Candida albicans gains azole resistance by altering sphingolipid composition

Jiaxin Gao1,2,3, Haitao Wang4,5, Zeyao Li1,6, Ada Hang-Heng Wong4, Yi-Zheng Wang1, Yahui Guo7, Xin Lin7, Guisheng Zeng5, Haoping Liu3, Yue Wang5,8 & Jianbin Wang1,2

Fungal infections by drug-resistant Candida albicans pose a global public health threat. However, the pathogen’s diploid genome greatly hinders genome-wide investigations of resistance mechanisms. Here, we develop an efficient piggyBac transposon-mediated mutagenesis system using stable haploid C. albicans to conduct genome-wide genetic screens. We find that null mutants in either gene FEN1 or FEN12 (encoding enzymes for the synthesis of very-long-chain fatty acids as precursors of sphingolipids) exhibit resistance to fluconazole, a first-line antifungal drug. Mass-spectrometry analyses demonstrate changes in cellular sphingolipid composition in both mutants, including substantially increased levels of several mannosylinositolphosphoceramides with shorter fatty-acid chains. Treatment with fluconazole induces similar changes in wild-type cells, suggesting a natural response mechanism. Furthermore, the resistance relies on a robust upregulation of sphingolipid biosynthesis genes. Our results shed light into the mechanisms underlying azole resistance, and the new transposon-mediated mutagenesis system should facilitate future genome-wide studies of C. albicans.
**Canidida albicans** is a major fungal pathogen of humans, affecting millions of people and kills hundreds of thousands worldwide annually.1 Mortality rates of invasive C. albicans infection remain high despite the treatment with existing antifungal therapies. Multidrug-resistant * Candida* species and strains are rapidly emerging and spreading globally, threatening to render our entire antifungal arsenal obsolete.2 Azoles are common first-line drugs against most * Candida* species in systemic infections, which act by inhibiting the 14α-demethylase Erg11 in the ergosterol biosynthesis pathway and causing the membrane damage.3 Extensive research has demonstrated that fungal pathogens can quickly evolve various resistance strategies after exposure to an antifungal agent. C. albicans can gain resistance to azoles via genetic alteration of the drug target Erg11, upregulation of efflux pumps Cdr1, Cdr2, and Mdr1, and inactivation of Erg3 that synthesizes the toxic sterol.4,5 However, our mechanistic understanding of antifungal resistance is incomplete.6 It remains unclear why some fungal species are intrinsically resistant or easily acquire resistance to multiple antifungal drugs. Thus, it is imperative to identify and understand the molecular mechanisms that govern antifungal resistance.

Genome-wide genetic screening is a powerful tool to identify genes involved in a biological process. However, it has been difficult to apply these technologies to *C. albicans* due to its diploid genome. Although several recent studies conducting large-scale genetic screens of *C. albicans* mutant libraries resulted in important findings,6–10 investigation of many aspects of *C. albicans* biology still relies on inference from data obtained in the studies of *Saccharomyces cerevisiae*.11,12 This approach inevitably misses out genes that perform specific functions and determine unique traits in *C. albicans*. For example, *S. cerevisiae* is non-pathogenic and does not grow true hyphae which are the predominant morphology of *C. albicans* in host tissues and a key virulence factor.11 Currently, most *C. albicans* genes with limited homology to *S. cerevisiae* remain uncharacterized.13

Recently, some haploid *C. albicans* strains were isolated.14 Although avirulent, the haploids possess most traits that define the species including the ability to undergo the yeast–hyphae transition, the white-opaque phenotypic switch, the formation of chlamydospores, and mating.14 The haploids have opened up opportunities for scientists to explore large-scale, genome-wide genetic screening strategies in this pathogen. The recent application of CRISPR-cas9 technology in haploid *C. albicans* in the identification of new virulence regulators and genetic networks demonstrates the massive potential of using haploid *C. albicans* in genetic screens.15

Transposon-mediated mutagenesis is a widely used genetic tool. Transposons are movable genetic units and have two essential components, a transposase and a pair of transposon-specific inverted terminal repeats (ITRs). One example is the *piggyBac* (PB) transposable element, first isolated from the moth *Trichoplusia ni* and later adapted to other organisms from bacteria to mammals.16,17 PB transposase recognizes the ITRs and moves the entire content from one chromosomal locus and integrates it at a TTAA motif in a new locus.18 PB transposition occurs via a precise cut-and-paste mechanism which leaves no footprints behind, resulting in a full restoration of the donor site.19,20 Thus, a phenotype caused by a PB insertion can be easily verified by phenotype reversion. Furthermore, PB transposon inserts randomly throughout the genome.20

In this study, we develop a highly efficient, PB-based mutagenesis system using stable haploid *C. albicans* strains. We apply this technology to conduct genome-wide genetic screens for genes involved in resistance to the antifungal drug fluconazole and discover a new resistance mechanism.

**Results**

**Construction of the piggyBac transposition system in *C. albicans***. We first optimized the piggyBac transposase (PBase) gene for use in *C. albicans*. We replaced all CUG codons with UUG because CUG encodes serine instead of the conventional leucine in *C. albicans*, added a 3xSV40 nuclear localization signal to its N-terminus, and controlled the expression using the tetracycline-inducible (Tet-On) promoter (Fig. 1a). Also, we mutated seven amino-acid residues to generate a hyperactive transposase according to previous studies.21 We then integrated this CaPBase gene, using SAT1 as the selectable marker, at the ADH1 locus of the stable, uridine auxotrophic (Ura-) haploid *C. albicans* strain GZY80322 to generate the YW01 CaPBase-expressing strain.

To test whether PB transposition would occur in *C. albicans*, we transformed the donor plasmid pPB[URA3] carrying the ITR-URA3-ITR cassette into YW01 cells that had been grown in the absence or presence of doxycycline (Dox) for 48 h and then spread the cells onto uridine-deficient (-U1) plates. The transformation of Dox-induced cells led to the growth of numerous colonies, while the uninduced cells produced only a few likely due to the basal expression of CaPBase (Fig. 1b), an event that happened in <0.001% of the cells. This result indicated that the Dox-induced CaPBase excised the PB cassette from the plasmid and integrated it into the genome.

Previous studies in other organisms showed that PB excision did not leave any footprint behind.19 To confirm this characteristic in *C. albicans*, we constructed a *C. albicans* strain in which we inserted the PB[URA3] cassette at a TTAA site (n.t. 709–712) within the open reading frame (ORF) of ARG4 (Fig. 1c), yielding YW02 that is arginine auxotrophic (Arg−) and uridine prototrophic (Ura+) and also expresses CaPBase from the Tet-On promoter at the ADH1 locus. Precise excision of PB would convert YW02 cells from Arg− to Arg+, After Dox induction for 48 h, we spread the YW02 cells onto glucose minimal medium (GMM) plates, picked 20 independent colonies, and confirmed that all 20 clones grew well on -Arg-U1 plates (Fig. 1d), indicating that the cells had excised PB[URA3] out of ARG4 precisely. This was confirmed by site-specific PCR using two pairs of primers as shown in Fig. 1c (Fig. 1e shows examples of 5 clones) and DNA sequencing analysis of the PCR products. To map PB insertion sites, we performed inverse PCR as described in Fig. 1f which allowed amplification of the DNA fragment encompassing a transposon junction. DNA sequencing analysis of the PCR products and homology search of the *Candida* Genome Database (CGD) located the PB insertion sites on all eight chromosomes (Supplementary Table 1), seven within ORFs and 13 in intergenic regions (Fig. 1g). Our results demonstrated random PB transposon insertion and precise excision in *C. albicans*.

