Supplementary Materials for

RELATe enables genome-scale engineering in fungal genomics

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Supplementary Methods
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Other Supplementary Material for this manuscript includes the following:

(available at advances.sciencemag.org/cgi/content/full/6/38/eabb8783/DC1)

Tables S2 to S4
Supplementary methods

sgRNA pool construction with restriction/ligation method

Linker preparation

All linkers are ordered from IDT as oligonucleotides. Linkers 1 and 3 are PAGE purified. Phosphorylation is using T4 PNK. Annealing is carried out in 1 x CutSmart buffer in a beaker with 1-liter boiling water which cools down to room temperature naturally. All completed adapters are drop-dialyzed against ddH₂O for 20 min before use using a dialysis disc.

Linker 1 (82 nt):

5’-
GTTGGATAGTGACTGCGGCTCCATAGACTAGCTCAGGACCAGGATCTTA
GATAGTAATCAACAGCCCCCTCTTAAATTCCAAAC-3’

3’-
CAACCTATCACATGACGCCGAGGATATCGAGGTCCTGTTCTAGAAT
CTATCATTAGTGGTCGGGGAGGATTAAGGTTG-5’

MmeI site
BsaXI site
ScrfI site

Preparation:
Phosphorylate each oligo separately, then anneal. Heat inactivate T4 PNK.

Linker 2 (40+2 nt):

Top: 5’-AAAGTGGAAACTCTACATTCACGAGGTGTATATACTGTGGNN-3’
Bottom: 3’-TTTCACCTTGGAGATGTAAGTGCTCCACATATATGACAAAC-5’

C. neoformans U6 promoter

Additional G

Preparation:
Phosphorylate “bottom” oligonucleotide only. Heat-inactivate T4 PNK and anneal to “top” oligo.

Linker 3 (80+3 nt):

Top:
5’-
TTAGAGCTAGAAATAGCAAGTTAAAATAAAGGCTAGTCCGTTATCAACTTG
AAAAAAAAAGTGGCACGGCAGGTCGGGTCTTTTTT-3’

Bottom:
sgRNA body
7-T terminator
Preparation:
Phosphorylate “top” oligonucleotide only. Heat-inactivate T4 PNK and anneal to “bottom” oligo.

Protocol:
1. Mix the following:
   - 143 µl DNA at 70 ng/µl (10 µg)
   - 20 µl 10 x CutSmart Buffer
   - 27 µl ddH2O
   - 2 µl rSAP

   Incubate at 37°C for 30 min. Heat inactivate at 65°C for 10 min. Treatment with rSAP prevents broken DNA from ligating to adapters.

2. Split the sample into 3 tubes, add the following restriction enzymes to each tube:
   - 1.5 µl HpaII (50 U)
   - 3 µl ScrFI (50 U)
   - 1.5 µl BfaI (50 U) (Store at -80°C)

3. Incubate for 1 h at 37°C. Inactivate the enzymes at 80°C, 20 min. Mix sample with 2 volumes of Oligo Binding Buffer and 8 volumes of ethanol, purify over 1 column each and elute in 10 µl ddH2O each.

4. In a 100 µl reaction volume, add 10 µl 10 x MBN reaction buffer and 5 µl Mung Bean Nuclease to the digested DNA sample. Incubate at 30°C for 30 min.

5. Stop MBN digestion with 1 µl 1% SDS.

6. Mix the sample with 2 volumes of Oligo Binding Buffer and 8 volumes of ethanol. Split over 2 Zymo columns and elute in 10 µl ddH2O each. Take 0.5 µl sample and label it 1.

7. Drop-dialyze sample for 20 min.

8. Drop-dialyze the phosphorylated MmeI linker (Linker 1, 82 nt) for 20 min and then ligate:
   - 20 µl DNA
   - 20 µl Linker 1 (5 pmol/µl)
   - 44 µl 2 x Quick Ligation Buffer
   - 3 µl Quick Ligase
   - 1 µl T4 Polynucleotide Kinase

9. Incubate for 15 min at room temperature.
10. Mix the sample with 5 volumes of Zymo DNA binding buffer. Split across 2 Zymo columns and elute in 10 µl ddH₂O each. Take 0.5 µl sample and label it 2.

11. Mix the sample (about 18 µl) together with 20 µl 10 x Cutsmart, 10 µl ScrFI and 6 µl AclI. Incubate at 37°C, 1 h. Take 5 µl sample and label it 3. ScrFI and AclI break up tandem adapter products into a ~40 bp size which will be removed by Ampure selection.

