MICROBIOLOGY

# RELATe enables genome-scale engineering in fungal genomics

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**CRISPR-Cas9–based screening with single-guide RNA (sgRNA) libraries has emerged as a revolutionary tool for comprehensive analysis of genetic elements. However, genome-scale sgRNA libraries are currently available only in a few model organisms. The traditional approach is to synthesize thousands to tens of thousands of sgRNAs, which is laborious and expensive. We have developed a simple method, RELATe (restriction/ligation coupled with *Agrobacterium*-mediated transformation), to generate sgRNA libraries from 10 μg of genomic DNA, targeting over 98% of the protein-coding genes in the human fungal pathogen *Cryptococcus neoformans*. Functional screens identified 142 potential *C. neoformans* genes contributing to blood-brain barrier penetration. We selected two cryptococcal genes, *SFP1* and *WDR1*, for a proof-of-concept demonstration that RELATe-identified genes are relevant to *C. neoformans* central nervous system infection. Our RELATe method can be used in many other fungal species and is powerful and cost-effective for genome-wide high-throughput screening for elucidating functional genomics.**

## INTRODUCTION

CRISPR-Cas9–mediated gene editing occurs naturally in bacteria and has now been retooled to manipulate the genome of virtually any organism. CRISPR-Cas9–mediated mutagenesis requires Cas9 nuclease and a single-guide RNA (sgRNA). The sgRNA directs Cas9 to the complementary genomic sequence, where it makes a double-stranded break (DSB). Repairing the DSBs often results in insertions or deletions within the targeted locus. This simple and powerful tool is widely applied in fungi (*1*–*3*).

Cas9 nuclease combined with genome-scale sgRNA libraries has been developed for forward genetic screening in a few model organisms (human, mouse, *Toxoplasma gondii*, *Saccharomyces cerevisiae*, and *Escherichia coli*) (*4*–*8*). The traditional method to constructing an sgRNA library is to design and synthesize thousands to tens of thousands of sgRNAs based on known whole-genome sequences, which is a laborious and expensive process. The high cost of sgRNA library synthesis, as well as unknown whole-genome sequences for most fungal pathogens, has hindered the application of CRISPR-Cas9–based genome-wide screen in fungal pathogens. In this study, we developed a simple method, RELATe (restriction/ligation coupled with *Agrobacterium*-mediated transformation), to create a genome-scale sgRNA library in human fungal pathogen *Cryptococcus neoformans*.

*C. neoformans* causes life-threatening central nervous system (CNS) infection in immunocompromised individuals such as HIV-1–infected patients, resulting in over 180,000 deaths annually (*9*). *C. neoformans* is globally distributed and particularly abundant in avian excreta (*10*). Infection with *C. neoformans* starts with inhalation of fungal cells from the environment, followed by extrapulmonary spread, leading to hematogenous dissemination to target organs, most commonly resulting in CNS infection (*10*).

Several lines of evidence from human cases and experimental animal models of hematogenous *C. neoformans* CNS infection indicate that cerebral capillaries are the portal of entry into the brain (*11*–*14*). Because *C. neoformans* entry into the brain occurred in the cerebral microvasculature, we and others have used an in vitro blood-brain barrier model with human brain microvascular endothelial cells (HBMECs) to study *C. neoformans* penetration of the blood-brain barrier (*12*, *14*–*16*). *C. neoformans* strains exhibit the ability to traverse the HBMEC monolayer and penetrate into the brain in the animal models of experimental CNS infection (*12*–*16*). However, the *C. neoformans* factors contributing to penetration of the blood-brain barrier remain incompletely understood.

We explored the potential of using a CRISPR-Cas9 sgRNA library for genome-wide functional screening of cryptococcal factors contributing to penetration of the blood-brain barrier, the essential step in the development of *C. neoformans* CNS infection (Fig. 1). We demonstrated that RELATe enabled creation of a genome-scale CRISPR library covering 98.28% of the protein-coding genes in *C. neoformans*. Functional screens of in vitro and in vivo blood-brain barrier models revealed hundreds of potential cryptococcal factors in *C. neoformans* CNS infection.

## RESULTS

### *Agrobacterium*-based delivery of sgRNA produces efficient deletion of target gene

We first tested the efficacy of gene disruption with CRISPR-Cas9 in *C. neoformans*. *Agrobacterium*-mediated transformation is commonly applied for insertional mutagenesis in *C. neoformans* (*17*–*19*). *Agrobacterium*-mediated DNA delivery produces stable transformants with relatively low open reading frame (ORF) hit rate, and over 80% of the transformants contain a single transferred DNA (T-DNA) insertion (*17*–*19*). We designed a T-DNA vector to deliver a G418 selection marker, *NEO*, and sgRNAs into *C. neoformans* strain $H99_{CAS9}$, which stably expressed a derivative of the *Streptococcus pyogenes* Cas9 nuclease (Fig. 2A) (*20*). Delivery of T-DNA vector with sgRNAs into *C. neoformans* and subsequent editing of CRISPR-Cas9 will generate mutation. The unique sgRNA cassette in each T-DNA vector serves as a genetic barcode for mutation tracking by sequencing.

To examine the efficacy of this method, we designed sgRNAs targeting laccase-encoding gene *LAC1* (fig. S1). Laccase is a fungal

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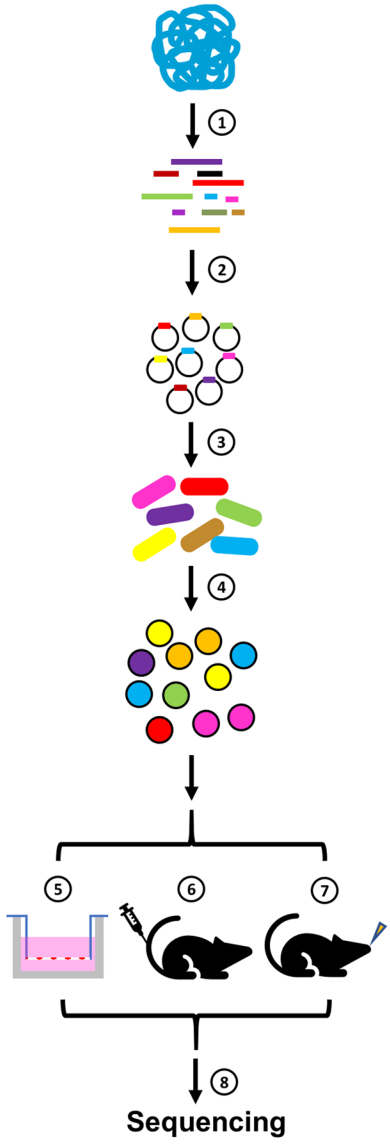


**Fig. 1. Workflow of sgRNA library design and functional screen.** (1) *C. neoformans* genomic DNA was digested with restriction enzyme cocktail, Hpa II, Scr FI, and Bfa I; (2) the sgRNA library was cloned into T-DNA vector pDHt-SK-NEO-CnU6; (3) the sgRNA library was introduced into *A. tumefaciens*; (4) the sgRNA library was delivered into *C. neoformans* $H99_{CAS9}$; (5) transcytosis assay in HBMEC; (6) intravenous inoculation in mice; (7) intranasal inoculation in mice; (8) the recovered sgRNA library was amplified by polymerase chain reaction (PCR) and submitted for deep sequencing.