**Ploidy and PB transposition efficiency analysis**. Autodiploidization observed in *C. albicans* haploids has been a major concern regarding their applicability in genetic screens.14 To evaluate ploidy stability during transposition, we first performed qPCR analysis to measure CaPBase expression levels at timed intervals in YW02 cells grown on solid media with or without Dox. The results demonstrated a sharp increase of CaPBase expression upon Dox addition (Fig. 2a). In parallel, we examined cell ploidy by flow cytometry and found that the cells retained IN DNA content for at least 72 h (Fig. 2b) regardless of Dox addition, indicating a stable ploidy during the robust expression of...
Tests in liquid medium produced similar results (Supplementary Figure 1a and 1b).

We next estimated \( \text{PB} \) transposition frequency. Aliquots of YW02 cells grown on solid medium containing Dox were harvested at intervals, and each was divided into two equal parts, and one was spread onto a YPD plate that allows all cells to grow and the other onto GMM\( +\)histidine (His) plates where only Arg\( +\)Ura\( +\)transposants can grow. Transposition frequency was calculated by dividing the number of colonies on the GMM plate with that on the YPD plate. Figure 2c shows that \( \text{PB} \) transposition frequency increased with time during Dox induction, reaching \(~5\%\) at 72 h. We also achieved a similar frequency in liquid medium (Supplementary Figure 1c).

Multiple transposon insertions in a genome complicate subsequent identification of the mutation responsible for a phenotype. Although a single copy of \( \text{PB} \) transposon exists at the ARG4 locus in YW02, the copy number may increase when transposition happens in S or G2 cells\(^2\). To test whether \( \text{PB} \) copy number changes during transposition, we analyzed the copy number of \( \text{PB}[\text{URA3}] \) in transposants. After 24 h of CaPBase induction, we spread the culture onto GMM\( +\)His plates to select transposants and randomly picked 10 colonies.

Fig. 1 \( \text{PB} \) transposition in haploid \( \text{C. albicans} \). a Schematic description of the CaPBase construct inserted at the ADH1 locus. Ptet, Tet-On promoter; 3XSV40, SV40 nuclear localization signal; and \( P_{\text{ADH1}} \), ADH1 promoter. Hyperactive mutations are indicated along the top. b Selection of transformants with \( \text{PB}[\text{URA3}] \) integration on plates. YW01 cells were grown in YPD for 48 h in the presence (+) or absence (−) of Dox before transformation with pPB[URA3]. Transformation products were spread onto GMM plates (90 mm Petri dishes). c Schematic description of the PB[URA3] cassette integrated within ARG4. Arrows indicate the PCR primers used to detect \( \text{PB} \) excision. d Confirmation of the auxotrophic phenotypes of transposition products of YW02. Strains grown on a GMM\(+\)Uri\(+\)Arg\(+\)His plate were replica-transferred onto a GMM\(+\)Uri\(+\)His\(+(\text{Arg}) \) or GMM\(+\)Arg\(+\)His\(+(\text{Uri}) \) plate and then incubated at 30 °C overnight. YW01 and YW02 were included as controls. e Precise excision of \( \text{PB} \) from the ARG4 locus. Representative gel electrophoresis results of PCR products amplified from YW02 transposition products using primers shown in c. f Schematic description of the identification of \( \text{PB} \) insertion sites by inverse PCR. Arrows indicate the PCR (PBLf and PBLr) and sequencing (Seq1) primers used. g Distribution of \( \text{PB} \) insertion sites in and around ORFs in YW02 transposition products. Positions in the ORF were determined by treating the entire length of an ORF as 100 and then determining the relative position of an insertion in the ORF. Positions in the 5′ or 3′ region were determined by whether the insertion was closest to the 5′ or 3′ end of an ORF.
qPCR to determine the level of PB DNA in comparison with a control gene CDC28 in the genome. All clones were found to contain a single copy of PB (Fig. 2d and Supplementary Figure 1d), indicating that the vast majority of transposants harbored a single PB insertion in the genome.

**Genome-wide analysis of PB transposon distribution.** Next, we analyzed PB distribution in the genome using next-generation sequencing (NGS). Transposition was induced with Dox for 24 h, and then ARG^+Ura^+ transposants were selected on GMM+His plates. Genomic DNA was extracted from the transposant pool.
and subjected to NGS. We acquired 11,595,368 overlapping read pairs, each containing one part matching the PB element and the other unambiguously matching a sequence of the *C. albicans* genome. The PB transposon distribution in 10-kb non-overlapping windows is largely uniform throughout the genome (Fig. 2e and Supplementary Figure 1e). On average, there were 20 unique insertion sites in a 10-kb window (Fig. 2f), i.e., ~1 every 500 bp. We detected PB inserts in nearly 5000 genes out of a total of 6466 annotated and predicted genes in CGD. The failure to find PB insertion in the rest of the genes is likely due to their essentiality or the fitness cost caused by the PB insertion, consistent with the ~1100 essential genes in *S. cerevisiae*.

Nucleotide motif analysis of the insertion sites revealed 82.4% at TTAA sites, 4.5% at TTAG, 4.4% at CTAA, 3.6% at TTGT, and 5.1% at other non-specific sites (Fig. 2g). Also, we found significant enrichment of Ts and As in the 5 nucleotides immediately upstream and downstream of the PB inserts, respectively (Fig. 2g). PB insertions occurred more frequently in promoter regions than in ORFs. This could be due to the enrichment of Ts and As nucleotides in promoters, which facilitates the unwinding of double-stranded DNA during transcription initiation. Another reason could be the fitness cost caused PB insertion in some ORFs, which may reduce their presence in the transposant population.

