# *CANDIDA ALBICANS*: A MOLECULAR REVOLUTION BUILT ON LESSONS FROM BUDDING YEAST

*Judith Berman* and Peter E. Sudbery‡*

*Candida albicans* is an opportunistic fungal pathogen that is found in the normal gastrointestinal flora of most healthy humans. However, in immunocompromised patients, blood-stream infections often cause death, despite the use of anti-fungal therapies. The recent completion of the *C. albicans* genome sequence, the availability of whole-genome microarrays and the development of tools for rapid molecular-genetic manipulations of the *C. albicans* genome are generating an explosion of information about the intriguing biology of this pathogen and about its mechanisms of virulence. They also reveal the extent of similarities and differences between *C. albicans* and its benign relative, *Saccharomyces cerevisiae.*

OPPORTUNIST
An organism that usually does not cause disease but, under circumstances such as immune deficiency, can become a pathogen.

COMMENSAL
An organism that lives in another without causing injury to its host.

**Department of Genetics, Cell Biology and Development, and Department of Microbiology, 6–160 Jackson Hall, 321 Church Street SE, Minneapolis, Minnesota 55455, USA. ‡Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2TN, UK. Correspondence to J.B. e-mail: judith@cbs.umn.edu*
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*Candida albicans* is an OPPORTUNISTIC COMMENSAL. Virtually all of us carry it in our gastrointestinal and genitourinary tracts and, to a lesser extent, on our skin. When immune systems are weak (for example, as a result of cancer chemotherapy, HIV infection or in neonates) or when the competing flora are eliminated (for example, after antibiotic treatment), *C. albicans* colonizes and invades host tissues. Although HIV patients frequently suffer from recurring oral CANDIDIASIS and sometimes die from advanced oesophageal colonization, infections (such as thrush and vaginitis) of mucosal tissues are usually not life threatening. However, if the organism gains access to the blood stream (a condition known as candidaemia), by invasion of host tissues or by contamination of in-dwelling catheters, the infection can progress to the growth of fungal masses in the kidney, heart or brain.

*C. albicans* is the fourth most common hospital-acquired infection in the United States, the treatment of which is estimated to cost more than US $1 billion annually[1,2]. Because *C. albicans* and other fungal pathogens are eukaryotes and therefore share many of their biological processes with humans, most anti-fungal drugs cause deleterious side effects and, at the doses used, are FUNGISTATIC rather than FUNGICIDAL. So, it is an important goal of *C. albicans* research to identify appropriate targets for anti-fungal technologies. Understanding the biology of this opportunist, in all its morphological and biochemical states, is necessary for the development of therapies that will prevent or treat candidiasis in susceptible patients.

Rapid advances in the understanding of many basic biological processes in *C. albicans* have been made as a result of their similarity to well-studied processes in *Saccharomyces cerevisiae*. *S. cerevisiae*, which diverged from *C. albicans* 140–841 million years ago[3,4], is an indispensible guide for studying aspects of cell-cycle progression, signal transduction, mating, metabolism and cell-wall biosynthesis in *C. albicans*. In addition, *S. cerevisiae* has been used for preliminary testing of hypotheses that were later addressed directly in *C. albicans* (for example, see REF. 5).

Despite the many processes that are conserved between these distant cousins, there are also significant differences. For example, *S. cerevisiae* grows exclusively by budding off round yeast cells or elongated pseudo-hyphal cells, whereas *C. albicans* is more morphologically diverse, forming true hyphae (BOX 1) and CHLAMYDOSPORES. This diversity is thought to aid its survival, growth and dissemination in the mammalian host. Such features are less readily studied using *S. cerevisiae* as a model and highlight the importance of studying *C. albicans* itself.



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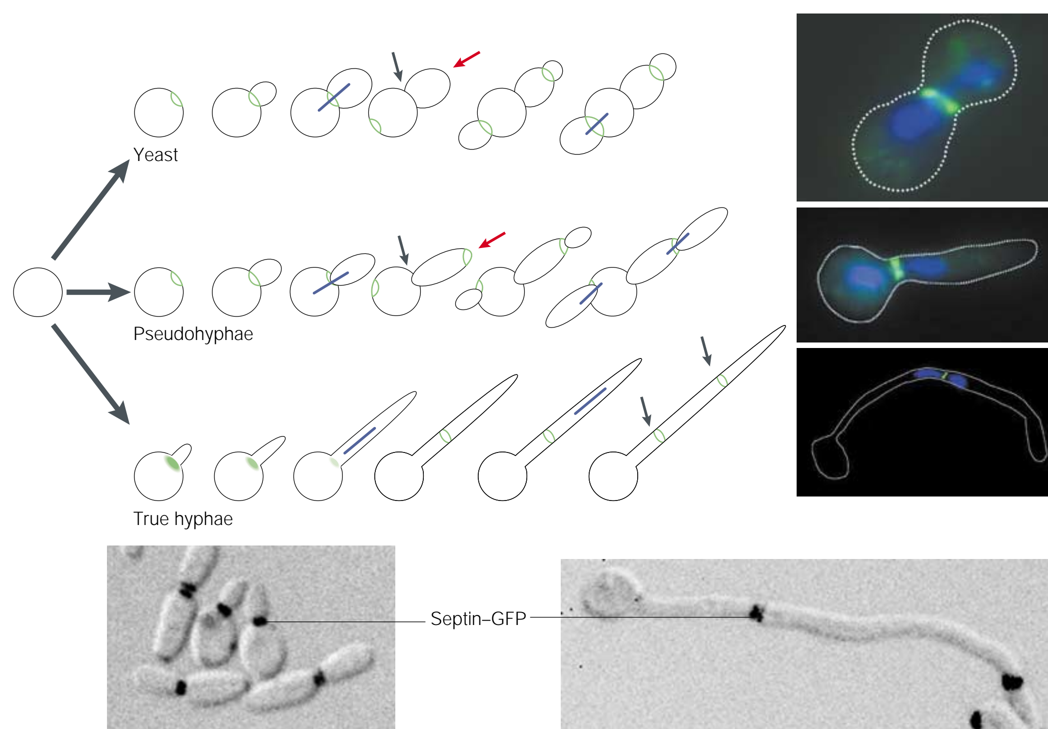
Box 1 | **Differences between yeast, pseudohyphae and true hyphae**