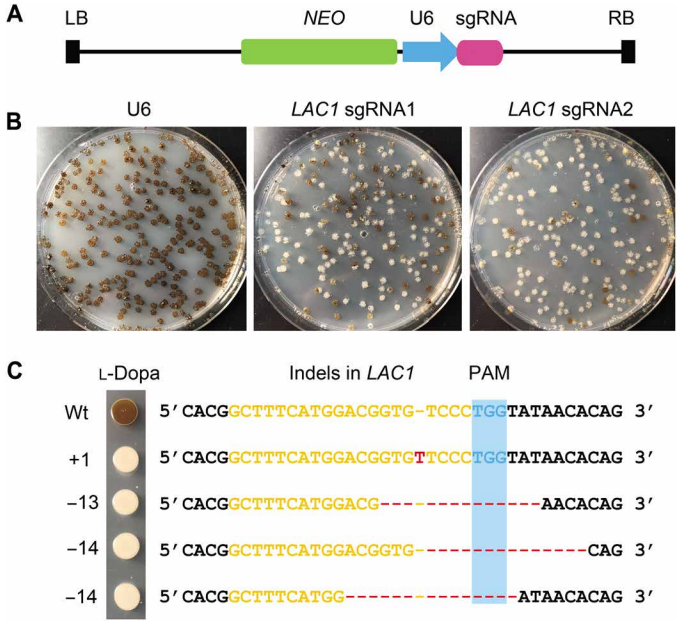


**Fig. 2. *Agrobacterium*-based delivery of sgRNA produces efficient deletion of target gene.** (**A**) T-DNA expression vector for sgRNA. LB, left border of T-DNA; RB, right border of T-DNA; *NEO*, drug G418 resistance gene; U6, *C. neoformans* U6 promoter. (**B**) Melanization assay. U6, transformants containing only *C. neoformans* U6 promoter; *LAC1* sgRNA1 and sgRNA2, transformants containing sgRNAs expressed under U6 promoter. Dark brown colony, melanization; white colony, no melanization. Photo credit: Z. Li, Johns Hopkins University. (**C**) Indel mutation in the targeting site of *LAC1*. Sequence targeted by *LAC1* sgRNA2, highlighted in orange; PAM (protospacer adjacent motif) site, highlighted in blue; red dashes, deleted bases; red bases, insertion. Photo credit: Z. Li, Johns Hopkins University.

factor responsible for melanin formation (*21*). Under glucose-free condition, wild-type strain H99 produces dark brown colonies, whereas *LAC1* mutant forms white colonies. About 400 to 500 transformants for each cassette were obtained after selection with the drug G418 for 3 days and then cultured in 200 ml of YPD (yeast extract, peptone, and dextrose) broth for additional 18 days ($^1/_{100}$ of the culture was transferred to 200 ml of fresh YPD broth every 2 days), which gave CRISPR-Cas9 enough time for efficient editing. The final culture was plated on YPD agar plates, cultured for 3 days, and then replica-spotted to melanin-inducing medium for melanization. CRISPR-Cas9 abolished melanin formation in 59 ± 1.3% of cells expressing *LAC1* sgRNA1 and in 89 ± 0.1% of cells expressing *LAC1* sgRNA2 (Fig. 2B). In contrast, all the colonies from cells with U6 promoter only were dark brown (Fig. 2B). Sequencing of *LAC1* locus from white colonies expressing *LAC1* sgRNA2 revealed that bases were inserted or deleted, causing a frameshift within *LAC1* (Fig. 2C). These results indicated that *Agrobacterium*-based delivery of sgRNAs provided efficient gene disruption in *C. neoformans*.

## RELATe generates CRISPR-Cas9 sgRNA library covering over 98% of protein-coding genes in *C. neoformans*

We developed a simple approach, RELATe, to generate genome-wide gene disruption in *C. neoformans* (Fig. 1 and figs. S2 and S3). *C. neoformans* genomic DNA was digested with a cocktail of restriction enzymes (Scr FI, CC/NGG; Hpa II, C/CGG; Bfa I, C/TAG), which separated the potential protospacers from PAM (protospacer adjacent motif) sites. All the potential protospacers, together with their native PAM sites, were mapped to the protein-coding sequences using Bowtie 2 with exact match (*22*). The protospacers aligned to protein-coding sequences two or more times were discarded from analysis because of off-target sites. In theory, 160,212 sgRNAs without multiple target sties could be generated with RELATe.

After blunting the ends, the DNA fragments were ligated to adaptor containing the Mme I site. Digestion with Mme I produced 20- or 21-nucleotide (nt) protospacer (fig. S2). After ligating the protospacers to a *C. neoformans* U6 promoter (*23*), the adaptor was removed. The resulting products were ligated to sgRNA constant

region. The sgRNA pool was assembled into T-DNA vector and then delivered into *C. neoformans* strain $H99_{CAS9}$ by *Agrobacterium*-mediated transformation.

In total, ~$4.40 \times 10^5$ transformants were obtained after 3-day selection with the drug G418. Amplification by polymerase chain reaction (PCR) and deep sequencing revealed that 57,356 sgRNAs were created with RELATe, accounting for 35.80% of the predicted sgRNAs (160,212 sgRNAs) (table S1). Of the total sequence reads, 43.43% represented protospacer length = 20 base pairs (bp) and 52.55% represented protospacer length = 21 bp, which was consistent with the intended design (Fig. 3A). Of the reads representing protospacer length 20 and 21 bp, 52.55% were sgRNAs targeting the coding sequence, 9.90% were sgRNAs targeting noncoding region, and 1.25% represented mitochondrial genomic sequence (Fig. 3B). In addition, 30.45% were genomic sequences not followed by the PAM site, likely due to promiscuous activity of mung bean nuclease (Fig. 3B). The reads were not equally distributed within the sgRNA library, which were likely introduced during amplification (Fig. 3C). Over 80% of the genes were targeted by three or more individual sgRNAs (Fig. 3D). The 57,356 sgRNAs targeted the coding region of 6842 genes, resulting in 98.28% coverage of the genome (table S1). These results demonstrated that RELATe represents a simple approach to generate genome-wide CRISPR-Cas9 sgRNA library.