**PB mutagenesis for forward genetic screens of 5-FOA resistant mutants.** To evaluate the effectiveness of the PB transposon system in genetic screens in *C. albicans*, we performed a proof-of-principle screen for mutants resistant to 5-fluoroorotic acid (5-FOA). As 5-FOA is converted to the toxic 5-fluorouracil in strains expressing a functional URA3, we wanted to determine how frequently we can identify *ura3* mutants in the transposon insertion library. We constructed a starting strain YW05 by using URA3 to delete HIS1 in GYZ803 and then inserting a PB[HIS1] cassette in ARG4 ORF. YW05 also expresses CapBase from the ADH1 locus. Figure 3a describes the steps of the screen. YW05 cells were grown as patches on YPD plates containing Dox for 48 h to induce the transposition. Then, the cells were replica-transferred to GMM+Uri plates to enrich Arg+His+ transposants which were then transferred to GMM+Uri+5-FOA plates to select for 5-FOA-resistant mutants. Among 30 colonies randomly picked from the 5-FOA plates were 26 true 5-FOA-resistant mutants (Fig. 3b) and 4 clones (#3, #7, #18, and #21) were false and thus excluded from further characterization. Colony PCR using a pair of primers flanking URA3 failed to detect the 1.4-kb amplicon expected from the WT strain in 22 of the 26 5-FOA-resistant mutants (Fig. 3c), suggesting PB[HIS1] insertion in *URA3*. URA3 seemed intact in four clones, #4, #9, #15, and #26, suggesting PB disruption of other genes leading to 5-FOA resistance (Fig. 3c). Next, we performed PCR analysis of the 22 *ura3* mutants by pairing a URA3-flanking primer, URA3F or URA3R, with the PB-specific PBLR primer (Fig. 3d), followed by DNA sequencing analysis of the PCR products. We identified 4 different insertion sites in the URA3 locus (Fig. 3e). One was located in the promoter region, and all the others were located in the ORF. Also, 19 PB insertions occurred at TTAA, one at TTAG, and two at CTAA motifs.

We also performed an NGS comparison of the mutant pools before and after 5-FOA treatment. We obtained 3,266,688 and 3,865,123 read pairs of PB insertion junctions from 5-FOA-treated and untreated samples respectively. Ranking the fold changes of genes after 5-FOA treatment, URA5 and URA3 occupied the first and second positions (Fig. 3f), exceeding the third by ~10 times. URA5 and URA3 also occupied the top eight places in the rank of fold changes of insertion sites after 5-FOA treatment (Supplementary Figure 2a). Consistently, inverse PCR and sequencing analysis of 5-FOA resistant clones #4, #9, #15, and #26 confirmed PB[HIS1] insertion at the same position in URA5 ORF (Fig. 3g). Deletion of URA5 was found previously to cause partial resistance to 5-FOA in *S. cerevisiae*. However, deleting URA5 in both haploid and diploid *C. albicans* caused comparable 5-FOA resistance as deleting URA3 (Supplementary Figure 2b). Ura3 and Ura5 catalyze two consecutive steps in the de novo biosynthesis of pyrimidines. Taken together, this proof-of-principle screen demonstrated the superb power of this PB-mediated mutagenesis system in identifying genes required for a cellular process.

**Genome-wide genetic screening for fluconazole-resistant genes.** To identify new antifungal resistance mechanisms, we applied the PB-transposon mutagenesis system to perform genome-wide profiling of genes whose inactivation causes resistance to fluconazole. The rationale of the screen is: if PB insertion into a gene causes resistance to fluconazole, cells carrying this mutation will outcompete sensitive cells when grown in the presence of fluconazole and become overrepresented in the population. Thus, the subsequent NGS reads of PB insertions in this gene in the pooled genomic DNA will increase correspondingly. The PB insertion library was prepared by first growing YW02 cells in YPD+Dox medium and then dividing the culture into two equal parts for further cultivation in Dox-free medium with or without fluconazole. Cells were harvested after doubling 10 times and processed for NGS analysis.

We detected, in three independent experiments, an average of 5250 and 5122 genes from fluconazole-treated and untreated samples and ranked the genes according to the levels of enrichment after fluconazole treatment. Among the top 10 were three genes that either had been linked toazole susceptibility or are involved in ergosterol biosynthesis including ERG3, ERG6, and ERG251 (Fig. 4a and Supplementary Data 2). We randomly selected 10 genes ranked higher than PMCI, which is the lowest ranking known resistant genes in our list, to construct haploid deletion mutants and found that they all showed increased resistance to fluconazole (Fig. 4b). Gene Ontology (GO) analyses of the genes that showed a significant increase in abundance in fluconazole-treated cells (log2 fold change > 1 and p-value < 0.05) revealed an overrepresentation of genes associated with ergosterol and sphingolipid biosynthesis (Fig. 4c). We also performed Gene Set Enrichment Analysis (GSEA) and found significant enrichment of genes associated with ergosterol and sphingolipid biosynthesis in fluconazole-treated cells in the leading edge subset that has the highest correlation with fluconazole resistance (Fig. 4d). The latter group included FEN1, FEN12, and ARV1 that play important roles in sphingolipid biosynthesis and trafficking. Like ergosterol, sphingolipids are the main components of the fungal plasma membrane which is both the target of azole and polyene classes of antifungal drugs and site of drug transporters. We reasoned that the discovery of these genes in our screen likely reflects some previously unknown mechanisms of drug resistance. Thus, we further characterized FEN1 and FEN12, both encoding fatty-acid elongases that synthesize very-long-chain fatty acids as precursors of sphingolipid biosynthesis. We verified that fen1ΔΔ and fen12ΔΔ mutants constructed in the diploid BWPI7 background also exhibited significantly higher resistance to fluconazole than the WT strain (Fig. 4e).

**fen1ΔΔ mutants can tolerate higher levels of the toxic sterol.** Since blocking DMCD synthesis by inactivating ERG3 is a known resistance mechanism to fluconazole, we quantified...
ergosterol and DMCDD in whole cell lysates and the plasma membrane using LC-MS. Due to the lack of standard DMCDD, we included an ergΔ strain to ensure correct identification of this molecule by combining the detected molecular mass and the absence of the compound in ergΔ cells (Fig. 5a, top and Supplementary Figure 3). LC-MS detected a ~90% decrease of ergosterol in the whole cell lysates of both fen1ΔΔ and fen12ΔΔ mutants compared to WT controls grown in drug-free media (Fig. 5a, middle), indicating that lacking either FEN1 or FEN12 impairs ergosterol biosynthesis severely. As expected, fluconazole treatment caused a dramatic drop of ergosterol levels in all strains. Unexpectedly, we detected a ~270% increase in the amount of DMCDD in both fen1ΔΔ and fen12ΔΔ cell lysates compared with WT cells upon fluconazole treatment (Fig. 5a, bottom). However, the DMCDD level in the plasma membrane fraction of fen1ΔΔ and fen12ΔΔ cells was found to be 35 and 29% of that in WT cells (Fig. 5a, bottom). The results suggest that, unlike ergΔΔ, the higher fluconazole resistance of fenΔΔ mutants is not due to decreased production of the toxic sterol.