12. Mix with 1.8 volumes Ampure XP beads. Collect the sample on magnet, wash three times with fresh 70% ethanol, dry and elute in 30 µl ddH₂O. Take 1 µl sample and label it 4.

13. Digest with MmeI:

\[
\begin{align*}
&30 \mu l & &\text{DNA} \\
&14 \mu l & &\text{H}_2\text{O} \\
&5 \mu l & &10 \times \text{Cutsmart} \\
&0.8 \mu l & &3.2 \text{mM SAM} \\
&1 \mu l & &\text{MmeI (MmeI prefers two copies of its recognition sequence for cleavage to occur, excess MmeI blocks cleavage)}
\end{align*}
\]

14. Incubate at 37°C for 1 h and then inactivate MmeI at 65°C for 20 min. Take 2 µl sample and label it 5. Dialyze sample for 20 min.

15. Drop-dialyze the phosphorylated Linker 2 (U6 promoter) for 20 min and then ligate:

\[
\begin{align*}
&50 \mu l & &\text{DNA} \\
&12 \mu l & &\text{Linker 2 (5 pmol/µl)} \\
&67 \mu l & &2 \times \text{Quick Ligation Buffer} \\
&5 \mu l & &\text{Quick Ligase}
\end{align*}
\]

16. Incubate for 15 min at RT. Mix the sample with 5 volumes of Zymo buffer, purify on a Zymo column and elute in 10 µl ddH₂O. Take 0.5 µl sample and label it 6.

17. To the 9 µl recovered, add 35 µl ddH₂O, 5 µl 10 x Cutsmart and 1 µl BsaXI (BsaXI cannot be heat-inactivated, addition of greater than 2 units of BsaXI in a 16 h incubation is not recommended due to DNA binding.). Incubate at 37°C for 1 h.

18. Drop-dialyze sample for 20 min. Wash dialysis drop with additional 5 µl ddH₂O, Take 1 µl sample and label it 7.

19. Drop-dialyze the phosphorylated Linker 3 (sgRNA body) and then ligate:

\[
\begin{align*}
&50 \mu l & &\text{DNA} \\
&8 \mu l & &\text{Linker 3 (5 pmo/µl)} \\
&63 \mu l & &2 \times \text{Quick Ligation Buffer} \\
&5 \mu l & &\text{Quick Ligase}
\end{align*}
\]

20. Incubate for 15 min at RT. Mix the sample with 5 volumes of Zymo PCR purification buffer. Purify over a column and elute in 10 µl ddH₂O. Take 0.5 µl sample and label it 8.

21. Amplify the ligation product:

\[
\begin{align*}
\text{PCR mixture:} \\
&2 \mu l & &\text{Ligation product}
\end{align*}
\]
0.25 µl sgRNA library forward and sgRNA body reverse (0.5 µM final) 
(table S4)

12.5 µl 2 x Q5 HotStart Master Mix
10.25 µl ddH₂O

PCR cycle:
98°C 30 s
98°C 10 s \n60°C 10 s | x 10
72°C 20 s /
72°C 2 min

22. Take 1 µl PCR product and label it 9. Run gel to check library for presence of 177 bp band (fig. S3).

23. Mix the 24 µl remaining PCR product with 20 µl Orange DNA Loading Dye (6 x). Run the sample across 4 lanes of a 10-well gel and stain with 1 µl GelStar (sensitivity, 0.02 ng) in 10 ml 1xTBE buffer for 30 min.

24. Cut out 177 bp band under blue light illuminator. Crush gel slice with pellet pestle and mix with 2 volumes of diffusion buffer (1 mM EDTA 8.0, 0.1% SDS, 0.5 M ammonium acetate, 10 mM magnesium acetate). Incubate at 50°C for 30 min.

25. Microcentrifuge the sample for 1 min. Remove the supernatant to a clean tube. Spin through Corning® Costar® Spin-X® centrifuge tube filters, Nylon membrane, pore size 0.2 µm (3 min, max speed). Column-purify using QIAEX II Gel Extraction Kit, elute in 20 µl ddH₂O (4 ng/µl).