## Functional screen reveals cryptococcal factors contributing to penetration of the blood-brain barrier

To test the feasibility of genome-wide screen for determining cryptococcal factors in penetration of the blood-brain barrier, we performed pooled sgRNA library screen both in vitro and in vivo (Fig. 1). Screen was accomplished by isolating sgRNA pool from both the inoculum and recovered populations and quantifying the abundance of each sgRNAs in the samples. The final sgRNA candidates were selected based on the following criteria: (i) defect in transcytosis assay in HBMEC, (ii) defect in brain invasion after intravenous infection in mice, (iii) defect in dissemination from lung to brain after intranasal infection in mice, (iv) no defect in in vitro fitness, and (v) no defect in lung survival. The sgRNA library was cultured in YPD broth for additional 18 days ($^1/_{100}$ of the culture was transferred to 2 liters of fresh YPD broth every 2 days).

For in vitro screen, we used fungal transcytosis assay with HBMEC (*12*), which forms a monolayer of brain microvascular endothelial cells mimicking the in vivo blood-brain barrier and separates the blood side (upper chamber) from the brain side (lower chamber). HBMEC was seeded on transwell insert to form a monolayer and then incubated with sgRNA library ($1 \times 10^6$ *C. neoformans* cells per insert) for 4 to 6 hours. We collected the sgRNA pool from the lower chamber. Five hundred transwell inserts were used, and about $1.43 \times 10^7$ colony-forming units (CFUs) were recovered from

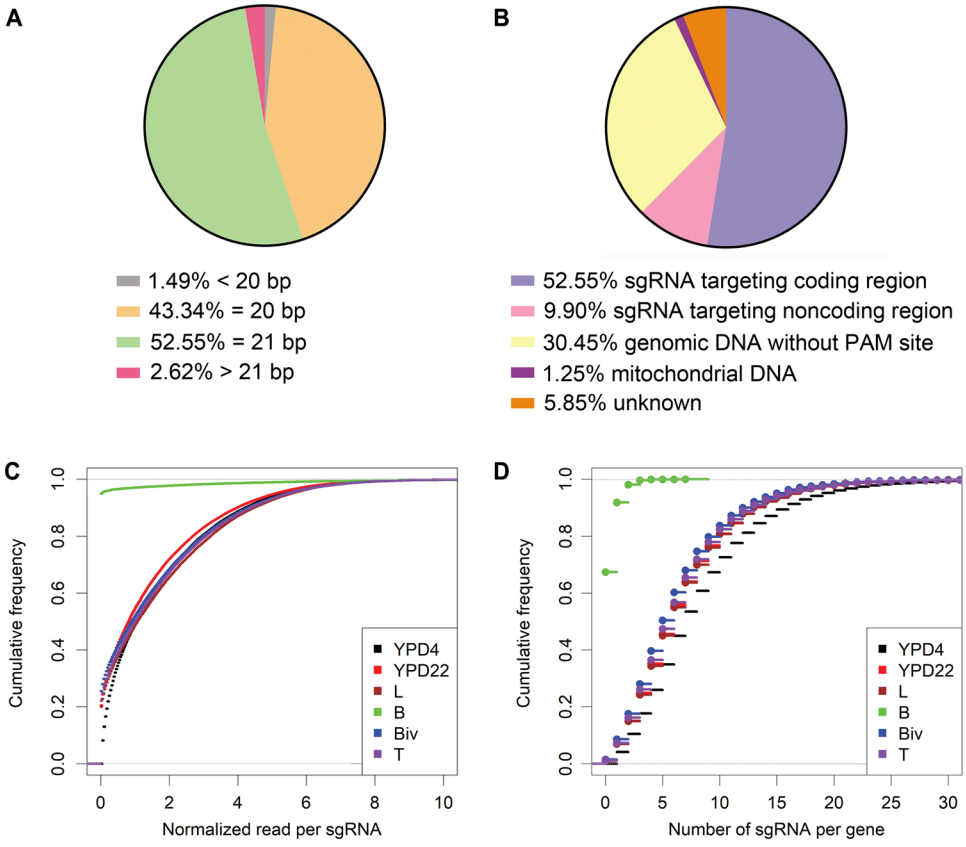


**Fig. 3. sgRNA library targeting *C. neoformans* genomic sequences.** (**A**) Length distribution of the protospacers (region between U6 promoter and sgRNA guide body). (**B**) Distribution of the protospacers within *C. neoformans*. (**C**) Cumulative frequency of sgRNAs. YPD4, initial sgRNA library; YPD22, sgRNA library cultured for additional 18 days; L, sgRNA library recovered from lung after intranasal infection; B, sgRNA recovered from brain after intranasal infection; Biv, sgRNA recovered from brain after intravenous infection; T, sgRNA library recovered from transcytosis assay. (**D**) Cumulative frequency of genes.

the lower chamber, achieving ~250-fold coverage of the sgRNA library.

For in vivo screen, we infected the mice both intravenously and intranasally (mimicking systemic and inhalational inoculations, respectively). For intravenous inoculation, each mouse was challenged with $2 \times 10^7$ cells. Infection was allowed to progress until the mice displayed signs of morbidity (2 to 3 days after infection). The sgRNA pool was recovered from the brain. In total, ~$7.26 \times 10^7$ CFUs were recovered from the brains of 30 mice, with ~1200-fold coverage of sgRNA library. For intranasal inoculation, each mouse was challenged with $2 \times 10^7$ cells. sgRNA pool was recovered from the brains and lungs separately when mice showed signs of morbidity (6 to 16 days after infection). Of the 30 infected mice, $2.60 \times 10^8$ CFUs were recovered from the brains and $4.60 \times 10^9$ CFUs were obtained from the lungs.

The sgRNA pools from each sample were amplified by PCR and then submitted for deep sequencing (table S1). Deep sequencing revealed a substantial reduction in sgRNA diversity after 18-day culture in YPD broth (YPD22 versus YPD4), indicating that the potential genes for in vitro fitness were eliminated by sgRNAs (Fig. 3, C and D, and table S1). About 3292 sgRNAs showed normalized $\log_2$ fold change < −1 [normalized reads per sgRNA = (reads per sgRNA/total reads for all sgRNAs in sample) × $10^6$ + 1], targeting 2447 potential genes for in vitro fitness. A total of 4572 pairs of sequence orthologs between *C. neoformans* and *S. cerevisiae* were reported (*24*). About 1105 known essential genes in *S. cerevisiae* was documented (*25*), and *C. neoformans* contained 942 essential *S. cerevisiae* orthologs. The 2447 potential genes for in vitro fitness from the sgRNA library screen covered 357 essential *S. cerevisiae* orthologs, accounting for ~38% of the essential *S. cerevisiae* orthologs (fig. S4).

