Supporting Information

Becker et al. 10.1073/pnas.1009845107

SI Results

Virulence Determinants Among other Cellular Pathways. Like ergosterol, cell wall biosynthesis is essential for growth and has long been recognized as an ideal fungal-specific drug target to which novel antifungal agents have been developed. Accordingly, eleven genes essential for normal growth in vitro and participating in various aspects of cell wall biogenesis, including 1,3-beta-glucan (*FKS1*, *PHR2*, *EXG1*), 1,6-beta-glucan (*KRE9*), chitin (*CHS2*, *GNA1*, *GFA1*), glucosylphosphatidylinositol biosynthesis (*GPI16*), as well key regulatory factors involved in polarized growth and cell wall organization (*RHO1*, *ROM2*, *CDC24*) were evaluated in the in vivo screen. In all but two cases (*PHR2* and *EXG1*), conditional mutants comprising this gene set produced marked avirulence phenotypes ($p < 0.001$) in the dox − 3D infection model that were largely reproduced in the dox + 2D model. We determined that *PHR1* is essential for growth in YNBD, but not in YPD, and that *EXG1* is nonessential (Tables S1 and S2). On the other hand, failure to identify a virulence phenotype associated with *pTET-PHR2* is consistent with the regulation of this gene. *PHR2* and its paralog, *PHR1*, are genes for 1,3-beta-glucanosyltransferases uniquely expressed at low pH (≤5.5) and neutral to acid pH (≥5.5), respectively (1). Accordingly, at physiological pH in a murine model of infection, strains deleted of *PHR2* remained fully virulent whereas a *PHR1* deletion strain was avirulent (2). Similarly, *EXG1* and *XOG1* comprise a second gene family encoding exo-beta-(1,3)-glucanases (3), suggesting they too may be differentially regulated in a host environment. However, as *Candida albicans* virulence is unaltered among strains individually defective in *EXG1* or *XOG1* (4), depletion of both paralogs is required to further assess their combined role as potential virulence determinants.

C. albicans survival and pathogenesis requires effective nitrogen and carbon utilization and requisite metabolic processes to sustain growth. Whereas some amino acid biosynthetic pathways are dispensable in a host environment (e.g., methionine, histidine and lysine), others are essential (e.g., isoleucine and valine, both are essential amino acids; Tables S1 and S2). In vivo characterization of *ARO1*, *ARO2*, and *ARO7* demonstrated the importance of the shikimate pathway and chorismate biosynthesis as virulence determinants of *C. albicans*. Genetic inactivation of the shikimate pathway effects biosynthesis of multiple amino acids (e.g., biosynthesis of phenylalanine, tryptophan and tyrosine, the first two of which are essential amino acids). Therefore, localized concentrations of these amino acids are either insufficient to suppress the auxotrophic requirements of *C. albicans* strains defective in this pathway, and/or the pathogen is inefficient in scavenging these multiple requirements during infection. Furthermore, multiple steps in amino acid, fatty acid and heme biosynthesis are compartmentalized in the mitochondrion. Conditional mutants corresponding to 14 genes involved in various aspects of mitochondrial function, including its ribosome biogenesis (*YML6*, *MRPL3*, *MRPL6*, *MRPL7*, *MRPL10*, *MRPL16*, *MRPL23*), protein targeting (*TIM23*, *TIM44*, *TIM50*, *TOM40*), genome maintenance (*MGM101*), transcription (*RPO41*) and maintenance of organelle shape and structure (*MMM1*) all displayed dramatic avirulence phenotypes ($p < 0.001$) under the dox − 3D treatment regimen that were reproduced in the dox + 2D model (Tables S1 and S2), with only two exceptions (*MGM101*, $p < 0.01$; *YML6*, $p < 0.05$). Similarly, *Saccharomyces cerevisiae* mitochondrial gene deletion mutants, *MIP1* and *COX15*, display dramatically reduced fitness in vivo (5). These results highlight the broad importance of mitochondrial function for fungal survival in a host environment.

The protein secretory pathway mediates multiple aspects of growth, including polarized cell wall biogenesis, morphogenesis, and cell surface targeting of receptors and permeases, some of which are virulence factors required for the cell to sense its growth environment and survival. Nine genes were evaluated that directly function in various stages of protein secretion, including protein retention in the ER lumen (*ERD2*, *RET3*), ER to Golgi vesicle-mediated transport (*YIP1*, *BOS1*, *TRS20*, *TRS23*), intra-Golgi vesicle-mediated transport (*VTI1*), or Golgi to plasma membrane transport (*SEC6*, *SEC9*). In all but one instance (*TRS20*), the corresponding conditional mutants demonstrated an essential terminal phenotype in vitro (consistent with the yeast ortholog) that presumably reflects their essential role in *C. albicans* pathogenesis (Table S1).