***Candida albicans*** **can exist in three forms that have distinct shapes: yeast cells (also known as blastospores), pseudohyphal cells and true hyphal cells. Yeast cells are round to ovoid in shape and separate readily from each other. Pseudohyphae resemble elongated, ellipsoid yeast cells that remain attached to one another at the constricted septation site and usually grow in a branching pattern that is thought to facilitate foraging for nutrients away from the parental cell and colony. True hyphal cells are long and highly polarized, with parallel sides and no obvious constrictions between cells. Actin is always localized at the tip of the growing hypha[89]. A basal SEPTIN band (green) forms transiently at the junction of the mother cell and the evaginating GERM TUBE; the first true hyphal septum forms distal to the mother cell and well within the germ tube[66]. The sub-apical cells become highly vacuolated and do not branch or bud until the ratio of cytoplasm to vacuolar material increases significantly[63]. All three cell types have a single nucleus per cell before mitosis. Important differences between yeast, pseudohyphal and true hyphal cells include the degree of polarized growth, the positioning of the septin ring (green in diagram and micrographs, and black in light microscope images) and of the true septum relative to the mother cell, the movement of the nucleus (blue line in diagram; stained with DAPI, blue in micrographs) relative to the mother cell and the degree to which daughter cells are able to separate into individuals. GFP, green fluorescent protein.**




CANDIDIASIS
Infection with a *Candida* species. It often refers to the infection of mucosal surfaces, such as the mouth, vagina, skin or oesophagus.

FUNGISTATIC
The ability to inhibit the growth of fungi. Fungistatic agents can keep an infection in check but usually do not completely eliminate the fungus from the host.

FUNGICIDAL
The ability to kill fungi. Fungicides have the potential to clear a fungal infection from the host.

CHLAMYDOSPORES
Thick-walled round cells that sometimes form at the ends of hyphae or pseudohyphae in response to nutrient stress or other stresses.

SEPTIN
A protein that forms a ring-shaped scaffold-like structure at the incipient bud site in yeast cells and pseudohyphal cells and at the incipient site of septation in true hyphae.

GERM TUBE
The elongating structure that evaginates from a round yeast cell when it is induced to form true hyphae.

Genetic manipulations that are carried out easily in *S. cerevisiae* are much more laborious in *C. albicans* because of the lack of a complete sexual cycle in this presumed obligate diploid. Both conventional and molecular-genetic analysis have therefore proved difficult. However, recent advances in molecular-genetic techniques, together with the availability of the genome sequence, have revolutionized research in this organism. Moreover, data from the *Candida* Genome Sequencing Project[6], together with sophisticated cloning approaches[7], have revealed the existence of 'mating-type-like' loci that, when homozygous, can direct the formation of recombinants between diploid strains[7–9]. So, conventional genetic techniques might soon be available in *C. albicans*.

This review provides examples of how *S. cerevisiae* models guided the early molecular studies of *C. albicans* biology and how new tools are facilitating the direct analysis of biological questions in *C. albicans*. How does this pathogen respond to environmental stimuli? What alters its morphogenesis programmes? What possible mating interactions does it undergo? And how does it organize and reorganize its genome while growing *in vitro* or in mammalian host cells and tissues?

## Technical challenges and solutions

Genetic manipulations of *C. albicans* have been fraught with difficulties that stem from the lack of a useful sexual cycle and a lack of molecular tools. Today, reverse-genetic approaches, in which genes are first identified by their sequence and then both genomic copies are sequentially deleted or mutated, are commonly used. Clearly, this approach requires some previous knowledge of the biological process of interest, emphasizing the benefit of using a well-established model organism such as *S. cerevisiae*.



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Laboratory studies of *C. albicans* use a small number of strains that have been engineered with one or more AUXOTROPHIC markers. Plasmids that carry autonomously replicating sequences (ARSs) are available for transformation at high frequency and for expressing genes in a non-chromosome-specific context[10]. Although it is desirable that these plasmids remain extrachromosomal, even when they carry two ARSs, they integrate into the genome primarily by homologous recombination.

Another significant challenge is posed by the unconventional *C. albicans* codon usage — *C. albicans* translates the CUG codon as serine, rather than the 'universal' leucine[11]. For this reason, many heterologous markers do not function in *C. albicans* unless the CUG codons are first modified. Nonetheless, many *C. albicans* genes are at least partially functional in *S. cerevisiae*, which facilitated their identification by complementation studies.