There was a drastic reduction in sgRNA diversity in the brain from intranasal infection. Only 2944 sgRNAs were detected from sgRNA library ($2.60 \times 10^8$ CFUs) recovered from brain after intranasal infection, reflecting that there is a bottleneck in dissemination from lungs to brains (Fig. 3, C and D, and table S1). Two hundred sixty-two sgRNAs (normalized $\log_2$ fold change < −1) were depleted from HBMEC transcytosis as well as brain infection following intravenous and intranasal inoculations (Fig. 4A). After removing the potential genes for in vitro fitness (YPD22 versus YPD4) and pulmonary survival (lung versus YPD22), 144 sgRNAs remained, which targeted 142 genes (Fig. 4, B and C, and table S2). Their putative function from gene annotation implicated that they represent a broad variety of cellular processes (Fig. 4D and table S2).

### Sfp1 and Wdr1 are required in *C. neoformans* penetration of the blood-brain barrier

The RELATe-identified genes (57.7%, 82 of 142) are available from *C. neoformans* deletion library in Fungal Genetics Stock Center (http://www.fgsc.net/), which were generated by the group of H. Madhani (table S3) (*26*). We tested 82 deletion strains by diagnostic PCR and restriction enzyme digestion of the PCR fragments. We observed that 17.1% (14 of 82) of the deletion strains (CNAG_02878, CNAG_04469, CNAG_04501, CNAG_05908, CNAG_06873, CNAG_06920, CNAG_01150, CNAG_02553, CNAG_03177, CNAG_00531, CNAG_03764, CNAG_00876, CNAG_03102, and CNAG_05340) were incorrect based on restriction fragments (table S3). Of the 68 deletion strains verified by diagnostic PCR, 4 deletion strains (CNAG_00467, CNAG_01536, CNAG_02490, and CNAG_05404) showed growth defect at host temperature (table S3). Of the remaining 64 deletion strains, 16 deletion strains showed a defect in transcytosis assay (Fig. 5, A and B, and fig. S5A). These include CNAG_03174 designated *SFP1*, 4′-phosphopantetheinyl transferase; CNAG_02402 designated *WDR1*, WD40 repeat protein; CNAG_02449, long-chain fatty acid coenzyme A ligase; CNAG_01941, glucan synthesis regulatory protein; CNAG_01954, aldo-keto reductase; CNAG_02468, AP-3 complex subunit delta-1; CNAG_03262, myo-inositol-1(or 4)-monophosphatase; CNAG_06738, exo70 exocyst complex subunit; CNAG_01878, myo-inositol 2-dehydrogenase; CNAG_00794, hypothetical protein; CNAG_03308, hypothetical protein; CNAG_07903, hypothetical protein; CNAG_05141, EamA-like transporter; CNAG_00805, hypothetical protein; CNAG_03912, membrane protein; and CNAG_04696, DNA clamp loader. All the 16 deletion strains had no growth defect at host temperature, excluding the possibility that defect in penetration of the blood-brain barrier was due to growth defect (Fig. 5, E and F, and fig. S5B). Of the 16 deletion strains with defect in transcytosis assay, 4 deletion strains (*sfp1*, *wdr1*, CNAG_02449, and CNAG_01941), verified by Southern blot, had a significant defect in blood-brain barrier penetration in vivo (Fig. 5, C and D, and figs. S5C and S6).

*SFP1* encodes a 4′-phosphopantetheinyl transferase, belonging to SFP-type phosphopantetheinyl transferase. Phosphopantetheinyl transferases posttranslationally modify modular and iterative synthases including fatty acid synthases, polyketide synthases (PKSs), and nonribosomal peptide synthetases (NRPSs) (*27*). Phosphopantetheinyl transferases are crucial for the biosynthesis of a multitude of metabolites and virulence factors synthesized via PKS and NRPS pathways (*27*). Phosphopantetheinyl transferases have emerged as a promising drug target against fungal pathogens, as phosphopantetheinyl transferases are involved in virulence (*28*). *WDR1* encodes a WD40 repeat protein. Wdr1 contains two WD40 repeat (WDR) domains and a predicted signal peptide, indicating that Wdr1 represents a potential secreted protein. The WDR domains are involved in a wide spectrum of cellular networks and may function as a scaffold for protein-protein interactions (*29*). Complemented strains *sfp1*Δ::*SFP1* and *wdr1*Δ::*WDR1* were generated by introducing a copy of wild-type gene into respective mutant strains (fig. S7). Functional analysis revealed that a copy of wild-type gene rescued the defects of mutants (*sfp1*Δ and *wdr1*Δ) in penetration of the blood-brain barrier in vitro (Fig. 5, A and B) and in vivo (Fig. 5, C and D). Two deletion strains that exhibited a defect in blood-brain barrier penetration both in vitro and in vivo (CNAG_01941 and CNAG_02449) will be further examined by complementation. The remaining 12 deletion strains will be validated by Southern blot, intravenous infection, and complementation, as shown with mutants *sfp1*Δ and *wdr1*Δ.

## DISCUSSION

Genome-scale high-throughput screen enables comprehensive analysis of genetic elements. *Agrobacterium*-mediated transformation was commonly applied to generate T-DNA insertion library for forward genetic screen in fungal species, but efficient genome-wide high-throughput screen was not reported due to biased T-DNA integration with relatively low ORF hits and challenge in identifying the genes disrupted by T-DNA (*30*). CRISPR-Cas9–mediated gene editing can virtually work for any organisms. The CRISPR-Cas9 system has been applied for gene editing in *C. neoformans* (*20*, *23*, *31*, *32*). Wang *et al.* (*23*) characterized the endogenous U6 promoter for expressing sgRNA and first demonstrated the CRISPR-Cas9–mediated