**Significant changes in sphingolipid composition in fenΔΔ mutants.** As ergosterol and DMCDD insert themselves into the cell membrane mainly through interaction with sphingolipids, we speculated that changes in the sphingolipid profile in fen1ΔΔ and fen12ΔΔ might be responsible for the tolerance of DMCDD. To test this hypothesis, we performed lipidomics profiling by LC-MS. We detected rather different fatty-acid profiles between fen1ΔΔ and fen12ΔΔ mutants (Fig. 5b and Supplementary Figure 4). These data suggest that changes in the sphingolipid composition might contribute to the increased tolerance of fen1ΔΔ and fen12ΔΔ mutants to DMCDD.
these fatty acids while fen12Δ cells showed 164% and 419% increase in FA(20:0) and FA(22:0), respectively (Fig. 5b). Similar fatty-acid profiles were detected in fluconazole-treated cells. Consistently, we detected a marked decrease in the levels of α-hydroxyphytoceramide (αHPC), inositolphosphoceramide (IPC), and mannosylinositolphosphoceramide (MIPC) species with a 26-carbon fatty acid chain in both fen12Δ and fen122Δ cells grown in drug-free media (Fig. 5b and Supplementary Figures 5, 6 and 7); in particular, there was a 20-fold decrease of d18:0/26:0 αHPC and ~50-fold decrease of both d18:0/26:0 IPC and d18:0/26:0 MIPC in fen12Δ cells. In contrast, several aHPC, IPC, and MIPC molecules with a 20–24-carbon fatty acid chain showed an increase over a broad range; for example, d16:0/24:0 aHPC increased by 37.4 fold, 18:0/22:0 or d16:0/24:0 (the two molecules have identical mass) IPC by 73.3 fold, and d18:0/22:0 or d16:0/24:0 MIPC by almost 300 fold in fen12Δ/Δ cells (Fig. 5b). Strikingly, in response to fluconazole treatment, all species of aHPC and MIPC increased significantly in WT cells from 5.48 to 99.3 fold. This fluconazole-induced increase was more dramatic in fen1Δ cells; for example, d16:0/20:0 αHPC increased by 50.5 fold in fen1Δ/Δ and d16:0/24:0 aHPC increased by 361 fold in fen1Δ/Δ cells. The most significant changes occurred in d18:0/22:0 or d16:0/24:0 and d18:0/20:0 or d16:0/22:0 MIPC which increased by >3 orders of magnitude (Fig. 5b). The results suggest that increasing the cellular levels of some species of sphingolipids might be a natural stress response to fluconazole. We speculate that high levels of sphingolipids could improve cell membrane strength leading to higher tolerance of the toxic sterol.

Fig. 4 Genetic screens for fluconazole-resistant mutants. a Volcano plot of fold changes of genes with PB inserts in PB transposition mutants grown in media containing 100 μg/ml fluconazole in comparison with the same mutants grown in normal media. p-Values were calculated using the Wald test. Ten candidates selected for phenotype confirmation are indicated. Genes known to be associated with fluconazole resistance and ergosterol and sphingolipid biosynthesis are shown with different colors. b Gene deletion of 10 candidates randomly selected from the top-ranked genes in a confirms increased resistance to fluconazole. Fluconazole susceptibility was evaluated using the standard CLSI broth microdilution protocol. Cultures were incubated for 24 h, and growth was measured as OD_{600} and expressed as a percentage of the growth in the control wells with no drug (DMSO alone) for each strain. c Gene Ontology (GO) analysis of the PB-mutated genes that led to statistically significant fold increases (p<0.05) after fluconazole treatment. Red bars highlight the ergosterol and sphingolipid biosynthesis pathway. p-Values were from Fisher’s exact test. d Gene Set Enrichment Analysis (GSEA) of the NGS data from a. p-Values were determined using the Kolmogorov-Smirnov test. e Homozygous deletion of FEN1 or FEN12 in BWP17 also results in increased fluconazole resistance. The assay was done as described in b.
Mechanisms of sphingolipid overproduction in fenΔ/Δ mutants. We further investigated the mechanisms behind the fluconazole-induced sphingolipid overproduction. Fungal cells possess a remarkable capacity to counterbalance a disturbance to maintain cellular homeostasis. In response to fluconazole treatment, C. albicans reacts by overexpressing UPC2 which encodes a transcription factor that regulates ergosterol biosynthesis (Fig. 6a, summarizing the biosynthetic pathways of ergosterol and sphingolipids) and sterol uptake. Indeed, we detected the overexpression of UPC2, ERG3, and ERG11 in both WT and fenΔ/Δ strains after fluconazole treatment (Fig. 6b). UPC2 also regulates genes involved in sphingolipid biosynthesis. Consistently, we detected overexpression of the ceramide synthase gene LAG1, the IPC synthase gene AUR1, the MIPC synthase gene MIT1, and the M(IP)_2C synthase gene IPT1 upon fluconazole treatment (Fig. 6b). Importantly, the fluconazole-induced UPC2 overexpression is much stronger in fenΔ/Δ cells than in WT cells. The results are consistent with the higher amounts of sphingolipids found in the mutants grown in the presence of fluconazole.

To determine whether the upregulation of sphingolipid biosynthesis is responsible for the increased resistance to fluconazole in fen1Δ/Δ and fen2Δ/Δ mutants, we attempted to delete UPC2 and LAG1 in fen1Δ/Δ and fen2Δ/Δ strains. Despite repeated efforts, we could not delete both copies of LAG1 in either mutant and those of UPC2 in fen1Δ/Δ, suggesting synthetic lethality. However, we found that deleting one copy of LAG1 or one copy of UPC2 in fen1Δ/Δ and fen2Δ/Δ appeared to increase their sensitivity to fluconazole (Fig. 6c). Also, deleting both copies of UPC2 in fen2Δ/Δ abolished its resistance to fluconazole (Fig. 6c). The results suggest that UPC2-induced upregulation of sphingolipid biosynthesis may underlie the increased fluconazole resistance of fen1Δ/Δ and fen2Δ/Δ mutants. Consistently, the fluconazole-induced upregulation of ERG3, ERG11, LAG1, AUR1, MIT1, and IPT1 was significantly reduced or abolished when one or both copies of UPC2 were deleted (Fig. 6b). Together, the results corroborate the hypothesis that fen1Δ/Δ and fen2Δ/Δ mutants increase the cellular levels of sphingolipids in response to fluconazole by strongly upregulating the expression of genes.
The standard CLSI broth microdilution protocol. Following 24 h of incubation, growth was measured as OD600 and expressed as a percentage of the growth in the control wells with no drug (DMSO alone) for each strain. 

**Fig. 6** Sphingolipid overproduction underlies fluconazole resistance of fenΔ/Δ cells. **a** Illustration of ergosterol and sphingolipid biosynthesis pathways. **b** qPCR analysis of UPC2, ERG3, ERG11, LAG1, AUR1, MIT1, and IPT1 expression in WT, fen1Δ/Δ, and fen2Δ/Δ mutants with UPC2 single or double knockout in the presence or absence of fluconazole. Error bars represent s.d. from the mean of three independent experiments. Significance was measured using unpaired t-test (*p < 0.05, **p < 0.01, ***p < 0.001, n.s., not significant). **c** Fluconazole susceptibility of WT and the indicated mutants was evaluated using the standard CLSI broth microdilution protocol. Following 24 h of incubation, growth was measured as OD600 and expressed as a percentage of the growth in the control wells with no drug (DMSO alone) for each strain.
involved in sphingolipid biosynthesis, leading to tolerance of the toxic sterol.