***Transformation and mutagenesis.*** In the past few years, several crucial tools have greatly enhanced our ability to manipulate *C. albicans* genetically (reviewed in REF. 12). Methods for transformation were modified from protocols for transformation of *S. cerevisiae* and *Pichia pastoris*, which is the methanol-trophic yeast that is used primarily for protein production[13]. In 1993, a recyclable *URA3* cassette was adapted for multiple sequential transformations of *C. albicans* strains that were auxotrophic for *URA3* (REF. 14; FIG. 1a). The numerous single and double mutant strains that were generated using this 'Ura-blaster' strategy provided the first genetic evidence of signalling pathways that were important for *C. albicans* morphogenesis and virulence. More recently, it has become clear that uracil auxotrophy affects the ability of *C. albicans* cells to adhere to human tissues. It also affects the virulence of *C. albicans* in a mouse model of systemic candidiasis[15–17].

A PCR-mediated transformation system similar to that used in *S. cerevisiae*[18] has been developed for use in *C. albicans*[19,20], obviating the need to clone a gene before disrupting it (FIG. 1b). Strain BWP17, which is triple auxotrophic (*ura3*, *his1* and *arg4*) has made the generation of double mutants simpler by allowing sequential transformation steps without the need to regenerate a single selectable marker.

Other tools, including a plasmid that can be used to test whether a gene is essential (FIG. 1c) and dominant selectable markers coupled with an excision system that is based on the *S. cerevisiae* 2-μm *FLP*/FRT SYSTEM[21], have also been developed in the past few years (TABLE 1). Furthermore, systems that rely on *in vitro* transposition[22] into *C. albicans* genomic DNA to generate heterozygous or homozygous mutant strains are being developed (D. Davis, V. Bruno, L. Loza, S. Filler and A. Mitchell, personal communication; A. Uhl and A. D. Johnson, personal communication), and antisense mRNA expression can be used to generate mutant growth phenotypes[23]. In addition, promoters that are designed to study gene expression and several heterologous reporter genes that are designed to monitor gene expression are now available (TABLE 1). For example, green fluorescent protein (GFP) has been codon optimized for expression in *C. albicans*[24,25], and more recently, codon-modified GFP has been altered to generate the cyan (CFP) and yellow (YFP) versions of the fluorescent proteins. Codon-optimized *GFP*, *YFP* and *CFP* genes have also been coupled with one of two selectable markers (*URA3* or *HIS1*). This generated cassettes that, when amplified by PCR, can be inserted in-frame at the carboxyl terminus of any gene of interest to tag its product and visualize it *in vivo*[26].

***Use of* S. cerevisiae *to study* C. albicans.** To circumvent the difficulties of carrying out genetic studies directly in *C. albicans*, many *C. albicans* genes have been identified and/or analysed using *S. cerevisiae* as a 'surrogate'. For example, many *C. albicans* genes were cloned by their ability to complement a mutation in *S. cerevisiae*. This approach is not as important as it once was, because homologues can be identified on the basis of their sequence similarity, as a result of the *C. albicans* genome sequencing project. Nonetheless, if a gene does function in *S. cerevisiae*, then the effects of mutant alleles can now be tested in *S. cerevisiae* before the more laborious process of testing them in *C. albicans* (see, for example, REF. 27).

*C. albicans* genes have also been cloned on the basis of their ability to interfere with an *S. cerevisiae* process. Czf1, a putative transcription factor, interferes in a dominant manner with the cell-cycle arrest that is induced normally in *S. cerevisiae* in response to mating pheromone[28]. The *C. albicans* *INT1* gene encodes a protein that is present at the septin rings of yeast and hyphal cells. When expressed in *S. cerevisiae*, *INT1* induces the formation of highly elongated cells that resemble hyphal germ tubes and are much more elongated than *S. cerevisiae* pseudohyphae[29]. In these cells, Int1 associates with septin proteins, causing them to form abnormal spiral structures[30]. Int1 also affects morphogenesis and virulence in *C. albicans*[31].

Some *C. albicans* genes were cloned on the basis of their ability to confer new properties to *S. cerevisiae*, such as the ability to make *S. cerevisiae* cells adhere to human cells[32–34]. Of these, two encode cell-wall proteins that are important for adhesion in *C. albicans*, whereas one affects adhesion of *S. cerevisiae* cells through an indirect mechanism. Another important example was the isolation of Cph1, the *C. albicans* homologue of *S. cerevisiae* Ste12 — the transcription factor that is activated by the mating pheromone resonse MAP kinase cascade during mating and filamentous growth. It was isolated in a screen for genes that, when overexpressed in *S. cerevisiae*, enhanced pseudohyphal formation[35].

*S. cerevisiae* is also used as a substitute for *C. albicans* in studies of host interaction. In the human host, neutrophils represent the first line of defence against *C. albicans*, although macrophages and dendritic cells also have a role in the immune response to candidial infection[36]. When cultured macrophages are incubated with *C. albicans* cells, the macrophages ingest the yeast cells. However, wild-type *C. albicans* strains proceed to grow hyphae that lyse the macrophage membranes and escape from the cells[37]. Before *C. albicans* microarrays

AUXOTROPHIC
Requiring a nutritional supplement to grow.