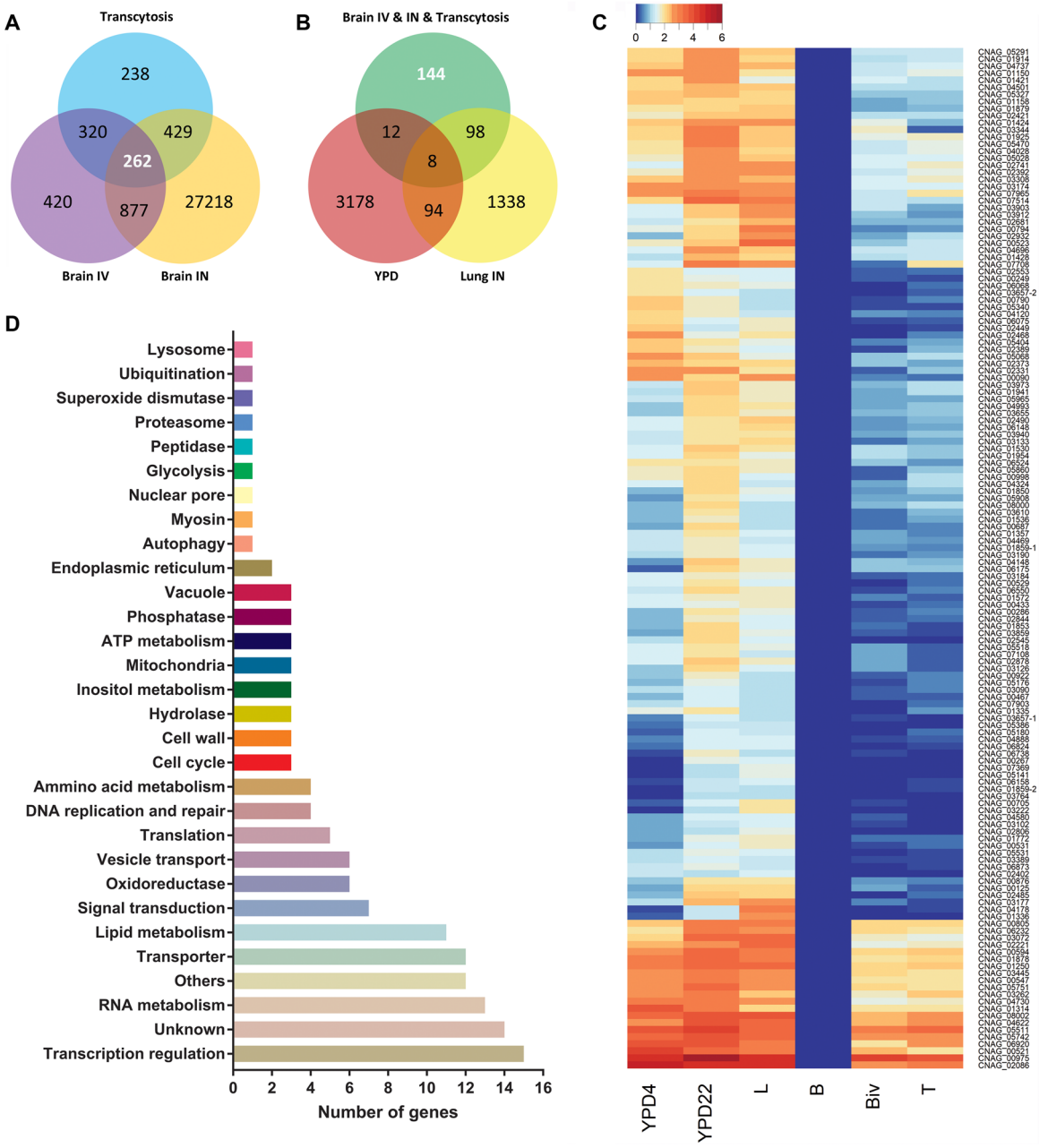


**Fig. 4. Functional screen reveals cryptococcal factors contributing to penetration of the blood-brain barrier.** (**A** and **B**) Venn diagram on distribution of the depleted sgRNAs. Transcytosis, sgRNAs depleted in penetration of the HBMEC monolayer in vitro; Brain IV, sgRNAs depleted in brain invasion after intravenous infection; Brain IN, sgRNAs depleted in disseminating from lung to brain after intranasal infection; Brain IV & IN & Transcytosis, overlap of depleted sgRNAs between Transcytosis, Brain IV, and Brain IN; YPD, sgRNAs depleted in in vitro growth; Lung IN, sgRNAs depleted in lung survival. Threshold of depletion defined as normalized $\log_2$ fold change $\leq -1$. (**C**) Heat map showing the enrichment change of the depleted sgRNAs. Each column corresponds to a sample, and rows represent the 144 sgRNAs that were depleted during transcytosis and intravenous and intranasal infection (normalized $\log_2$ fold change $\leq -1$). Sequencing read counts of the sgRNA were normalized and $\log_2$-transformed and are displayed as colors ranging from blue to red as shown in the key. The sgRNA order was determined by hierarchical clustering, and their corresponding gene IDs are denoted at the right. (**D**) Function categories of the 142 depleted genes. The number of genes in each functional category is shown on the *x* axis.

gene editing in *C. neoformans*. Arras *et al.* (*20*) stably expressed a derivative of the *S. pyogenes* Cas9 nuclease in *C. neoformans* and showed that expression of Cas9 in *C. neoformans* has no effect on growth, production of virulence factors, or virulence. Arras *et al.* (*20*) expressed the sgRNA sequence flanked by the hammerhead and hepatitis delta virus ribozymes. Following transcription, the 5′ capped, polyadenylated transcript undergoes self-cleavage, liberating the sgRNA. Fan and Lin (*31*) developed a transient CRISPR-Cas9 coupled with electroporation (TRACE) system for deleting multiple genes in a single transformation. Wang (*32*) developed a ribonucleoprotein-mediated CRISPR-Cas9 system for genome editing in *C. neoformans*. However, only a few model organisms have

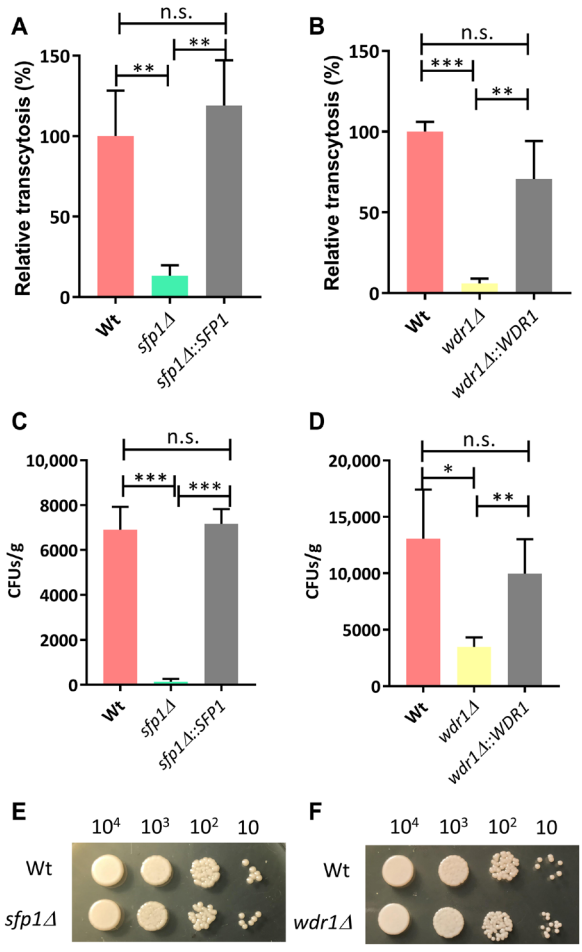


**Fig. 5. Sfp1 and Wdr1 promote *C. neoformans* penetration of the blood-brain barrier in vitro and in vivo.** (**A** and **B**) Transcytosis assay using HBMEC monolayer. HBMEC monolayer was incubated with $1 \times 10^6$ *C. neoformans* for 4 hours. Transcytosis frequency (%) was determined [(total CFUs recovered from the lower chamber/total number of cryptococcal cells added to the upper chamber) × 100]. Relative transcytosis (%) was determined [(transcytosis frequency of mutant or complemented strain/transcytosis frequency of wild-type strain) × 100]. (**C** and **D**) *C. neoformans* penetration into brain. CFUs from the brains were determined 24 hours after intravenous infection with $1 \times 10^5$ cells. Wild type (Wt) ($n = 3$); Mutants *sfp1*Δ and *wdr1*Δ ($n = 3$). Complemented strains *sfp1*Δ*::SFP1* and *wdr1*Δ*::WDR1* ($n = 4$). *P* value is determined by Student's *t* test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s., no significant difference. Data shown are means ± SEM. (**E** and **F**) Growth test of mutants *sfp1*Δ and *wdr1*Δ on YPD agar for 3 days at 37°C. Photo credit: Z. Li, Johns Hopkins University.

genome-scale sgRNA libraries due to high budget for sgRNA library synthesis and unknown genome sequences. We developed a simple method RELATe, which allowed us to generate genome-wide sgRNA library targeting over 98% of protein-coding genes in *C. neoformans* from 10 μg of genome DNA instead of synthesizing thousands to tens of thousands of sgRNAs.