Discussion
In recent years, considerable efforts have been made to introduce transposon mutagenesis strategies to create libraries of heterozygous 36,37 or homozygous 38,39 mutants in C. albicans. In general, these methods start with in vitro transposition of a transposon-containing cassette, Tn5-UAA1 or Tn7-UAA1, into a C. albicans genomic DNA library 36,37. Then, the mutagenized library is transformed into Escherichia coli for amplification before transformation into C. albicans. These methods are tedious, labor-intensive, and unsuitable for high-throughput genome-wide genetic studies. Also, their use in diploid C. albicans creates heterozygous mutations which do not result in readily detectable phenotypes except for a small number of haploinsufficient genes 40,41. Mielich et al. recently introduced the Activator/Dissociation transposon of maize into haploid C. albicans 32, but the system is not very useful at its current state because of frequent autodiploidization.

Here, we introduce a simple and efficient PB transposon-based mutagenesis system in haploid C. albicans, which has the following advantageous features. First, a single transposon insertion in the promoter or ORF of a gene is, in most cases, sufficient to inactivate a gene and produce a phenotype. Second, the transposon donor is inserted into a selectable marker gene, i.e., ARG4, and the transposase is expressed from a different locus under the control of the Tet-On promoter. Thus, a mutant library can be generated merely by growing the cells in a medium containing Dox to express the transposase and then selecting Arg+ transposants in -Arg media. Third, transposition occurs at high frequencies and in a random manner. Our NGS analysis showed an even distribution of PB insertions on all chromosomes and identified PB inserts in ~5000 genes (Fig. 2e). Fourth, each mutant cell contains a single PB insertion, which allows straightforward mapping of the affected gene by inverse PCR and DNA sequencing. Fifth, the precise excision of PB from the insertion site restores the coding sequence allowing easy confirmation of the phenotype-genotype relationship. Our successful identification of URA3 and URA5 in 26 out of 30 random 5-FOA-resistant mutants demonstrates the superb power of the PB transposon mutagenesis system (Fig. 3). Furthermore, the PB-mediated mutagenesis system is readily applicable to whole-genome epistasis analysis of gene networks by first deleting a gene of interest before activating the transposition and screening for synthetic phenotypes. We anticipate that this PB transposon-based mutagenesis platform will significantly accelerate discoveries to advance our understanding of the biology and pathogenesis of this pathogen.

Frequent isolation of multidrug-resistant Candida worldwide is a cause for concern because currently only three classes of drugs are available approved for the treatment of systemic fungal infections 43,44. Hard lessons learned since the discovery of penicillin have taught us that microbial pathogens are extremely innovative in quickly finding tricks to defeat every one of our therapies. To win the war against infectious disease, it is imperative to elucidate how a pathogen has acquired resistance and even to predict how it can do it. The development of the PB transposon mutagenesis system has allowed us to conduct genome-wide screening in C. albicans to identify genes underlying antifungal susceptibility. Our NGS analyses of PB transposon insertion sites in mutants selected by growth in the presence of fluconazole in comparison with mutants grown in drug-free media revealed significant enrichment of genes involved in ergosterol biosynthesis, in particular, ERG3 and ERG6 whose mutation is well known to increase fungal resistance to azoles 45-48. Deleting any one of 10 genes randomly picked from our resistant mutant list all led to increased fluconazole resistance (Fig. 4b), indicating that the screen was successful. Among the highest ranking genes were FEN1, FEN12, and ARV1 which play important roles in sphingolipid biosynthesis 49,50. The understanding that sphingolipids are major components of fungal cell membrane and interact both physically and functionally with ergosterol 50,51,52, the target of azole and polyene antifungal drugs, prompted us to conduct a comprehensive analysis of FEN1 and FEN12. Our first informative finding was a severalfold increase in the cellular levels of the toxic sterol in both fen1Δ mutants compared to WT cells (Fig. 5a), which indicated a resistance mechanism distinct from that of the erg3 mutant 2,48. Interestingly, the level of the toxic sterol in the plasma membrane is significantly lower in the mutants than in the wild type (Fig. 5a), suggesting that the mutants pack fewer toxic sterol molecules into the membrane. Furthermore, we detected dramatic changes in the sphingolipid composition in whole cell extracts (Fig. 5b). The most striking change was the increase of more than orders of magnitude of some MIPC species in fen12Δ cells upon fluconazole treatment, which also increased the total cellular level of sphingolipids. An illuminating observation was the sharp increase of the same MIPC species in WT cells after fluconazole treatment (Fig. 5b), indicating that C. albicans alters its sphingolipid composition as a natural stress response mechanism to the disruption of ergosterol biosynthesis. This could be a conserved survival strategy because eukaryotic cells are known to have a huge capacity to preferentially modify the cellular levels and make-up of sphingolipids in response to changes in sterol composition in their membrane as an adaptation mechanism to maintain membrane structure and function 52. In S. cerevisiae, mutation of ELO3, a homolog of FEN12, also affected sphingolipid composition significantly 53. Many previous studies have revealed intricate relationships between ergosterol and sphingolipid biosynthesis and resistance to azoles. Particularly consistent with our findings, Prasad et al. found that blocking M(1P)C synthesis by deleting the IPT1 gene significantly increased C. albicans susceptibility to azoles 54; furthermore, the level of the drug efflux pump Cdr1 in the plasma membrane was reduced in ipt1ΔΔ cells as well as in cells treated with fumonisin B1, an inhibitor which blocks the synthesis of phytoceramide 55. Consistently, we demonstrated that blocking sphingolipid biosynthesis reduced the fluconazole resistance in fen1ΔΔ and fen12ΔΔ cells. Sphingolipids are abundant components of the fungal cell membrane and play both structural and signaling roles 56. Numerous experiments have shown that changes of sphingolipid composition can dramatically alter the properties of cell membranes, such as rigidity, fluidity, and domain organization which further influences cellular functions 57. Thus, the marked changes of the cellular sphingolipid composition in fen1ΔΔ mutants might lead to fluconazole resistance by reducing the incorporation of the toxic sterol into the cell membrane and limiting subsequent structural and functional damage of the cell membrane. Although we do not have direct evidence confirming repulsion of the toxic sterol from the cell membrane by high levels of sphingolipids, this can be expected based on the chemical properties of sterols and sphingolipids and is indirectly supported by various previous studies. Cell membranes have a complex organization featuring domains with distinct lipid compositions known as rafts whose formation is determined by the hydrophobic interaction between sterols and sphingolipids 58,59. However, the toxic sterol is structurally different from ergosterol in terms of the absence of B-ring unsaturation, the presence of the 6-OH group and the additional C-14 methyl group, and side-chain modification. In particular, the polar 6-OH group is thought to interfere with sterol–sphingolipid
packing in the plasma membrane, causing membrane damage. The high level of sphingolipids in fen1ΔΔ mutants could strengthen the cell membrane by restraining membrane deformation. When the additional free energy required for incorporating polar groups of the toxic sterol cannot be compensated by membrane deformation, they must be pushed out to reach a lower energy status. Thus, more toxic sterol molecules with a polar side chain would be repelled from the membrane by their hydrophilic lipophilic heptaene part is directing outward to hydroxy side is pointing inward to constitute the pore lining and penetrate cell membranes, in which their hydrophilic poly-
to ergosterol in a parallel manner to form barrel-stave type pores and interact with the membrane interior. While the high levels of sphingolipids in the membrane hinder the insertion of the toxic sterol, the binding of amphotericin B to ergosterol is not affected; and on the contrary, the shorter sphingolipids in fen1ΔΔ mutants could expose ergosterol molecules and thus facilitate the binding to amphotericin B, making mutant cells more sensitive to this drug. In future studies, it would be interesting to know whether high cellular levels of sphingolipids contribute to azole resistance in clinical isolates of C. albicans and other fungal pathogens.