Genes participating in other fundamental cellular processes including, chromosome condensation (*MCD1*, *BRN1*), mitotic spindle organization (*TUB1*, *SPC97*), and nuclear pore organization (*NUP192*, *NSP1*, *MEX67*, *SRM1*) were all critical for growth in vitro and survival in vivo ($p < 0.001$ in the dox − 3D and dox + 2D infection models; Table S1), as expected based on their known essentiality in *S. cerevisiae*. Similar conclusions are also drawn for genes involved in RNA polymerase II-based transcription (*RPB8*, *RPB10*, *TFA2*, *TGF1*, *SRB4*, *TFB1*), mRNA polyadenylation (*PAP1*, *MPE1*, *YSH1*, *CFT2*, *PFS2*, *CLP1*, *PTA1*), and translation (orf19.7057, *CDC60*, *EFT2*, *YEF3*, *TIF35*, *GCD6*, *SUI2*), despite the existence of homologous genes in some instances, which could have redundant or overlapping functions (Tables S1 and S2). Further, 26 essential genes involved in yeast rRNA processing (largely encoding subunits of the 80S U3 snoRNA complex or SSU processome) and ribosome biogenesis were all essential for *C. albicans* growth in vitro and all but one (*POL5*) were required for survival and virulence in both infection models. Relatively few genes of unknown function were examined, however, *orf19.2362*, *orf19.5356*, and *orf19.4479* were required for growth in vitro and survival in vivo, with significant avirulence phenotypes determined under the dox − 3D and dox + 2D infection models ($p < 0.001$), whereas *orf19.764* was dispensable for survival and virulence in both infection models (Table S1). Only one gene without a homolog in *S. cerevisiae*, *orf19.1143* was characterized in our study. However, it was not required for viability in vitro or virulence in vivo (Table S1).

SI Discussion.

Multiple approaches have been taken to evaluate the reliability of the Tet-off system and the resulting in vivo phenotypes of genes characterized in this screen. We compared the virulence phenotypes summarized in our gene set versus those independently studied in an analogous immunocompetent systemic infection model of candidiasis where mutants were constructed using either an alternative conditional promoter or a homozygous deletion strain was conditionally viable. In total, 11 genes were identified, of which 10 (*RHO1*, *CDC24*, *CHS1*, *GNA1*, *FAS2*, *FBA1*, *ADE2*, *ERG3*, *ERG24*, and *YEF3*) displayed highly reproducible avirulence phenotypes in our dox − 3D model ($p < 0.001$) and are consistent with previous studies (Table S2; see footnotes for references). Only *PHR2* proved dispensable for virulence in our infection model, as independently demonstrated (6). Virulence phenotypes of conditional mutants were also compared against orthologous mutants constructed in a virulent strain of *S. cerevisiae* (7, 8). Although we note significant technical differences in

the infection model used, these studies identified important yeast virulence determinants that are likely common to *C. albicans* pathogenesis. For example, *C. albicans pTET-ARO7* and *pTET-ADE2* displayed dramatic avirulence phenotypes, paralleling their reduced fitness as homozygote deletion mutants in *S. cerevisiae* (7, 8). A strong concordance between in vivo survival phenotypes was also determined among 11 additional *C. albicans* conditional mutants in distinct steps of conserved metabolic pathways as similarly evaluated in a pathogenic isolate of *S. cerevisiae*, including amino acid biosynthesis, as well as purine and pyrimidine biosynthesis and mitochondrial respiration (relevant genes are highlighted and references provided in Table S2). Further, homozygote deletion mutants of *C. albicans HIS1* (9) and *LYS2* (10) remain pathogenic, whereas *ILV2* (11), *ADE2* (12, 13), and *URA3* (13) null mutants are avirulent, consistent with our results using conditional mutants of these genes or other genes participating in these metabolic pathways and corroborating the view that *S. cerevisiae* and *C. albicans* effectively scavenge specific amino acids but not adenine or uridine in a murine host. Finally, among genes for which no comparative analysis is available, we demonstrate a strong correlation between *C. albicans* gene essentiality for growth in an in vitro environment and survival and virulence in the murine host (Fig. 2*C*).