In this report, we demonstrated that RELATe represents a simple and biologically relevant method to generate genome-scale mutants in human fungal pathogen *C. neoformans*. *C. neoformans* exploits fungal factors for penetration of the blood-brain barrier, the essential step in the development of *C. neoformans* CNS infection, but determinations of such cryptococcal factors remain incomplete. Our high-throughput screen revealed 142 *C. neoformans* genes with potential roles in the pathogenesis of CNS infection. A proof-of-concept demonstration was shown with two such targets, Sfp1 and Wdr1. The knockout mutants of Sfp1 and Wdr1 were defective in penetration of the blood-brain barrier in vitro and in vivo, and these defects were restored by complementation with wild-type genes. Fourteen additional genes were shown to be involved in penetration of HBMEC monolayer, and studies are in progress for validation of those mutants in penetration of the blood-brain barrier in vitro and in vivo.

The current usage of sgRNA library generated with RELATe has its limitations. First, the current sgRNA library only accounts for 35.80% of the predicted sgRNAs that could be generated with RELATe. The number of *Agrobacterium*-mediated transformation can be scaled up to obtain an even larger library size. Second, the current sgRNA library does not contain equal numbers of read representing each sgRNA, resulting in 16.56% sgRNA with raw read <3. A deeper sequencing can increase the read number for each sgRNA as well as the library coverage. Third, the sgRNAs generated by RELATe are randomly distributed within *C. neoformans* genome. If the sgRNA targets the downstream of functional domain of the protein (e.g., close to C terminus), the indel introduced by sgRNA is less likely to disrupt the gene function. This could explain that library construction generated an average of eight sgRNAs per coding sequence, but the final identified 142 target genes were covered only by a total of 144 sgRNAs. Seven cryptococcal factors [metalloprotease Mpr1 (*33*), urease Ure1 (*13*, *14*), hyaluronic acid synthase Cps1 (*34*), phospholipase Plb1 (*15*), Itr1a and Itr3c (*35*), and Fnx1 (*16*)] known to contribute to penetration into the brain were not identified from our screen. Cps1 is required for growth at host temperature (*36*). Plb1 is involved in pulmonary survival (*37*). Therefore, Cps1 and Plb1 do not meet our screen criteria. Itr1a and Itr3c have functional overlap, and single deletion did not show a defect in penetration of the blood-brain barrier (*35*). Itr1a and Itr3c are two major inositol transporters. In our screen, we identified that two genes [CNAG_03262, myo-inositol-1(or 4)-monophosphatase; CNAG_01878, myo-inositol 2-dehydrogenase] in inositol metabolism were required for HBMEC monolayer penetration. Ure1 was reported to contribute blood-brain barrier penetration in vivo, but it is not involved in penetration of the HBMEC monolayer in vitro within 4 to 6 hours of incubation (*38*). Recently, Lee *et al.* (*39*) identified 14 kinases (Cex1, Alk1, Pbs2, Yfh701, Pkh201, Met3, Hsl101, Snf1, Sch9, Irk5, Vrk1, Gal83, Urk1, and Irk2) and 9 transcription factors (Hap2, Ada2, Jjj1, Pho4, Sre1, Pdr802, Fzc9, Hob1, and Fzc1) for the blood-brain barrier penetration. Among them, nine kinases (Met3, Hsl101, Snf1, Sch9, Irk5, Vrk1, Gal83, Urk1, and Irk2) and five transcription factors (Sre1, Pdr802, Fzc9, Hob1, and Fzc1) are also required for pulmonary survival (*39*). The 142 target genes we identified contain five kinases (Tco1, Bub1, Pro1, Ypk101, and Lcb5) and one transcription factor (Hob3). Tco1 was required for brain infection (*40*, *41*). None of them were reported to be involved in the blood-brain barrier traversal (*39*). Failure to identify known targets in the blood-brain barrier traversal was likely due to low coverage of the sgRNA library and/or low sensitivity of negative selection. Negative selection with genome-scale sgRNA library usually produces more background noise than positive selection. To reduce the false positive background, we used biological triplicates relevant to penetration of the blood-brain barrier: HBMEC transcytosis, intravenous infection, and intranasal infection.

In summary, we showed that CRISPR-Cas9–based genome-wide screen is feasible for identification of fungal factors contributing to

*C. neoformans* CNS infection, as exemplified with two such targets, Sfp1 and Wdr1. We expect that many other genes identified from our screen represent promising candidates contributing to the pathogenesis of *C. neoformans* CNS infection. Characterization of such cryptococcal factors will uncover previously unknown strategies exploited by *C. neoformans* for penetration of the blood-brain barrier. The CRISPR-Cas9 system has been successfully used for single gene editing in many fungal organisms. The simple approach, RELATe, we developed will expand the application of the CRISPR-Cas9 system and enable converting genomic DNA into CRISPR sgRNA library for genome-scale mutagenesis screen in fungi as well as other organisms at a low cost, even in organisms with poorly annotated genomic information. Recently, an engineered CRISPR enzyme, *Streptococcus canis* Cas9-$Sc^{++}$, was developed with robust DNA-cleavage activity, minimal off-target activity, and broad PAM compatibility (NNG) (*42*). The current approach RELATe relied on three restriction enzymes (Scr FI, Hpa II, and Bfa I). We found that 30 more restriction enzymes are compatible with *S. canis* Cas9-$Sc^{++}$, which will extend the application of RELATe, increase the coverage of sgRNA library developed via RELATe, and decrease the off-target effect.

## MATERIALS AND METHODS

### Strains

*C. neoformans* strain $H99_{CAS9}$ (*20*) was a recipient strain for generating sgRNA library. *Agrobacterium tumefaciens* strain EHA105 (*17*) was used for delivery of sgRNAs into *C. neoformans*. The knockout mutants and their wild-type strain KN99 were obtained from Fungal Genetics Stock Center.