Methods

Plasmid construction. pPB[URA3] was constructed as follows: the URA3 marker was PCR-amplified from a pBluescript-based plasmid with primers URA3Apal and URA3SalI, and cloned into the Apel and SacII sites of pPB[ura4] to generate pPB[URA3]. Construction of pARG4-PB[URA3]: the EcoRI-PstI fragment including the intact PB[URA3] transposon cassette was PCR-amplified from pPB[URA3] with primers PB[URA3]Ecorl and PB[URA3]PstI, and inserted into the EcoRI and PstI sites of a pBluescript-based plasmid containing ARG4 selectable marker. Construction of pARG4-PB[HI5]: the HIS1 marker was PCR-amplified from a pBluescript-based plasmid with primers HIS1Apal and HIS1SalI, and cloned into the Apel and SacII sites of pARG4-PB[URA3] to generate pARG4-PB[HI5]. Construction of pNIM1-CaPBase: site-directed mutagenesis of the PBase coding sequence in plasmid pDUAL-PBase was performed using Quick-Change Site-Directed Mutagenesis Kit (Stratagene, 200514) to generate pDUAL-CaPBase which contains a codon-optimized PBase with hyperactivity. Then a PCR fragment containing the CaPBase was amplified from pDUAL-CaPBase with primers PBXOH and PBBaseSamII, and cloned into the SacII and BglII sites of pNIM1 to generate pNIM1-CaPBase. pPB[ura4], pDUAL-PBase, and pNIM1 were described previously.20,65.

Construction of strains YW02 and YW05. YW02 was constructed from GZ/803 by introducing the linear DNA fragment from pNIM1-CaPBase containing the PTet-On-CaPBase fusion and the Xhol-NotI fragment from pARG4-PB[URA3] containing the ARG4-PB[URA3] transposon cassette to transform GZ/803 to become nourseothricin resistance and histidine prototrophic. Correct integration was confirmed by PCR.

YW05 was constructed from YW11 by introducing the linear DNA fragment from pNIM1-CaPBase containing the PTet-On-CaPBase fusion and the Xhol-NotI fragment from pARG4-PB[HI5] containing the ARG4-PB[HI5] fusion to transform YW11 to become nourseothricin resistance and histidine prototrophic. Correct integration was confirmed by PCR.

Inverse PCR for mapping PB insertion sites. Genomic DNA was prepared using MasterPure Yeast DNA Purification Kit (Epicentre Technologies, MPV8200) and digested with BfuCI overnight. After heat inactivation of BfuCI, the digested genomic DNA was self-ligated with T4 DNA ligase at 16 °C overnight. Primers used to recover the flanking sequence of the left side of the PB transposon were PBfl1 and PBfl2. PCR products were purified from agarose gel and sequenced using Seq1 as the sequencing primer. The sequencing results were used to BLAST-search Candida Genome Database (www.candidagenome.org) to determine the PB insertion sites.

Quantitative PCR. To determine the copy number of PB[URA3], genomic DNA was purified using MasterPure yeast DNA purification kit and analyzed on STRATAGENE MX3000p using Maxima SYBR Green qPCR Master Mix (2X) with separate ROX vial (Thermo Fisher Scientific, K0253) according to the manufacturer’s instructions. PCR was carried out with primers CDC28 sense and CDC28 antisense to amplify CDC28, URA3 sense and URA3 antisense to amplify PB[URA3]. PB copy number was determined by ΔΔCT method using the genomic DNA of strain YW02 as a control, which has a single copy of PB[URA3]. Since the ratios of PB versus CDC28 were usually not integral numbers, we estimated the copy number to be the integral closest to the ratios.

Transposition induction. In the experiments using integrated PB[URA3] or PB[HI5] as the transposon donor, a single colony of YW02 or YW05 was first cultured in liquid YPD medium at 30 °C overnight. Then, the cells were transferred to liquid or solid YPD medium supplemented with 50 μg/mL Dox to induce the expression of CaPBase and undergo transposition. To calculate transposition efficiency, aliquots of cells were harvested at timed intervals and spread on GMM plates to count the number of cells that had undergone transposition and on YPD plates to obtain the total number of cells. Transposition efficiency was calculated as the number of colonies on GMM plates divided by the number of colonies on YPD plates.

Flow cytometry. Flow cytometry analysis was performed using a BD FACSCalibur as described previously.12,22. Mid-log phase cells were collected, washed, and fixed with 70% (vol/vol) ethanol (Sigma, E7023-500ML). Cells were washed with solution I (2% glucose and 6.79 g/L yeast nitrogen base without amino acids) and cultured in liquid YPD medium at 30 °C overnight. Then, the cells were transferred to liquid or solid YPD medium supplemented with 50 μg/mL Dox to induce the expression of CaPBase and undergo transposition. To calculate transposition efficiency, aliquots of cells were harvested at timed intervals and spread on GMM plates. Expression levels of target genes were analyzed using the Maxima SYBR Green qPCR Master Mix (2X) once quantitated with 10 mg/mL RNase A (Sigma, R8475-100MG) (1:100 dilution in solution I). Cells were washed with PBS and resuspended in 5 mg/mL propidium iodide (Sigma, 81845-25MG) (1:50 dilution in PBS) incubated in the dark at 4 °C overnight. Stained cells were collected and resuspended in PBS and sonicated before loading.

Mapping PB insertion sites by next-generation sequencing. A single colony of YW02 or YW05 cells were inoculated into 5 mL of YPD and grown at 30 °C to the exponential phase. Eighteen equal aliquots of the culture, each containing about 10^8 cells, were used for sequencing library preparations. Site-directed mutagenesis of the PBase coding sequence in plasmid pDUAL-PBase was performed using Quick-Change Site-Directed Mutagenesis Kit (Stratagene, 200514) to generate pDUAL-CaPBase which contains a codon-optimized PBase with hyperactivity. Then a PCR fragment containing the CaPBase was amplified from pDUAL-CaPBase with primers PBXOH and PBBaseSamII, and cloned into the SacII and BglII sites of pNIM1 to generate pNIM1-CaPBase. pPB[ura4], pDUAL-PBase, and pNIM1 were described previously.20,65.