This study, together with previous reports evaluating *C. albicans* virulence (Table S4), captures only ~6% of the pathogen genome. Expanding this target space by current means is limited by significant time and resource constraints. Therefore, an appropriate high throughput screening strategy is required. One approach to address these issues is termed signature-tagged mutagenesis (STM), and involves screening for virulence determinants using pools of defined mutants whose growth and survival in an in vivo setting can be monitored using strain specific markers by DNA sequencing or microarray methods (14). STM has been widely used to identify virulence determinants among bacterial pathogens (15) and more recently the haploid fungal pathogen, *Cryptococcus neoformans* (16). As Tet-based conditional mutants are each uniquely marked with two independent strain identifying DNA barcodes (17) and can be used to robustly discriminate strains growth in complex mixtures (18), a similar STM strategy could be applied to *C. albicans*. Preliminary data demonstrate *C. albicans* strain pools comprising between 12–24 individual conditional mutants coinfected in a murine infection model reliably reproduce in vivo survival phenotypes identified among individual strains when examined separately. Thus, in vivo screens to rapidly identify *C. albicans* virulence-associated factor may be performed in significantly larger throughput, either in rodent or invertebrate infection models, such as *Caenorhabditis elegans* or *Drosophilia melanogaster*. Future studies could also include genetic analysis of interactions between *C. albicans* and innate immunity of the host (e.g., Tol-like receptor (TLR) activation, signaling, and cytokine induction) as well as other host-pathogen interactions, thereby broadening the antifungal target set.

SI Materials and Methods.

An immunocompetent murine model of systemic infection was performed using male imprinting control region mice. Mice were housed five per cage with food and water supplied ad libitum. All experiments were performed according to the National Institutes of Health guidelines for the ethical treatment of animals. Mice (15 mice per group) were infected via the tail vein using 1×10^6 *C. albicans* Tet conditional mutant cells/mouse (19, 20). Three days prior to the inoculation, five mice were given drinking water containing sucrose (5%) and doxycycline (2 mg/mL) to repress gene expression at the onset of infection. The 10 remaining mice were given drinking water with sucrose (5%) only. Five of the mice receiving sucrose water were maintained on this water throughout the infection, whereas the remaining five mice were switched to doxycycline-containing sucrose water on day 2 after injection of *C. albicans* Tet conditional mutant cells. The drinking water regimen for all mice receiving doxycycline was maintained throughout the course of the experimental infection. After 21 days post infection, two mice from each group were taken for necropsy and fungal burden assessed by enumerating *C. albicans* colony forming units (CFU) from the infected kidneys. For all remaining mice, doxycycline-supplemented water was replaced with sucrose water and their survival was monitored for two additional weeks followed by additional necropsies and CFU enumerations thereafter. Signs of infection (torticollis, lethargy, ataxia) were monitored three times daily throughout the experimental time course. Moribund mice, along with those surviving the end of the experiment, were killed by anesthetization followed by cervical dislocation. All *C. albicans* strains were coded prior to injection so that the laboratory workers did not know the identity of the strains. Strains were decoded at the termination of the experiment. Control experiments demonstrated that the doxycycline regimen does not affect survival of mice infected with the parental *C. albicans* strain, CaSS1, when transformed and maintaining an integrated and functional copy of *HIS3* (17). Single factor ANOVA was used to determine statistic significance, with $p < 0.05$ deemed significant.

