin and Crz1 in antifungal susceptibility and virulence of *Candida glabrata*. *Antimicrob. Agents Chemother.* 54, 1639–1643 (2010).

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

31. Okagaki, L. H. et al. Cryptococcal cell morphology affects host cell interactions and pathogenicity. *PLoS Pathog.* 6, e1000953 (2010).

32. Zaragoza, O. et al. Fungal cell gigantism during mammalian infection. *PLoS Pathog.* 6, e1000945 (2010).

33. Okagaki, L. H. et al. Cryptococcal titan cell formation is regulated by G-protein signaling in response to multiple stimuli. *Eukaryot. Cell* 10, 1306–1316 (2011).

34. Kim, S. M., Kim, S. Y., Yoon, J. K., Lee, Y. W. & Bahn, Y. S. An efficient gene-disruption method in *Candida neoformans* homozygous deletion library decouple morphogenetic switching and pathogenicity of *Cryptococcus neoformans*. *MBio* 2, e00103–e00111 (2011).

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

36. Biebert, M. et al. CFGBP 2.0: a versatile web-based platform for supporting comparative and evolutionary genomics of fungi and Oomycetes. *Nucleic Acids Res.* 41, D714–D719 (2013).

37. Park, J. et al. FTFFD: an informatics pipeline supporting phylogenomic analysis of fungal transcription factors. *Bioinformatics* 24, 1024–1025 (2008).

38. Hunter, S. et al. InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acids Res.* 40, D306–D312 (2012).

39. Bendtsen, J. D., Nielsen, H., Nielsen, K. & Bahn, Y. S. Ste50 adaptor protein governs sexual differentiation of *Cryptococcus neoformans* via the phenolone-response MAPK signaling pathway. *Fungal Genet. Biol.* 48, 154–165 (2011).

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

41. Biebert, M. et al. CFGBP 2.0: a versatile web-based platform for supporting comparative and evolutionary genomics of fungi and Oomycetes. *Nucleic Acids Res.* 41, D714–D719 (2013).

42. Park, J. et al. FTFFD: an informatics pipeline supporting phylogenomic analysis of fungal transcription factors. *Bioinformatics* 24, 1024–1025 (2008).

43. Hunter, S. et al. InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acids Res.* 40, D306–D312 (2012).

44. Bendtsen, J. D., Jensen, L. J., Brien, N., Von Heijne, G. & Brunak, S. Feature-based prediction of eukaryotic and leaderless protein secretion. *Protein Eng. Des. Sel.* 17, 349–356 (2004).

45. Cokol, M., Nair, R. & Rost, B. Finding nuclear localization signals. *EMBO Rep.* 1, 411–415 (2000).

46. Sonnhammer, E. L., von Heijne, G. & Krogh, A. A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 6, 175–182 (1998).

47. Pulakh, et al. SNUGB: a versatile genome browser supporting comparative and functional fungal genomes. *BMC Genomics* 9, 586 (2008).
Acknowledgements

We thank Hanna Na, Moonyoung Park, Yongiae Song, Yoojeon Kim, Kyeongjeon Ryu, Changhee Lee, Ja-Kyung Yoon, Seung-Yool Lee, Heejung Ahn, Jiwhan Lee, Hyunsoo Kim and Sunghyun Kim for their technical assistance in constructing the TF mutant library. This work was supported by National Research Foundation of Korea grants (nos 2008-0061963 and 2010-0029117) from MEST (to Y.-S.B.). This work was also supported in part by NIH/NIAID R01 AI050438-10 to J.H. and ANR (2010-BLAN-1620-01 program YeastIntrons) to G.J.

Author contributions

Y.-S.B. conceived the project. K.-W.J., D.-H.Y., S.M., K.-T.L., Y.-S.S., J.H., H.-J.B., H.K., S.B., M.-H.S., J.-W.L., M.S.K., S.-Y.K., J.-H.J., G.P., H.K., S.C., G.L.M., L.I.W., J.J., G.A., T.K., A.K.A. and Y.-S.B. performed the experiments and analysed the data. K.-W.J. contributed to the overall TF mutant construction, mating analysis and animal studies. D.-H.Y. contributed to the overall TF mutant construction and virulence-factor analysis. J.C. and Y.-H.L. contributed to the TF web database. K.-W.J., D.-H.Y., G.J., E.C., J.H., Y.-H.L., Y.-W.L. and Y.-S.B. supervised the experimental analysis and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Jung, K.-W. et al. Systematic functional profiling of transcription factor networks in Cryptococcus neoformans. Nat. Commun. 6:6757 doi: 10.1038/ncomms7757 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/