PROTOTROPH
A cell that can grow in the absence of nutritional supplements.

*FLP*/FRT SYSTEM
A recombination system that is adapted from the *Saccharomyces cerevisiae* 2-μm plasmid. *FLP* encodes a site-specific recombinase, and Frt is the *FLP* recombinase target site. Expression of *FLP* mediates excision of any sequence that is flanked by Frt sites.



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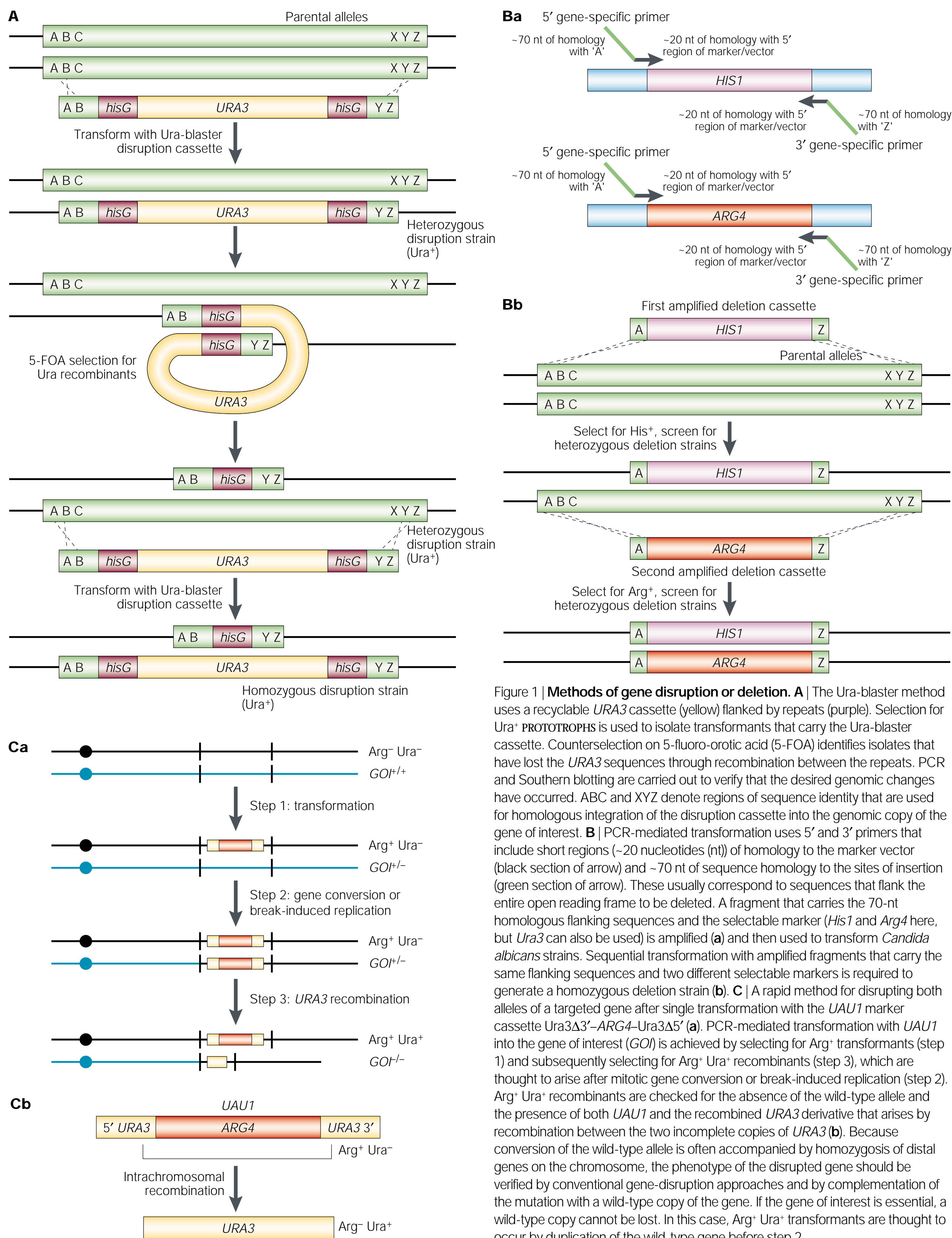