1. Mühlschlegel FA, Fonzi WA (1997) *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated gene PHR1 with an inverted pattern of pH-dependent expression. *Mol Cell Biol* 17:5960–7.
2. De Bernardis F, Mühlschlegel FA, Cassone A, Fonzi WA (1998) The pH of the host niche controls gene expression in and virulence of *Candida albicans*. *Infect Immun* 66:3317–25.
3. Larriba G, Andaluz E, Cueva R, Basco RD (1995) Molecular biology of yeast exoglucanases. *FEMS Microbiol Lett* 125:121–6.
4. González MM et al. (1997) Phenotypic characterization of a *Candida albicans* strain deficient in its major exoglucanase. *Microbiology* 143:3023–32.
5. Goldstein AL, McCusker JH (2001) Development of *Saccharomyces cerevisiae* as a model pathogen. A system for the genetic identification of gene products required for survival in the mammalian host environment. *Genetics* 159:499–513.
6. De Bernardis F, Mühlschlegel FA, Cassone A, Fonzi WA (1998) The pH of the host niche controls gene expression in and virulence of *Candida albicans*. *Infect Immun* 66:3317–25.
7. Goldstein AL, McCusker JH (2001) Development of *Saccharomyces cerevisiae* as a model pathogen. A system for the genetic identification of gene products required for survival in the mammalian host environment. *Genetics* 159:499–513.
8. Kingsbury JM, Goldstein AL, McCusker JH (2006) Role of nitrogen and carbon transport, regulation, and metabolism genes for *Saccharomyces cerevisiae* survival in vivo. *Eukaryot Cell* 5:816–24.
9. Alonso-Monge R, et al. (1999) Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J Bacteriol* 181:3058–68.
10. Bhattacherjee V, Bhattacharjee JK (1999) Characterization of a double gene disruption in the LYS2 locus of the pathogenic yeast, *Candida albicans*. *Med Mycol* 37:411–7.
11. Kingsbury JM, McCusker JH (2009) Cytocidal amino acid starvation of *Saccharomyces cerevisiae* and *Candida albicans* acetolactate synthase (ilv2{Delta}) mutants is influenced by the carbon source and rapamycin. *Microbiology* 156:929–39.
12. Donovan M et al. (2001) Virulence of a phosphoribosylaminoimidazole carboxylase-deficient *Candida albicans* strain in an immunosuppressed murine model of systemic candidiasis. *Infect Immun* 69:2542–8.
13. Kirsch DR, Whitney RR (1991) Pathogenicity of *Candida albicans* auxotrophic mutants in experimental infections. *Infect Immun* 59:3297–300.
14. Mazurkiewicz P, Tang CM, Boone C, Holden DW (2006) Signature-tagged mutagenesis: Barcoding mutants for genome-wide screens. *Nat Rev Genet* 7:929–39.
15. Autret N, Charbit A (2005) Lessons from signature-tagged mutagenesis on the infectious mechanimsms of pathogenic bacteria. *FEMS Microbiol Rev* 29:703–717.
16. Liu OW, et al. (2008) Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. *Cell* 135:174–88.
17. Roemer T, et al. (2003) Large-scale essential gene identification in *Candida albicans* and applications to antifungal drug discovery. *Mol Microbiol* 50:167–81.
18. Xu D, et al. (2007) Genome-wide fitness test and mechanism-of-action studies of inhibitory compounds in *Candida albicans*. *PLoS Pathog* 3:e92.
19. Becker JM, Henry LK, Jiang W, Koltin Y (1995) Reduced virulence of *Candida albicans* mutants affected in multidrug resistance. *Infect Immun* 63:4515–8.
20. Nakayama H, et al. (2000) Tetracycline-regulatable system to tightly control gene expression in the pathogenic fungus *Candida albicans*. *Infect Immun* 68:6712–9.