Construction of strains YW02 and YW05. YW02 was constructed from GZ/803 by introducing the linear DNA fragment from pNIM1-CaPBase containing the PTet-On-CaPBase fusion and the Xhol-NotI fragment from pARG4-PB[URA3] containing the ARG4-PB[URA3] transposon cassette to transform GZ/803 to become nourseothricin resistance and histidine prototrophic. Correct integration was confirmed by PCR.
In the screen for 5-FOA-resistant mutants, insertional mutagenesis in YW05 cells was induced by culturing the yeast cells in YPD plate containing Dox for 24 h and then replica-transferred to GMM plates containing uridine to select for Arg+His+ cells. After incubation at 30°C for 24 h, cells on the above GMM plates were replica-transferred to GMM plates supplemented with uridine and 5-FOA. The 5-FOA plates were then incubated at 30°C until resistant colonies had formed. For screening in the liquid medium, mutant cells were collected and washed three times with PBS. Two equal aliquots of cells were cultivated further in 20 mL GMM medium containing uridine with or without 5-FOA at 30°C for 48 h; then cells were harvested and processed for NGS analysis.

In order for fluorescence reporter–resistant mutants, transposition-mediated mutagenesis was performed using strain YW02 grown in YPD plate containing Dox for 24 h at 30°C. Then, the cells were replica-transferred to GMM plates and incubated at 30°C for 24 h to enrich Arg+URA+ cells that had undergone the transposition. Then, mutant cells were collected and washed three times with PBS. Approximately 2–4×10^6 cells/mL were cultivated further in 20 mL YPD medium in the presence or absence of fluconazole. Cell growth was monitored by measuring OD_{600} and cells were harvested after the population had doubled 10 times and processed for NGS analysis.

Flucconazole susceptibility test. Antifungal microtiter assay from the strains included in this study was performed by using the broth microdilution method described in the CLSI document M27-A3® in a 96-well plate format.

Flucconazole susceptibilities of pure cultures (range: 0.04 to 2.25 μg/mL) from RPMI 1640 medium (Thermo Fisher Scientific, 21875159) were determined against the CLSI document. The medium was buffered with 0.165 M morpholinepropanesulfonic acid (MOPS), and its pH was adjusted using NaOH and HCl. Cell inoculum was ~1×10^7 cells per well. Plates were incubated without shaking at 30°C for 24 h. The content of each well was carefully resuspended by pipetting up and down before OD_{530} was measured using the Spark multimode microplate reader (TECAN, Switzerland).

Preparation of C. albicans plasma membrane. Highly purified plasma membrane was isolated as previously described for S. cerevisiae with minor modifications. C. albicans yeast cells were harvested by centrifugation (500g, 5 min), then washed twice with 0.4 M sucrose (Sigma, V90165-100G) in buffer A (25 mM imidazole, pH 7.0) and resuspended in buffer A. A 1% M cell suspension was added to the cell pellet and the mix was vortexed for 1 min 8–10 times, with 2 min on ice between rounds. Then the mix was centrifuged at 330g (2500 rpm in Sorvall SS34 rotor) for 20 min to pellet unbroken cells and glass beads, and the supernatant was centrifuged at 22,000g (16,000 rpm in Sorvall SS34 rotor) for 30 min to obtain a pellet that includes the plasma membranes and mitochondria. The pellet was resuspended in buffer A by gentle vortexing for 30 s and the resuspended membranes were loaded onto discontinuous sucrose gradients (overlaying three 4 mL layers of 2.25, 1.65, and 1.1 M sucrose in buffer A in 14 × 89 mm Beckman ultraclear tube) and centrifuged either overnight (14 h) at 80,000g (22,000 rpm) for 6 h at 4°C. The Beckman SW41 or SW40 Ti rotor. The membrane band at the 2.25/1.65 M sucrose interface is essentially pure plasma membrane, which was collected at these interfaces from the top of the gradient with a Pasteur pipette. The collected membrane was diluted four times with buffer A and pelleted at 30,000g (18,000 rpm Beckman 50 Ti rotor) for 40 min. The plasma membrane pellet was suspended in 80% methanol and stored at −80°C for sterol analysis.

Sterol analysis. Sterols were extracted using pre-chilled 80% (v/v) methanol (Sigma, 34860-1L-R) according to the method of Yuan et al.68. C. albicans strains were cultured in YPD medium with or without fluconazole for 24 h, and the PBS washed cells were harvested by centrifugation. Add pre-chilled 80% (v/v) methanol and glass beads to the cell pellets. Vortex 1 min, then keep on ice for 2 min. Repeat this step 8 or 10 more times. Centrifuge at 14,000g for 40 min using a refrigerated centrifuge at 4°C to obtain the supernatant. The extracts were vacuum-dried and stored at −80°C. The dried samples were solubilized in methanol prior to analysis by LC-MS. Equal amounts of cells were processed for protein extraction, and protein concentration was measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, 23227) to conduct normalization.

LC-MS analysis was performed with a UPLC system, which was coupled to a Q-Exactive orbitrap mass spectrometer (Thermo Fisher, CA) equipped with a heated electrospray ionization (HESI) probe. Lipid extracts were separated by a C18 (100 × 2.1 mm 1.9 μm column) Waters. A binary solvent system was used, in which mobile phase A consisted of ACN:H2O (80:20), 10 mM ammonium acetate, and mobile phase B of IPA:ACN (90:10), 10 mM ammonium acetate. A 35-min gradient with a flow rate of 220 μL/min was used. Column chamber and sample tray were held at 45°C and 10°C, respectively. Data with mass ranges of m/z 240–2000 and m/z 200–2000 was acquired at positive ion mode and negative ion mode with data dependent MSMS acquisition. The full scan and fragment spectra were collected with a resolution of 70,000 and 17,500, respectively. The source parameters are as follows: spray voltage: 3000 V; capillary temperature: 320°C; heater temperature: 300°C; sheath gas flow rate: 35 Ar; auxiliary gas flow rate: 15 Ar. Data analysis was performed by the software Xcalibur (Thermo Fisher, CA). Compounds were identified based on their retention times and the masses detected in the liquid chromatography-mass spectrometry system.

Lipid analysis. Lipids were extracted according to the method of Bligh and Dyer69. C. albicans strains were cultured in YPD medium with or without fluconazole for 24 h, and the PBS washed cells were harvested by centrifugation. The cell pellets were lysed in PBS by bead-beating mechanical disruption at 4°C. The supernatants were then extracted with chloroform (AMRESCO, 0757-500ML)/methanol (2:1) at a final ratio of 20% (v/v). Vortex 30 s, then keep for 2 min. Repeat this step 5 more times. Centrifuge at 3000g for 20 min using a refrigerated centrifuge at 4°C to obtain the supernatant. The extracts were evaporated to dryness under N2 at room temperature and stored at −80°C. The dried samples were solubilized in 100 μL of dichloromethane (Sigma, 650463-1L) methanol (2:1) before analysis by LC-MS. Equal amounts of cells were processed for protein extraction and protein concentration was measured by BCA assay to conduct normalization.

LC-MS analysis was performed with a UPLC system coupled to a Q-Exactive orbitrap mass spectrometer system.
13. Skrzypek, M. S. et al. The Candida Genome Database (CGD): incorporation of Assembly 22. systematic identifiers and visualization of high throughput functional genomics. Nat. Genet. 40, 312–316 (2017).