Figure 1 | **Methods of gene disruption or deletion. A** | The Ura-blaster method uses a recyclable *URA3* cassette (yellow) flanked by repeats (purple). Selection for Ura+ PROTOTROPHS is used to isolate transformants that carry the Ura-blaster cassette. Counterselection on 5-fluoro-orotic acid (5-FOA) identifies isolates that have lost the *URA3* sequences through recombination between the repeats. PCR and Southern blotting are carried out to verify that the desired genomic changes have occurred. ABC and XYZ denote regions of sequence identity that are used for homologous integration of the disruption cassette into the genomic copy of the gene of interest. **B** | PCR-mediated transformation uses 5′ and 3′ primers that include short regions (~20 nucleotides (nt)) of homology to the marker vector (black section of arrow) and ~70 nt of sequence homology to the sites of insertion (green section of arrow). These usually correspond to sequences that flank the entire open reading frame to be deleted. A fragment that carries the 70-nt homologous flanking sequences and the selectable marker (*His1* and *Arg4* here, but *Ura3* can also be used) is amplified (**a**) and then used to transform *Candida albicans* strains. Sequential transformation with amplified fragments that carry the same flanking sequences and two different selectable markers is required to generate a homozygous deletion strain (**b**). **C** | A rapid method for disrupting both alleles of a targeted gene after single transformation with the *UAU1* marker cassette Ura3Δ3′–*ARG4*–Ura3Δ5′ (**a**). PCR-mediated transformation with *UAU1* into the gene of interest (*GOI*) is achieved by selecting for Arg+ transformants (step 1) and subsequently selecting for Arg+ Ura+ recombinants (step 3), which are thought to arise after mitotic gene conversion or break-induced replication (step 2). Arg+ Ura+ recombinants are checked for the absence of the wild-type allele and the presence of both *UAU1* and the recombined *URA3* derivative that arises by recombination between the two incomplete copies of *URA3* (**b**). Because conversion of the wild-type allele is often accompanied by homozygosis of distal genes on the chromosome, the phenotype of the disrupted gene should be verified by conventional gene-disruption approaches and by complementation of the mutation with a wild-type copy of the gene. If the gene of interest is essential, a wild-type copy cannot be lost. In this case, Arg+ Ura+ transformants are thought to occur by duplication of the wild-type gene before step 2.



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PHAGOLYSOSOME
An organelle in a phagocytic cell that is formed by fusion of an ingested particle (for example, a *Candida* cell) with a lysosome, which has hydrolytic enzymes that are used to digest the particle.

GLYOXYLATE CYCLE
A metabolic pathway for converting two acetyl CoA molecules to a four-carbon dicarboxylic acid. The cycle is present in bacteria, plants and fungi, but not in mammals.

became available, *S. cerevisiae* microarrays were used to study the global expression profiles of *S. cerevisiae* cells during their phagocytosis by macrophages[38]. *S. cerevisiae* cells that were isolated from PHAGOLYSOSOMES had elevated expression of genes that encode enzymes of the GLYOXYLATE CYCLE. This prompted the study of *C. albicans* cells, in which the expression of genes that encode the principal enzymes of the glyoxylate cycle, isocitrate lyase (*ICL1*) and malate synthase (*MLS1*), was also elevated during phagocytosis. Subsequent deletion of *C. albicans ICL1* yielded a strain that was less virulent in the systemic mouse model of candidiasis[38].

Table 1 | **Molecular tools that are commonly used in the study of *Candida albicans***

| Tools | Properties/comments | References |
|---|---|---|
| ***Selectable markers*** | | |
| *URA3* | Selection: uridine prototrophy, counterselection on 5-FOA; Ura$^-$ cells have reduced virulence | 14,103 |
| *HIS1* | Selection: histidine prototrophy | 20 |
| *ARG4* | Selection: arginine prototrophy | 20 |
| *IMH3* | Wild-type allele effective only at high copy, resistant alleles function at single copy and homology with endogenous copy reduces targeted integration efficiency | 25,104–106 |
| *pUAU* — cassette that carries *URA3* flanked by 5′ and 3′ portions (including ~500 bp of overlap) of *ARG4* | PCR-mediated transformation for arginine prototrophy, followed by selection for recombination between the *URA3* fragments (while maintaining selection for Ura$^+$ cells), yields some isolates in which both copies of the gene of interest have been disrupted; mitotic recombination might make homozygous sequences distal to the insertion site | 107 |
| ***Promoters*** | | |
| *ADH1* | High levels of expression | 108,109 |
| *ACT1* | High levels of expression; stronger than *ADH1* | 25,78,110,111 |
| *GAL1* | Induced ~10–12-fold with galactose, repressed with glucose; 3–4-fold weaker than *ACT1* | 112 |
| *PCK1* | Induced on succinate or, at higher levels (up to 100-fold), with casamino acids (acid digests of casein treated to eliminate or reduce vitamins); repressed with glucose | 111,113 |
| *MAL2* | Induced ~3–4-fold by maltose and sucrose, repressed by glucose | 114,115 |
| *MET3* | Repressed up to 85-fold in the presence of methionine and/or cysteine | 116 |
| Tetracycline-regulatable *Escherichia coli tetR* fused to Hap4 (*Saccharomyces cerevisiae*) activation domain; promoter to be regulated contains *tetO* binding site | Up to 500-fold repression; requires two components (TetR and TetO) inserted in the genome; a lack of homology to the *C. albicans* genome improves the frequency with which non-homologous recombination generates the desired integrants | 117 |
| ***Heterologous reporter genes*** | | |
| *Kluyveromyces lactis LAC4* (β-galactosidase) | Does not work well as a single copy | 118 |
| *Streptococcus thermophilus lacZ* (β-galactosidase) | Expression levels much higher than those of *LAC4* in *K. lactis*; no *C. albicans* homologue | 119 |
| *Renilla reniformis* luciferase | Can be detected at low levels of expression; no *C. albicans* homologue; no CUG codons | 120 |
| *Aequorea victoria* GFP | Codon optimized for use in *C. albicans* | 24,25 |
| Modified GFPs, YFPs and CYPs | Codon optimized and available in cassettes for gene replacement or fusion protein construction through PCR-mediated transformation | 26 |
| Flp/*FRT in vivo* expression system | Flp recombinase driven by a test promoter is used to excise a marker flanked by *FRT* sites; the timing of marker excision reflects the time when the test promoter was first active | 105 |