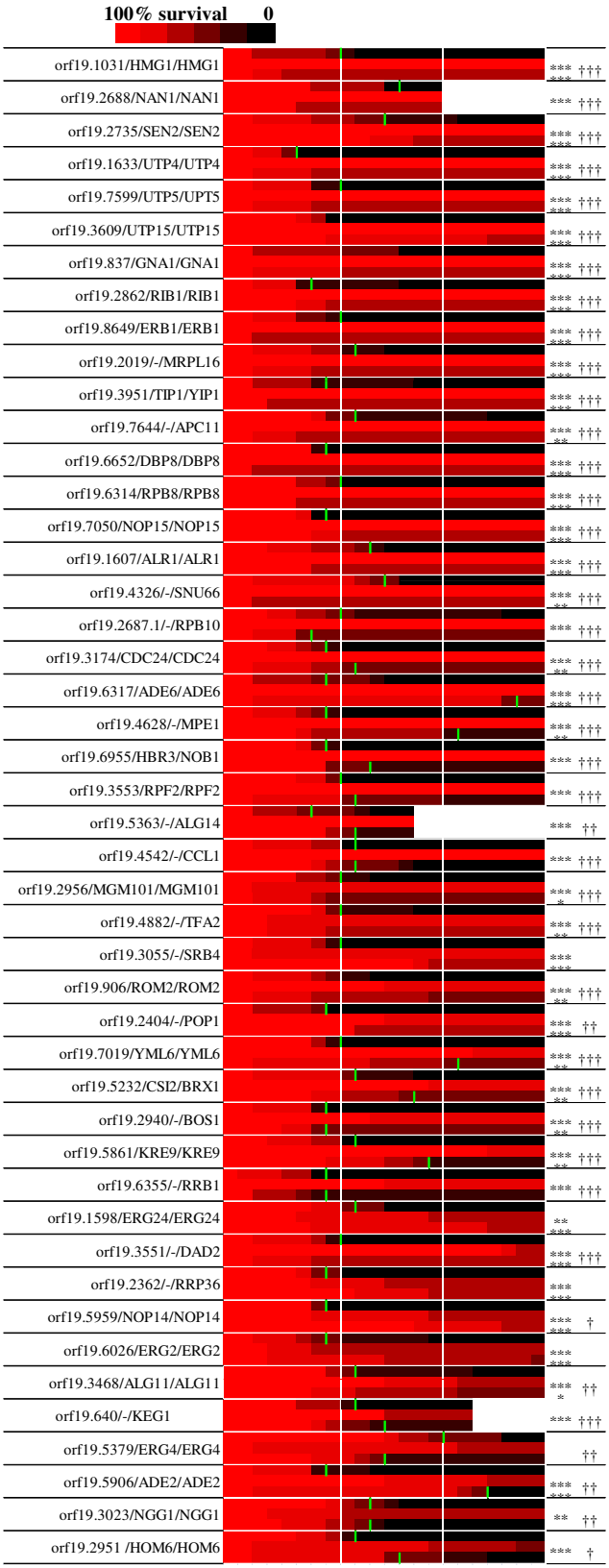

Fig. S1. Summary of survivals of animals infected with the *C. albicans* conditional shut-off strains of genes with attenuated virulence when repressed. The gene annotations are shown on the left, orf19 designation/*C. albicans* gene name/*S. cerevisiae* ortho-/homolog, as appeared in the *Candida* Genome Database. The survival data are summarized on the right. For each strain, the three conditions are as follows: (*Top*) Sugar (nonrepressing), (*Middle*) dox − 3D (repressing), and (*Bottom*) dox + 2D (repressing). With the exception of three strains, the survival of infected animals (red with different shades, with the scale shown on top) was monitored for 3 weeks (separated by white bars). Green bars indicate the day on which ≥60% of the animals were expired due to lethal infection. For statistic significance, survival under the each repressing condition was compared with that in the corresponding sugar group, ***, $p < 0.001$; **, $p < 0.01$, and *, $p < 0.05$. Those under two repressing conditions were also compared, with ††† indicating $p < 0.001$; †† $p < 0.01$; and † $p < 0.05$.