26. Clone into pDHt-SK-NEO-CnU6 with NEBuilder® HiFi DNA Assembly Master Mix.

Reagents needed:

• Ampure XP beads (Beckman A63880)
• DNA Clean and Concentrator Columns (Zymo Research D4003)
• Oligo Binding Buffer (Zymo Research D4060-1-10)
• Dialysis discs (Millipore VSWP04700)
• GelStar (Lonza 50535)
• Invitrogen™ Novex™ TBE Gels, 10%, 10 well,Catalog No.EC6275BOX
• Orange DNA Loading Dye (6X), Catalog number: R0631
• Agencourt SPRIStand Magnet PN A29182
• Kimble™ Kontes™ Pellet Pestle™, Catlog#, K749521-1500
• Corning® Costar® Spin-X® centrifuge tube filters, Nylon membrane, pore size 0.2 µm

The following are purchased from New England Biolabs
- Bfai (R0568S)
- HpaII (R0171S)
- ScrFI (R0110S)
- AclI (R0598S)
- Mung Bean Nuclease (M0250S)
- Quick ligation kit (M2200S)
- Mmel (R0637S)
- BsaXI (R0609S)
- rSAP (M0371S)
- T4 PNK (M0201S)
- 2X Q5 Hot Start Master Mix (M0494S)
- 50 bp DNA Ladder (N3236S)
- NEBuilder® HiFi DNA Assembly Master Mix (E2621L)
- NEB® 10-beta Competent E. coli (High Efficiency) (C3019H)