5-FOA, 5-fluoro-orotic acid; *ACT1*, actin 1; *ADH1*, alcohol dehydrogense 1; *ARG4*, arginine 4; CFP, cyan fluorescent protein, *GAL1*, galactose 1; GFP, green fluorescent protein; *HIS1*, histidine 1; *IMH3*, inosine 5′-monophosphate dehydrogenase 3; *PCK1*, phosphoenolpyruvate carboxykinase 1; *MAL2*, maltose 2; *MET3*, methionine 3; TetO, tetracycline operator; TetR, tetracycline repressor; *URA3*, uracil 3; YFP, yellow fluorescent protein.



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***DNA array analysis.*** The availability of the *C. albicans* genome sequence facilitated the development of DNA arrays for gene-expression analysis. Incyte, Inc., generated microarrays of 6,600 *C. albicans* open reading frames (ORFs) that had been determined on the basis of genomic and proprietary cDNA sequences. So far, these arrays have been used to analyse the expression patterns of cells exposed to Itraconazole, a broad-spectrum anti-fungal drug[39]. As discussed below (in the section entitled 'Hyphal-specific gene transcription'), groups led by Al Brown and Haoping Liu have used partial genome microarrays to analyse the regulatory pathways that orchestrate gene expression during the yeast-to-hyphal transition. Several groups are now constructing and using whole-genome microarrays. The first whole-genome arrays for *C. albicans* (6,334 ORFs) to be published came from Whiteway and co-workers, who used them to analyse the evolution of resistance to anti-fungals[40] and the yeast-to-hyphal transition[41]. Information about their production is available at the Online link to MicroArray Lab, National Research Council of Canada.

## Genome organization

*C. albicans* has a diploid genome that is split between eight pairs of chromosomes that can be separated by pulse-field gel electrophoresis[42]. At ~16 Mb, the haploid genome is slightly larger than that of *S. cerevisiae*, perhaps because of the greater number of retrotransposon families[6]. It contains several large families of genes that encode proteases, lipases and cell-wall proteins that are not present in such large gene families in *S. cerevisiae*. The genes of both yeasts usually lack introns[6]. Although centromere sequences have remained elusive, several ORFs that encode conserved centromere proteins are present in the genome sequence (K. Sanyal and J. Carbon, personal communication). Telomere sequences and telomerase homologues have also been identified[43–45].

An interesting, but poorly understood, property of *C. albicans* clinical isolates is their variable karyotype[46,47]. As has been observed in *S. cerevisiae*[48], the length of the chromosome that carries the ribosomal (r)DNA is highly variable, owing to changes in the number of rDNA repeats[49,50]. A set of nested repetitive sequences — multiple repeat sequences (MRSs) — seems to be the main site of the translocations that are found in clinical isolates. Karyotype changes are caused by the expansion and contraction of repeat sequences in the MRS, as well as by reciprocal translocation events between MRS repeats[51–53]. MRSs are found in one or two copies on all chromosomes except chromosome 3 (but, see REF. 54).

Although unintended, genome rearrangements occur in *S. cerevisiae* strains that go through several rounds of transformation[55]; non-disjunction apparently occurs with a higher frequency in *C. albicans*, perhaps as a mechanism to adapt to stressful conditions. For example, the loss of chromosome 5 occurs frequently in strains that are forced to grow on sorbose as the sole carbon source, presumably because a repressor of sorbose use resides on chromosome 5 (REF. 56). Similarly, strains that are resistant to the anti-fungal fluconozole have an increased frequency of chromosome 4 loss or chromosome 3 gain[57]. The mechanism by which these events affect fluconozole resistance is not clear. Although there are multidrug transporters on chromosomes 4 and 3, *ERG11* — the gene that is important for ergosterol biosynthesis and that, when mutated, confers resistance to fluconozole — is on chromosome 5. So, as for sorbose use, altered chromosome numbers might act by regulating the genes that are necessary for drug resistance.

***Genome sequence.*** The *C. albicans* genome was sequenced by the Stanford Genome Technology Center, and a draft of the assembled sequence can be downloaded and searched at their web site. An international, collaborative annotation group is now producing an annotated database. Information about progress of this effort is posted at the *Candida albicans* Genome Information web site. Partial annotation is also accessible at *Candida* DB, the European *Candida* Database web site.

Genome sequencing has uncovered many *C. albicans* ORFs that have obvious *S. cerevisiae* homologues. Among them are many of the putative homologues of *S. cerevisiae* genes that are required for sexual differentiation and meiosis[58]. *C. albicans* also contains many genes that have no obvious *S. cerevisiae* homologues, some of which are most similar to genes from other fungi, but others that encode novel gene products[6]. Because most *S. cerevisiae* strains do not adhere to, or invade, human tissues, gene products that have no homologues in *S. cerevisiae* are considered by some to be good candidates for genes that are important for host interactions. Homologues of genes that are common to all fungi, especially those that are essential for fungal growth, might be good candidates for broad-spectrum anti-fungal targets. *C. albicans* genes that lack human homologues are considered especially promising in this respect, because they are less likely to cause the negative side effects that are associated with most anti-fungal therapies.