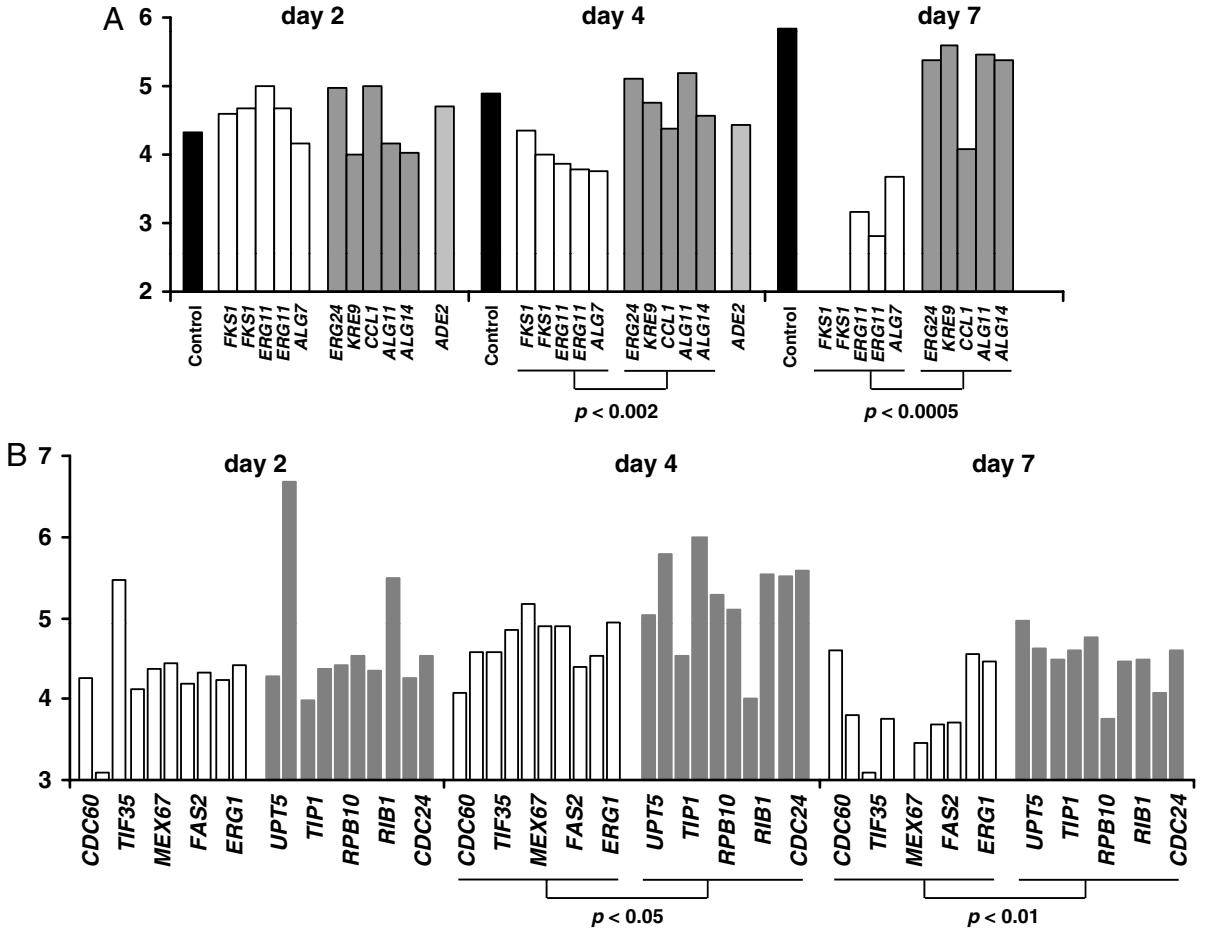

Fig. S2. Fungal burdens of [log10 (CFU/g Kidney)] animals infected with conditional shut-off strains for essential genes and those with attenuated virulence in vivo. (*A*) Selected strains corresponding to essential genes (open bards) and those with attenuated virulence (gray bars) as indicated, together with the starting parental strain (black bars), were tested in the dox + 2D model of infection. Prior to the establishment of the repressing conditions, no statistical difference was observed in the kidney fungal burdens between these two groups. However, the fungal burdens were significantly higher in animals infected with strains for genes of attenuated virulence on days 4 and day 7 (i.e., 2 and 5 days after the repressing conditions were established, with p values $\leq$0.02 and 0.0005, respectively). At least 16 colonies of each strain recovered from infected animals on days 2, 4, and 7 were tested for their phenotypes under both repressing and nonrepressing conditions on both YPD and YNBD media. No phonotypical variation was observed between these isolates and the original strains. Note that an animal infected with the *pTET-ADE2* strain (light gray bars) was expired before the end of the experiment, the day 2 and 4 fungal burdens were not included in the final analysis. We note that all of the colonies of this strain recovered on day 4 (not day 2) displayed red color on YPD (characteristic of *ADE2* repression) used in CFU enumeration suggesting that *ADE2* was fully repressed in vivo. (*B*) In a separate experiment, 10 additional strains, 5 corresponding to essential genes (open bars), and 5 genes of attenuated virulence (gray bars), were selected and tested in the dox + 2D model of infection as described in *A*, with each strain duplicated. Whereas no difference in the kidney fungal burdens was observed between the two groups on day 2, they were significantly higher in animals infected with strains from the second group on days 4 and 7, with p values $\leq$0.05 and 0.01, respectively. Note that the detection limits were 10^2 CFU/gram of kidney in *A*, and 10^3 in *B*, and that these experiments and those described elsewhere were performed independently. Our results confirm the quantitative difference between these two groups of genes in terms of the associated fungal burdens in the animal model of infection under the repressing conditions.

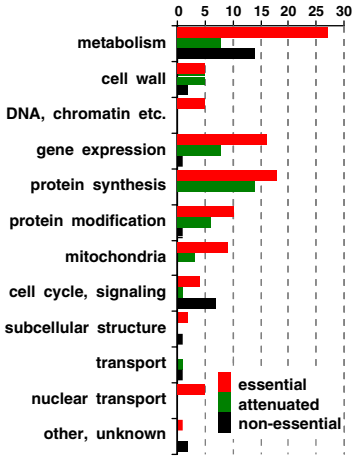

Fig. S3. Distribution of genes essential and nonessential for in vivo virulence, and genes with attenuated virulence when repressed according to functional groups. The identity of each gene is listed in Tables S1 and S2.