14. Hickman, M. A. et al. The 'obligate diploid' Candida albicans forms mating-competent haploids. Nature 494, 55–59 (2013).

15. Shapiro, R. S. et al. A CRISPR-Cas9-based gene drive platform for genetic interaction analysis in Candida albicans. Nat. Microbiol. 3, 73–82 (2018).

16. Caudill, L. C. et al. Caudill complex mutagenesis in Candida albicans. analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. Virolology 127, 156–169 (1989).

17. Ding, S. et al. Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. Cell 122, 473–483 (2005).

18. Mitra, R., Fain-Thomorton, J. & Craig, N. P. PiggyBac can bypass DNA synthesis during yeast-paste transposition. EMBO J. 27, 1107–1109 (2008).

19. Elick, T. A., Bauser, C. A. & Fraser, M. J. Excision of the piggyBac transposable element in vitro is a precise event that is enhanced by the expression of its encoded transposase. Genetica 98, 33–41 (1996).

20. Li, J. et al. A PiggyBac transposon-based mutagenesis system for the fission yeast Schizosaccharomyces pombe. Nucleic Acids Res. 39, e40 (2011).

21. Yusa, K., Zhou, L., Li, M. A., Bradley, A. & Craig, N. L. A hyperactive piggyBac transposase for mammalian applications. Proc. Natl. Acad. Sci. USA 108, 1513–1516 (2011).

22. Zeng, G., Wang, Y. M., Chan, F. Y. & Wang, Y. One-step targeted gene deletion in Candida albicans haploids. Nat. Protoc. 9, 464–473 (2014).

23. Caudill, L. C., Rosion-Schiltz, D. M., Eggleston, B. W. & Sved, J. High-frequency P element loss in Drosophila is homolog dependent. Cell 62, 515–520 (1990).

24. Giaever, G. et al. Functional profiling of the Saccharomyces cerevisiae genome. Nature 418, 387–391 (2002).

25. Boeke, J. D., LeCouteur, F. & Fink, G. R. A positive selection for mutants lacking orothic-5-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. 197, 345–346 (1984).

26. Jones, E. W. & Fink, G. R. Regulation of amino acid and nucleotide biosynthesis in yeast. In The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression (eds Strathern, J. N. et al.) 181–299 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1982).

27. David, D., Sundarababu, S. & Gerst, J. E. Involvement of long chain fatty acid elongation in the trafficking of secretory vesicles in yeast. J. Cell Biol. 143, 1167–1182 (1998).

28. Oh, C. S., Toke, D. A., Mandala, S. & Martin, C. E. ELO2 and ELO3, homologues of the Saccharomyces cerevisiae ELO1 gene, function in fatty acid elongation and are required for sphingolipid formation. J. Biol. Chem. 272, 17376–17384 (1997).

29. Kelly, S. L. et al. Resistance to fluconazole and cross-resistance to amphotericin B in Candida albicans from AIDS patients caused by defective sterol Δ5,6-desaturase. FEMS Lett. 400, 80–82 (1997).

30. Bittman, R., Kasirreddy, C. R., Mattijss, P. & Slotte, J. P. Interaction of cholesterol with sphingolipid in monolayers and vesicles. Biochemistry 33, 1176–1178 (1994).

31. Silver, P. M., Oliver, B. G. & White, T. C. Role of Candida albicans transcription factor Upc2α in drug resistance and sterol metabolism. Eukaryot. Cell 3, 1391–1397 (2004).

32. Hallström, T. C. et al. Coordinate control of sphingolipid biosynthesis and multidrug resistance in Saccharomyces cerevisiae. J. Biol. Chem. 276, 23674–23680 (2001).

33. Kihara, A. & Igarashi, Y. Cross talk between sphingolipids and glycosphingolipids in the establishment of plasma membrane asymmetry. Mol. Biol. Cell 15, 4949–4959 (2004).

34. Kolačzkowski, M., Kolačzkowa, A., Giau, B., Schneider, R. & Moye-Rowley, W. S. Differential regulation of ceramide synthase components LAC1 and LAG1 in Saccharomyces cerevisiae. Eukaryot. Cell 3, 880–892 (2004).

35. Panwar, S. L. & Moye-Rowley, W. S. Long chain base tolerance in Saccharomyces cerevisiae is induced by retrograde signals from the mitochondria. J. Biol. Chem. 281, 6376–6384 (2006).

36. Bla, M. A., Bien, M., Craig, N. & Johnson, A. D. Haplinsufficiency-based large-scale forward genetic analysis of filamentous growth in the diploid human fungal pathogen C. albicans. EMBO J. 22, 2668–2678 (2003).

37. Oh, J. et al. Gene annotation and drug target discovery in Candida albicans with a tagged transposon mutant collection. PLoS Pathog. 6, e1001400 (2010).

38. Davis, D. A., Bruno, V. M., Loza, L., Filler, S. G. & Mitchell, A. P. Candida albicans Mk3p, a conserved regulator of hyphal morphogenesis and virulence identified through insertional mutagenesis. Genetics 162, 1573–1581 (2002).

39. Epp, E. et al. Forward genetics in Candida albicans that reveals the Arp2/3 complex is required for hyphal formation, but not endocytosis. Mol. Microbiol. 75, 1182–1198 (2010).

40. Giaever, G. et al. Multidrug complex haplonsufficiency-based genetic analysis of Candida albicans transcription factors: tools and applications to virulence-associated phenotypes. G3 (Bethesda) 8, 1299–1314 (2018).
Acknowledgements
We thank Dr. Yang Chen from Dr. Li Yu’s lab and Dr. Feng Chen for technical assistance. We are grateful for the support of Metabolomics Facility in Technology Center for Protein Sciences and the advice of members of J.W.’s group and Yanyi Huang’s group at various stages of this work. J.G. thanks Jing Li for her support. This work was supported by the Thousand Young Talents Program (J.W.), Ministry of Science and Technology of China (2016YFC0900103 to J.W.), National Natural Science Foundation of China (21675098 to J.W.), THU-PKU Center for Life Sciences (J.G. and J.W.), and the Agency for Sciences, Technology and Research of Singapore (BMRC/BnB/0001b/2012 to Y.W.). Funding includes NIH GM117111 to H.L. We are grateful to Dr. Li-lin Du of NIBS for providing pPB[ura4] and pDUAL-PBase and Allan Bradley of Sanger for hyPBase.

Author contributions
J.G., J.W., and Y.W. conceptualized, designed, and supervised the project; H.L. conceptualized, designed and supervised the project and acquired funding; J.G. conducted experiments; H.W. and Z.L. contributed to the computational analysis; A.H.-H.W., Y.-Z. W., Y.G., and X.L. provided technical assistance; G.Z. generated haploid strains; J.G., H.W., J.W., and Y.W. analyzed data and wrote the paper; J.W. and Y.W. acquired funding.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-06944-1.

Competing interests: The authors declare no competing interests.

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

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

OPEN ACCESS
This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018