The *S. cerevisiae* genome is thought to have undergone a duplication ~100 million years ago[59]. The *C. albicans* genome contains fewer sets of duplicated genes with related or redundant functions[60], indicating that it might not have undergone this duplication. For example, the six B-cyclin genes of *S. cerevisiae* correspond to only two obvious B-cyclin homologues in *C. albicans*. Even if gene sequences indicate related functions, their roles might be different. For example, *spt3* mutants in *S. cerevisiae* are defective in filamentous growth, whereas *spt3* mutants in *C. albicans* are hyperfilamentous[61]. Furthermore, genes that are essential for viability in *S. cerevisiae* might not be essential in *C. albicans*, and vice versa. Accordingly, *S. cerevisiae* has two *RAS* homologues and together they are essential for viability, but the single obvious *RAS* homologue of *C. albicans* is not essential[62], indicating that a pathway controlled by Ras/cAMP in *S. cerevisiae* is either not controlled by Ras in *C. albicans* or is not important for *C. albicans* viability. Conversely, Abp1 in *S. cerevisiae*, an



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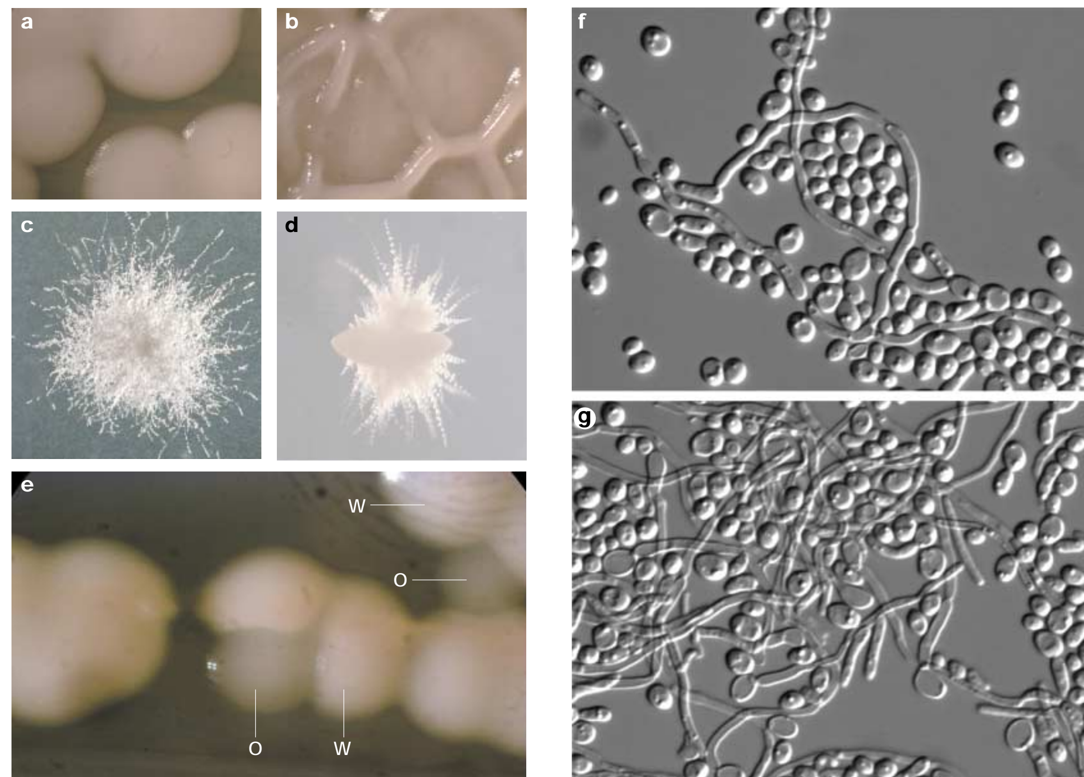


Figure 2 | **Colony morphologies of *Candida albicans*.** A single strain can take on different colony morphologies on different media or as a consequence of PHENOTYPIC SWITCHING. **a** | Smooth colonies grown on salt-dextrose complete (SDC) medium; **b** | wrinkled colonies grown on spider medium; **c** | fuzzy colonies grown on milk-Tween agar; and **d** | embedded colonies suspended in a matrix of rich medium that contains sucrose. **e** | White–opaque phenotypic switching is seen here on SDC medium maintained at 23 °C. White cells (W) of the WO-1 strain were plated at 23 °C for three days, and opaque colonies (O) and sectors appeared in the population. **f**,**g** | Cells in wrinked, embedded and fuzzy colonies are a mixture of yeast, pseudohyphal and true hyphal cells. A population of cells derived from different portions of wrinkled colonies is shown.

actin-binding protein, is not essential, but Abp1 in *C. albicans* seems to be required for growth[23]. So, knowing the role of a gene product in *S. cerevisiae* is not sufficient to infer its properties in *C. albicans*.