> pDHt-SK-NEO-CnU6-sgRNA

**T-DNA**

**NEO**

**U6 promoter**

**Protospacer**

**sgRNA body**

```
TGGCAGGATATATTTGTGGTGTAACAAAAATTTGACGCTTAGACAACTTAAATACA CATTGCGGACGTCTTAAATGTACTGAAATTAACGCGGAAATTACGCGGATC TGGATTTTAGTACTGGAATTGGTGGTGTTAGAATAGAATTTTACAGATAGAAT  
TATTTTGCATATAAAATACATATACCAATAGGTGGTCTTTATATGCTCAACACATGA 
GGCGAAACCTATAGGAAACCTAATTCCCTCTTTATCTGGGAACACTACTACACACAT  
TTATGGAAGAAACTCAGATTGGCAAGCTGCTCTAGCCCATACGAAACCCGACTCT CCCCAGCGCCTTGCGGCGATATTCTAAATGTGAGCTGGCTCTCTCTTCTCTCTCGGTAT TCCCCTGATTCTCTGTGGATAACCCTATACCCTCTTCTTTGGAGGTGGAGCGTAACCGCCT CCGCGACGCGAAGCAGCGACGGCGACGAGTCAGGAGCGAGCAGGAAACCGGAAG AGCGCCACTACGCAAACCCGCTCTCCCGCCTGCCTGGCGAATTACCGGCGAACGCAA TTAATGTGAGTTAGCCTACATATTGGAACGCCAGGATTTACACCTTACGGTAGC GGGCTGCTGATTGGTTGTAATGTGAGCTGGATACACACACACACACACACACAGGAAAC AGCTATGACCATGGATTACGCCAAGCGCGAATTAACCCTCACTAAAGGGAAC AAAAGCTTGGAGCTCGAGGATGTGAGCTGGAGAGCGCGACGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA GGAATGCTCATAAGCATGCCAGATCTGGAGCTGGAGAGCGCGACGCGAAGGGGA... ```
TATTCGTATAGGGACAATCCGATATGTCGATGGAGTGAAAGAGCCTGATGCAC
TCCGCATACAGCTCGATAATCTTTTCAGGGCTTTGTTCATCTTCATACTCTTCC
GAGCAAAAGGACGCCATCGGCCCTCACTCATGAGCAGATTTGCTCCAGCCATCATG
CCGTTCAAAGTGCAGGACCTTTGGAAACAGGCAGCTTTCCCTTGCAAGCCATAGCA
TCATGTCCCCCTCCGTCCACATCAGGTGGTCCCTTTTATACCGGCTGTCCG
TCACTTTTTAAATATAGTTTTTCATTTTCATCCCACCAGCTTATATACCTTAGCAG
GAGACATTCCTTTCCGTATCTTTTACGCAGCGGTATATTTCTCGATCAGTTTTTCA
ATTCCCGGTGATATTCTCATTTTCAGCCATTATTATTTCTCTCTCTTTTCTACAG
TATTTAAAGATACCCCAAGAAGCTAATTATAACAAGACGAACTCCAATTCAGT
GCCCTTTGCATTCTAAAACCTTTAATACCAAGAAAACAGCTTTTTCGAAAGTTGTT
TTCAAAAGTGGCGTATAACATAGTATCGACGGAGCCGATTTGGAAACCGCCTGT
GATCAGCGCAGCAGCTCTGCTATCGTTACAAATCAACATACGTACCCCTCCGC
GAGATCATCCGTGTCTAAAACCGCAGCTTAGTTGCCGTCTTTCCGAATAGC
ATCGGTAACCATGAGCAAAGTCTCGCCGCTATACAACGGCTCTCCCGCTGACGCC
GTCCCGGACTGAGGTGGGCTGCTGTTTGATATGGGCGAGCTGGC
GGTCGGGGAGCTGGTGCTGGCTGG
Supplementary codes

Codes for trimming the reads in Cutadapt:
cutadapt -a GTTTTAGAGCTAGAAATAGCAAG -o Sample1cut3.fasta.gz Sample1.fastq.gz
This was used to remove the 3’ adapter.

cutadapt -g GGAAACTCTACATTACGAGGTATATACTGTTG -o Sample1cut35.fasta.gz Sample1cut3.fasta.gz
This was used to remove the 5’ adapter.

Codes for read counting in R:
```r
> setwd("file route")
> Sample1<-read.table(file="Sample1cut35.fasta.gz.",header=FALSE,sep="\t")
> Sample1count=table(Sample1$V1)
> write.table(Sample1count,file=" Sample1count.txt",sep="\t")
```

Codes for mapping sgRNAs to protein-coding genes in Bowtie2:
```
./bowtie2-build Proteinncodinggenes.fa Proteinncodinggenes
./bowtie2 --very-sensitive -f -x Proteinncodinggenes -U sgRNAsInput.fa -S sgRNAsOutput.sam
```

Codes for cumulative frequency curve in R:
```r
> setwd("file route")
> Read<-read.table(file="Samples.txt",sep="\t", header=TRUE)
> tiff(filename="Samples.tiff", width=2000, height=2000,pointsize=70)
> plot(ecdf(Read$YPD4),xlim=c(0,30),xlab="Number of sgRNA per gene",ylab="Cumulative frequency",main="", col="black",cex=0,lwd=10)
> lines(ecdf(Read$YPD22),col="red", lwd=10)
> lines(ecdf(Read$L),col="brown", lwd=10)
> lines(ecdf(Read$B),col="green", lwd=10)
> lines(ecdf(Read$Biv),col="Blue", lwd=10)
> lines(ecdf(Read$T),col="Purple", lwd=10)
> legend('bottomright',legend=c("YPD4"","YPD22","L","B","Biv","T"),
col=c("black","red","brown","green","blue","purple"),pch=15)
> dev.off()
```

Codes for heatmap in R:
setwd("file route")
> Sample<-read.table(file="Sample1.txt", header=TRUE, sep="\t")
> colors=c(seq(0,1,length=10),seq(1.0001,2.5,length=10),seq(2.5001,6,length=10))
> col2<-colorRampPalette(rev(brewer.pal(10,"RdYlBu")))(29)
> tiff(filename="Heatmap.tiff",width=3000,height=8000,pointsize=80)
> heatmap.2(heatmap2,scale="none",col=col2,breaks=colors,dendrogram="none", Colv=FALSE, trace="none",density.info="none",symm=F, symkey=F,symbreaks=T, Rowv=TRUE)
> dev.off()

**Supplementary figures**

**fig. S1.** Design of sgRNAs to knockout LAC1. LAC1 sgRNA1, highlighted in red; LAC1 sgRNA2, highlighted in orange; HpaII (C/CGG) and ScrFI (CC/NGG), underlined.
fig. S2. **Outline of restriction/ligation method.** 1. Digest genomic DNA with ScrFI; 2. Blunt the ends with Mung Bean nuclease; 3. Ligate to linker 1 (82 nt), adaptor; 4. Break up tandem adapters into 41 nt half-adapters with ScrFI and AclI; 5. Remove 41 nt half-adapter with Ampure XP beads; 6. Digest with MmeI to produce 20-nt substrate fragment adjacent to 41 nt half-adapter; 7. Ligate to linker 2 (40+2 nt), U6 promoter; 8. Digest with BsaXI to complete removal of the adapter; 9. Ligate to linker 3 (80+3 nt), gRNA body.