### sgRNA pool construction by restriction/ligation

We generated an sgRNA pool in *C. neoformans* using a restriction/ligation method with some modification (figs. S2 and S3) (*43*). *C. neoformans* genomic DNA was digested with a restriction enzyme cocktail, Hpa II, Scr FI, and Bfa I, which recognize the sequences C/CGG, CC/NGG, and C/TAG separately. After digestion with these enzymes and removal of the single-strand overhangs, the remaining double-stranded DNA (dsDNA) is adjacent to a PAM motif (NGG or TAG) in the target DNA. To trim them into 20 or 21 nt, the blunt-ended PAM adjacent DNA fragments were ligated to an 82-nt dsDNA adapter with an Mme I site at each terminus, two internal Bsa XI sites, and one Scr FI site in the middle of the adapter. After Scr FI and Acl I digestion, DNA fragments are converted into 41-nt empty half-adapter, or half-adapters ligated successfully to substrate fragments. The 41-nt empty half-adapter fragments are removed with AMPure XP. Mme I digestion, which cuts 20 or 21 nt distant from its binding site, generates a 20- or 21-nt substrate fragment adjacent to a 41-nt half-adapter. The resulting fragments have a 2-nt overhang produced by Mme I on one end and a 1-nt overhang generated by Scr FI on the other end. The *C. neoformans* U6 promoter followed by a "G" that ensures proper RNA polymerase III initiation was ligated to the end produced by Mme I. The resulting products have a *C. neoformans* U6 promoter, a 20- or 21-nt PAM adjacent region, and a 41-nt half-adapter. To obtain the final sgRNA fragment, Bsa XI digestion removes the adapter portion, leaving only a 3-nt overhang for ligation of an 83-nt fragment containing the sgRNA constant region and a 7-T terminator. The resulting ~143-nt fragment contains a *C. neoformans* U6 promoter, 20 or 21 nt of sequence corresponding to a putative Cas9 targeting site in the substrate DNA sequence, 77 nt of sgRNA hairpin, and a 7-T terminator. The ~143-nt fragments were amplified by PCR using primers (sgRNA library forward and sgRNA body reverse) (table S4). The resulting ~177-nt band was isolated and purified using DNA–polyacrylamide gel electrophoresis (PAGE).

To create pDHt-SK-NEO-CnU6, the G418 resistance marker *NEO* and *C. neoformans* U6 promoter amplified by PCR using primers (NEO forward and NEO reverse, U6 forward and U6 reverse) were cloned into pDHt-SK, which was linearized with the restriction enzymes Sac I and Bam HI using NEBuilder HiFi DNA Assembly (table S4). pDHt-SK-NEO-CnU6 was digested with Bam HI and Kpn I and then treated with shrimp alkaline phosphatase (rSAP). In total, 350 ng of purified ~177-bp sgRNA pool was mixed with 10 μg of linearized pDHt-SK-NEO-CnU6 and 250 μl of NEBuilder HiFi DNA assembly master mix in a 500-μl volume. The reaction mixture was incubated at 50°C for 1 hour, then purified by ethanol precipitation, and dissolved in 30 μl of sterile water (240 ng/μl). The purified pDHt-SK-NEO-CnU6-sgRNA pool was electroporated into NEB 10-beta Competent *E. coli*. A small fraction of transformation was plated on LB agar supplemented with kanamycin (50 μg/ml), leading to an estimate of $2.05 \times 10^8$ total transformed clones.

### *A. tumefaciens*–mediated transformation of *C. neoformans*

The pDHt-SK-NEO-CnU6-sgRNA pool isolated from *E. coli* was electroporated into *A. tumefaciens* EHA105 using a Bio-Rad electroporator with a 1-mm cuvette and voltage at 1.7 kV. A small fraction of transformation was plated on LB agar supplemented with kanamycin (50 μg/ml), leading to an estimate of $2.00 \times 10^8$ total transformed clones. The sgRNA pool was introduced into *C. neoformans* strain $H99_{CAS9}$ via *A. tumefaciens*–mediated transformation as described previously with a minor modification (*17*). Equal aliquots of *A. tumefaciens* EHA105 [$OD_{660}$ (optical density at 600 nm) = 1.2] and *C. neoformans* ($1 \times 10^8$ cells/ml) were mixed together, and 1-ml fractions of the mixture were cocultivated on induction medium [2-(*N*-morpholino) ethane sulfonic acid (pH 5.3), 7.8 g; acetosyringone, 0.039 g; $K_2HPO_4{\cdot}3H_2O$, 26.9 g; $KH_2PO_4$, 14.5 g; NaCl, 1.5 g; $(NH_4)_2SO_4$, 5 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; $CaCl_2{\cdot}2H_2O$, 0.067 g; $FeSO_4{\cdot}7H_2O$, 0.0025 g; glucose, 0.9 g; glycerol, 5 ml; final volume, 1 liter] agar plate (petri dish, 150 mm by 15 mm) at room temperature for 72 hours. Following cocultivation, the bacteria/yeast mixture was removed from the induction medium agar with sterile water. *C. neoformans* was separated from *A. tumefaciens* by centrifugation for 10 min, 850 rpm. A small fraction of pellet was plated on YPD agar and G418 (100 μg/ml), gentamicin (50 μg/ml), and cefotaxime (100 μg/ml), leading to an estimate of $4.40 \times 10^5$ total transformed clones. The pellet was suspended in YPD broth and G418 (100 μg/ml), gentamicin (50 μg/ml), and cefotaxime (100 μg/ml) and then incubated at room temperature for 2 days, 200 rpm. *C. neoformans* was pelleted by centrifugation for 10 min at 2000 rpm. The pellet was suspended in fresh YPD broth and G418 (100 μg/ml), gentamicin (50 μg/ml), and cefotaxime (100 μg/ml) and then cultured for an additional day. The *C. neoformans* sgRNA pool was pelleted and stored at −80°C in 30% glycerol. The sgRNA pool was cultured in YPD broth overnight at room temperature, 200 rpm before genomic DNA preparation for sequencing analysis (designated YPD4). The sgRNA pool YPD4 was cultured in YPD broth for an additional 15 days ($^1/_{100}$ of the culture was transferred to 2 liters of fresh YPD broth every 2 days) before transcytosis assay and animal infection

(designated YPD19). The sgRNA pool YPD19 was cultured in YPD broth for 3 days before genomic DNA preparation for sequencing analysis (designated YPD22).