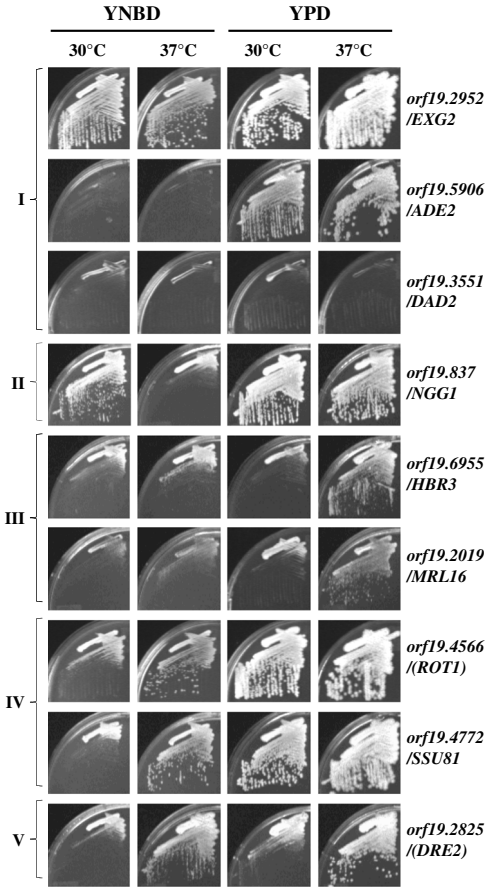

Fig. S4. Terminal growth phenotypes of conditional shut-off strains on solid media at 30 °C and 37 °C. We tested strains shown in Fig. 2 (genes involved in the ergosterol pathway) and additional 38 strains corresponding to genes that are essential in vivo for their terminal phenotypes under the repressing conditions at 30 and 37 °C, and found no differences between these two temperatures. We then tested all the remaining strains for genes with attenuated virulence and those that are nonessential in vivo. Of 73 strains (in these two groups) tested, the terminal phenotype of 67 strains at both temperatures were consistent—three examples are shown here (group I). Minor differences were observed with the remaining 6 strains. The *NGG1* strain failed to grow on YNBD only at 37 °C (II). The *HNR3* and the *MRL16* strains displayed marginally improved growth on YPD only at 37 °C (III), and the *ROT1* and *SSU81* strain on YNBD at the same temperature (IV). Improved growth was observed with the *DRE2* strain at 37 °C on both YNBD and YPD media (V). Whether such (marginal) phenotypic discrepancy is responsible for in vivo requirement of corresponding genes remains to be determined. These results indicate that for most *C. albicans* genes terminal phenotype associated with their transcriptional repression at 30 °C are consistent those at 37 °C.