![PAGE gel analysis of restriction/ligation fragments](image_url)

fig. S3. **PAGE gel analysis of restriction/ligation fragments.** DNA fragments were visualized under blue light after staining with GelStar. 1. Digest genomic DNA with ScrFI, HpaII, and BfaI; 2. Ligate to linker 1; 3. Digest with ScrFI and AclI; 4. Remove the half-adapter with Ampure XP beads; 5. Digest with MmeI; 6. Ligate to linker 2; 7. Digest with BsaXI; 8. Ligate to linker 3; 9. Amplify the ligation product by PCR; M, DNA ladder; *, the final sgRNA pool generated by restriction/ligation.
fig. S4. Venn diagram comparing potential genes for *in vitro* fitness with essential *S. cerevisiae* orthologs. Genes for *in vitro* fitness were defined as normalized log2 fold change < -1 (YPD22 vs YPD4). The orthologs of known essential genes of *S. cerevisiae* in *C. neoformans* were used for comparison.
**fig. S5. Fourteen additional genes contributing to *C. neoformans* penetration of HBMEC monolayer.** (A) Transcytosis assay using HBMEC monolayer. HBMEC monolayer was incubated with $1 \times 10^6$ *C. neoformans* for 4 h. Transcytosis frequency (%) was determined $\{(\text{total CFUs recovered from the lower chamber}/\text{total number of cryptococcal cells added to the upper chamber}) \times 100\}$. Relative transcytosis (%) was determined $\{(\text{transcytosis frequency of mutant or complemented strain}/\text{transcytosis frequency of wild type strain}) \times 100\}$. (B) Growth test on YPD agar for 3 days at 37°C. Photo credit: Z. Li, Johns Hopkins University. (C) *C. neoformans* penetration into brain. CFUs from the brains were determined 24 h after intravenous infection with $1 \times 10^5$ cells. Wild type strain KN99 and mutants (n=5). P value is determined by Student’s t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data shown are means ± SEM.
**fig. S6. Southern blot analysis.** (A) Mutant *sfp1Δ*. Genomic DNA, digested with KpnI; probe template, amplified by PCR with primers (CNAG_03174 forward and CNAG_03174 reverse). (B) Mutant *wdr1Δ*. Genomic DNA, digested with HindIII; probe template, amplified by PCR with primers (WDR1 forward 5 and WDR1 reverse 5). (C) Mutant CNAG_02449. Genomic DNA of mutant CNAG_02449, digested with EcoRI; probe template, amplified by PCR with primers (CANG_02449 forward and CANG_02449 reverse). (D) Mutant CNAG_01941. Genomic DNA of mutant CNAG_01941, digested with PstI; probe template, amplified by PCR with primers (CANG_01941 forward and CANG_01941 reverse).

**fig. S7. Complementation of mutants *sfp1Δ* and *wdr1Δ*.** (A) Complementation of mutant *sfp1Δ* was verified by PCR with primers (SFP1 forward 5 and SFP1 reverse 5). (B) Complementation of mutant *wdr1Δ* was verified by PCR with primers (WDR1 forward 5 and WDR1 reverse 5).

**Supplementary tables**

**table S1.** Summary of the sgRNA library coverage.
| Sample | CFUs     | Reads    | Number of sgRNA | Read per sgRNA on average | Number of targeted gene | sgRNA per gene on average | Coverage |
|--------|----------|----------|-----------------|---------------------------|------------------------|---------------------------|----------|
| YPD4   | 4.40E+05 | 33473526 | 57356           | 584                       | 6842                   | 8.4                       | 98%      |
| YPD22  | 4.40E+05 | 30202697 | 47751           | 633                       | 6770                   | 7.1                       | 97%      |
| L      | 4.60E+09 | 41082906 | 48228           | 852                       | 6779                   | 7.1                       | 97%      |
| B      | 2.60E+08 | 44745047 | 2944            | 15199                     | 2229                   | 1.3                       | 32%      |
| Biv    | 7.26E+07 | 35587441 | 44327           | 803                       | 6746                   | 6.6                       | 97%      |
| T      | 1.43E+07 | 34551855 | 46615           | 741                       | 6774                   | 6.9                       | 97%      |

table S2. Gene set enrichment analysis output for negative selection of genes contributing to blood-brain barrier penetration.

table S3. 82 deletion strains available in fungal genetics stock center.
“-”, not tested.

table S4. Primer sequences used in this research.