### Knockout of laccase-encoding gene *LAC1* by sgRNA-mediated mutagenesis

The T-DNA vector pDHt-SK-NEO-CnU6 was linearized with Bam HI and Kpn I. Two sgRNAs were designed to target the coding region of *LAC1* (fig. S1). The sgRNAs together with the sgRNA hairpin were amplified by PCR using primers (LAC1 sgRNA forward and sgRNA body reverse) (table S4) and then assembled into the linearized pDHt-SK-NEO-CnU6 using NEBuilder HiFi DNA Assembly. The T-DNA cassettes were delivered into *C. neoformans* strain $H99_{CAS9}$ by *A. tumefaciens*–mediated transformation. About 400 to 500 transformants for each cassette were obtained by selection with the drug G418 for 3 days and then cultured in YPD broth for additional 18 days ($^1/_{100}$ of the culture was transferred to 200 ml of fresh YPD broth every 2 days). The transformants were cultured on YPD agar plates for 3 days at 30°C and then replica-spotted to melanin-inducing medium (glucose, 1 g; L-asparagine, 1 g; $KH_2PO_4$, 3 g; $MgSO_4{\cdot}7H_2O$, 0.25 g; L-dopa, 0.1 g; final volume, 1 liter) agar plates (*44*). The melanin-inducing agar plates were incubated at 30°C for 3 days for melanization. pDHt-SK-NEO-CnU6 was used as a negative control. The *LAC1* sequence targeted by *LAC1* sgRNA2 was amplified from white colonies by PCR with primers (LAC1 seq forward and LAC1 seq reverse) and then submitted for sequencing (table S4).

### Transcytosis of *C. neoformans* across the HBMEC monolayer

Transcytosis assay was carried out as described previously (*12*). The primary HBMEC ($6 \times 10^4$ cells per insert) was seeded in Millicell cell culture insert with a pore size of 12 μm and a diameter of 12 mm (EMD Millipore) and then cultured for 4 days in RPMI medium supplemented with 10% fetal bovine serum, 10% Nu-serum, 1% minimum essential medium (MEM) vitamin, 1% MEM nonessential amino acids, 1 mM sodium pyruvate, and penicillin-streptomycin (100 U/ml). The HBMEC was incubated for 1 hour in experimental medium composed of Medium 199 and Ham's F12 supplemented with 5% human serum before transcytosis assay. *C. neoformans* sgRNA pool ($1 \times 10^6$ cells per insert) was loaded in the upper chamber and incubated for 4 to 6 hours. Samples from the lower chamber of 500 inserts were collected and cultured in YPD broth for 2 days before genomic DNA preparation for sequencing analysis. A small fraction of the samples was plated on YPD agar, leading to an estimate of $1.43 \times 10^7$ total CFUs. The integrity of HBMEC monolayer was assessed by measuring the transendothelial electrical resistance (TEER) with an epithelial voltohmmeter (EVOM, World Precision Instruments) before and after assays.

### *C. neoformans* penetration into the brain

Six- to 8-week-old female C57BL/6 mice were anesthetized in a small container with a conical tube containing isoflurane (60% isoflurane in propylene glycol)–soaked cotton pad. For intranasal infection, $2 \times 10^7$ cells in 50 μl of phosphate-buffered saline (PBS) were delivered into the nares using a pipette. Thirty mice were infected intranasally, monitored daily, and euthanized by $CO_2$ inhalation when they showed debilitating symptoms of infection, i.e., loss of appetite, moribund appearance, or labored breathing. The brains and lungs were collected separately, homogenized, and cultured in YPD broth for 2 days before genomic DNA preparation for sequencing analysis. Small fractions of the samples were plated on YPD agar, leading to an estimate of $4.60 \times 10^9$ total CFUs from lungs and $2.60 \times 10^8$ total CFUs from brains. For intravenous infection, $2 \times 10^7$ cells in 100 μl of PBS were injected into the tail vein. Thirty mice were infected intravenously, monitored daily, and euthanized via $CO_2$ inhalation once they exhibited debilitating symptoms of infection. The brains were collected, homogenized, and cultured in YPD broth for 2 days before genomic DNA preparation for sequencing analysis. A small fraction of the sample was plated on YPD agar, leading to an estimate of $7.26 \times 10^7$ total CFUs from brains.

### Deep sequencing

Frozen cell pellets were lyophilized and ground into powder in liquid nitrogen. Genomic DNA was purified independently from each sample using hexadecyltrimethylammonium bromide (Sigma-Aldrich). PCR was performed with barcoded primers (sgRNA seq forward and sgRNA seq reverse) using Q5 Hot Start High-Fidelity 2X Master Mix (NEB) with the following cycling parameters: 30 s of initial incubation at 98°C, 28 cycles at 98°C for 10 s and at 72°C for 30 s, and a final elongation step of 2 min at 72°C (table S4). For each sample, five separate 50-μl reactions with 2 μg of genomic DNA in each reaction were performed and then combined. The ~310-bp bands from each sample were purified from DNA-PAGE, quantified, mixed, and sequenced using NextSeq 500 (Illumina).

### Data analysis

All the 23-bp sequences with CCGG, CCNGG, or CTAG at 5′ or 3′ termini were identified from *C. neoformans* H99 genome sequence. To build the designed sgRNA library, the resulting 23-bp sgRNA + PAM sequences were aligned to the protein-coding sequences using Bowtie 2 with very sensitive mode (*22*). The sgRNAs targeting multiple sites within the *C. neoformans* H99 genome were eliminated from further investigation. Fastq files were demultiplexed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Demultiplexed reads were trimmed using Cutadapt (*45*), leaving only the 20- or 21-bp spacer (guide) sequences. The 21-bp spacer sequences were trimmed into 20 bp, leaving the 20-bp sgRNA adjacent to the PAM site. The 20-bp spacer sequences were then mapped to the designed sgRNA library. Reads for each sgRNA sequence were counted in *R*. Reads were normalized as follows (*46*): normalized reads per sgRNA = (reads per sgRNA/total reads for all sgRNAs in sample) $\times 10^6 + 1$. Figures were generated using the normalized reads in R.

### Southern blot

About 50 μg of genomic DNA was digested with appropriate restriction enzymes and then subjected to separation on 0.8% agarose gel. The blotting was conducted on nylon membranes, positively charged (Sigma-Aldrich, 11209272001). The PCR fragment of wild-type gene was used as the probe and labeled with DIG-High Prime DNA Labeling and Detection Starter Kit II (Sigma-Aldrich, 11585614910).

### Complementation of knockout mutants

The wild-type gene (including its native promoter, ORF, and terminator), hygromycin resistance gene *HYG* marker, and 3′ flanking regions (~1 kb) were amplified by PCR. The PCR fragments were assembled into plasmid pUC19 using NEBuilder HiFi DNA Assembly. The resulting cassettes were linearized with appropriate restriction enzymes and introduced into the knockout mutants by biolistic

transformation. The resulting complemented strain generated via homologous recombination was selected based on losing drug nourseothricin resistance. The complemented strains were further verified by PCR and phenotype analysis.

### Ethics statement

All mice were handled in strict accordance with good animal practice, as defined by the relevant national and/or local animal welfare bodies. All animal work was approved by Johns Hopkins University Animal Care and Use Program under protocol no. M019M42.

### Code availability

Custom batch scripts used for execution of these computational tools can be found in Supplementary Codes.

## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/38/eabb8783/DC1

View/request a protocol for this paper from *Bio-protocol*.

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