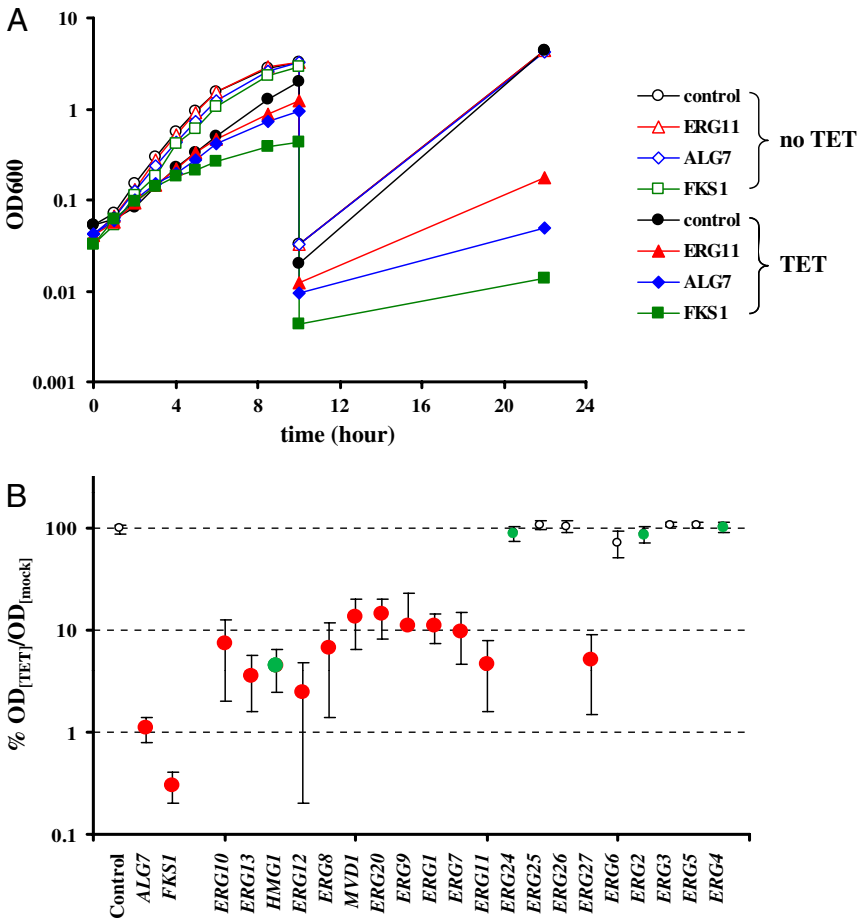

Fig. S5. Terminal phenotype of the conditional shut-off strains in liquid YPD under the repressing conditions. (*A*) In an attempt to quantify terminal growth phenotype, we monitored the growth of three representative (*ERG11*, *ALG7*, and *FKS1*) conditional shut-off strains and those for other genes involved in the ergosterol pathway in liquid YPD in the presence (filled symbols) and absence (open symbols) of tetracycline for 10 hours at 30 °C in flasks with constant shaking. Overnight cultures were first diluted to $OD_{600} \sim 0.02$. After two-hour growth, OD was determined for each culture, which was immediately divided into two parts (50 mL each). Tetracycline (a final concentration of 100 μg/mL, with 1% ethanol) was added to one of them, and ethanol (1%) to the mock. The growth of both cultures was monitored at the intervals indicated. After 10 hour growth, each culture was diluted 100-fold with the corresponding medium. Their growth was determined 12 hours later. For most strains, their growth in the absence of tetracycline (open symbols) was largely indistinguishable from the control. The control strain and most of those for nonessential genes displayed no pronounced difference in growth in the presence or absence of tetracycline for 22 hours. For genes deemed essential for growth in solid media, transcriptional repression resulted in growth inhibition ≥4 hours after the addition of tetracycline. Quantitative difference was also observed in the overnight growth of diluted cultures. In the cases of three representative (*ERG11*, *ALG7*, and *FKS1*) strains, the quantitative differences in the rate of shut-off and overnight residual growth bore no quantitative correlation with their essential role in the animal model of systemic candidiasis. (*B*) The overnight residual growth (in quadruplicates) was determined for all the indicated strains as described in *A*. Shown are the averages of growth of each strain under the repressing condition normalized by that of mock treatment, with bars indicating standard deviations. Although significant differences were observed with strains corresponding to genes from the three groups described in Fig. 2*A*, they did not necessarily reflect the requirement of these genes for in vivo virulence. The results presented in this and the previous figures suggest that the terminal phenotypes determined in solid media at both 30 and 37 °C are largely consistent, and are generally reflective of the requirement of corresponding genes in the animal model of candidiasis (also see Fig. 2*C*). However, any quantitative aspects of terminal phenotypes in liquid media do not necessarily reveal additional quantitative differences in vivo. Symbols correspond to essential genes (red), attenuated virulence (green), and nonessential genes (open) for survival and virulence.

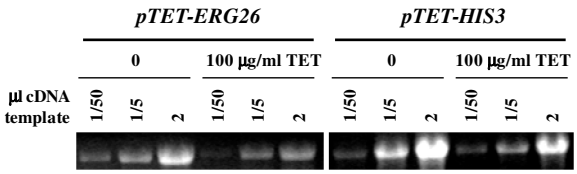

Fig. S6. RT-PCR determination of *ERG26* transcripts in the conditional shut-off strains for *ERG26* and *HIS3* under the nonrepressing (0 TET) and the repressing (100 μg/mL TET) conditions. Total RNA was extracted from both strains grown under the conditions indicated for 16 hours. Two microgram of total RNA was used in the standard synthesis of cDNA in the final volume of 20 μL, of which indicated amounts were used in PCR with *ERG26*-specific primer pairs (forward; CTACAAGAGACGACATTAGTGG, and reverse; GGCGCACACAACTTTAACTCTA) in the final volume of 20 μL. The PCR cycles consisted of initial denaturation for 3 min at 94 °C, amplification by 25 cycles of 40 seconds at 94 °C, 40 seconds at 54 °C, followed by 1.5 minutes at 72 °C, and a final extension for 3 minutes at 72 °C. The products (10 μL) were analyzed in 2% agarose gels with TAE buffer containing ethidium bromide. In the control (*pTET-HIS3*) strain, the abundance of *ERG26* specific transcript was marginally reduced. However, it remains to be determined if this is due the effect of tetracycline. In the *pTET-ERG26* strain, sufficient amount of *ERG26*-specific transcript was detected under the repressing conditions, suggesting that its expression was not sufficiently repressed from the heterologous tetracycline promoter. This could be responsible for the discrepancy between our data (Fig. 3) and published results (1).

1 Aaron KE, Pierson CA, Lees ND, Bard M (2001) The *Candida albicans ERG26* gene encoding the C-3 sterol dehydrogenase (C-4 decarboxylase) is essential for growth. *FEMS Yeast Res* 1:93–101.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (XLS)
Table S3 (XLS)
Table S4 (XLS)