**Morphogenesis**

Morphogenesis has been a focus of research in *C. albicans* because virulence is associated with the ability to switch between the yeast and hyphal morphologies. *C. albicans* grows vegetatively in at least three morphogenic forms: yeast, pseudohyphae and hyphae (BOX 1). The yeast form closely resembles the budding yeast *S. cerevisiae.* The pseudohyphal form consists of chains of elongated yeast cells that retain constrictions at the junctions between adjacent compartments, whereas hyphae are tube-like, with sides that are parallel along their entire length[63–66]. Pseudohyphae can sometimes superficially resemble hyphae; however, the two states are clearly different and should not be confused. The term filamentous is used here where it not clear whether cells are hyphal or pseudohyphal. Although mechanistic studies of pseudohyphal growth in *S. cerevisiae* have been informative about pseudohyphal growth in *C. albicans*, *S. cerevisiae* models are less relevant to true hyphal growth.

PHENOTYPIC SWITCHING
A change in cellular or colony properties that seems to be heritable, but reverses at a rate that is much higher than could be caused by mutation. Examples include colony switching and white–opaque switching in *Candida albicans*.

CRENULATED
Having an uneven 'saw-tooth'-like edge. Crenulated colonies have filamentous cells that protrude from the edges of them.

The *C. albicans* hyphal form is often found at sites of tissue invasion, and cells that do not readily form hyphae often have reduced virulence[64]. Importantly, other *Candida* spp. that do not readily form true hyphae are much less frequently isolated from the human host, indicating that they are less virulent. But strains that are unable to grow in the yeast form are also less virulent[37,61,64,67]. It is generally thought that hyphal cells expressing cell-wall proteins that facilitate adhesion to human tissues are important for tissue invasion, as well as for escape from phagocytosis mediated by neutrophils or macrophages. By contrast, the yeast form is thought to be important for dissemination of the pathogen through the blood stream. It is likely, therefore, that the ability to switch between the morphological forms is important for *C. albicans* virulence. However, proof that this is the case is still lacking, and the issue remains controversial among the *Candida* research community[68].

*C. albicans* cells that have different morphologies also contribute to the formation of colonies with different characteristics. First, colonies with hyphal and pseudohyphal cells invade the agar substratum. Second, the presence of hyphae and pseudohyphae causes colonies to have a CRENULATED appearance, in contrast to



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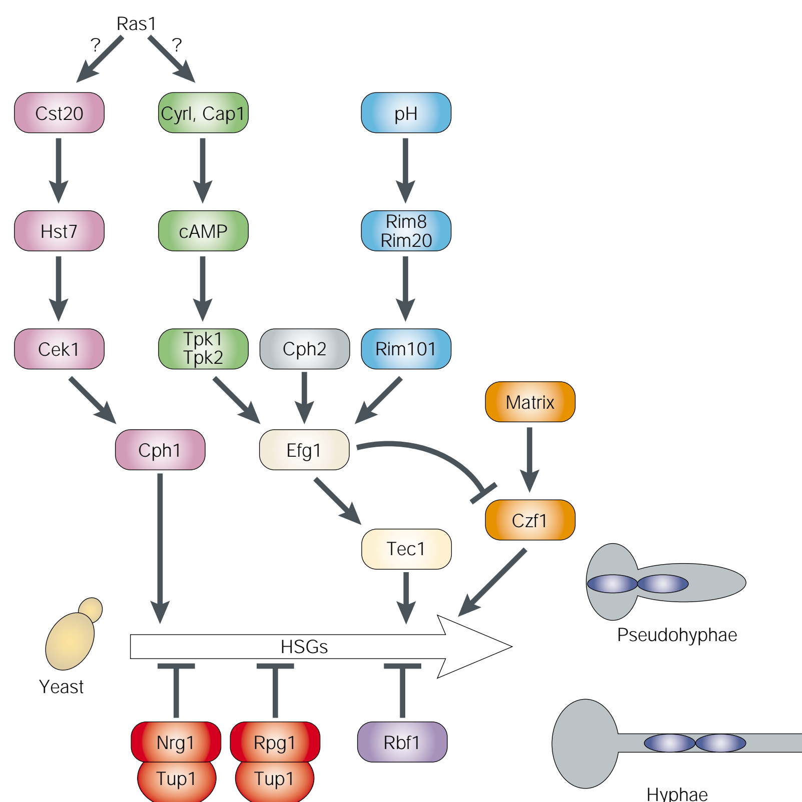


Figure 3 | **Signal-transduction pathways that regulate morphogenesis.** At least four positive (arrowheads) and two negative (bars) pathways control morphological transitions in *Candida albicans*. The pathways that promote the switch from yeast to pseudohyphal and hyphal growth are shown as follows: MAP-kinase pathway in pink, cAMP pathway in green, Cph2 pathway in grey, Rim101 pH response pathway in blue and Czf1 matrix pathway in orange. Pathways that inhibit this switch are the Tup1–Nrg1–Rpg1 pathway in red and the Rbf1 pathway in purple. HSGs, hyphal-specific genes. See TABLE 2 for *Saccharomyces cerevisiae* homologues and gene function.

the smooth appearance of yeast colonies (FIG. 2). Different colony shapes are a consequence of different proportions of yeast and filamentous cells in regions of the colony. Third, feathery projections often extend from the periphery of colonies that contain many hyphal cells (FIG. 2).

***Signal-transduction pathways.*** Several environmental factors (BOX 2) can induce yeast cells to form hyphae and pseudohyphae through several signal-transduction pathways (FIG. 3; TABLE 2). This probably reflects the variety of microenvironments in which this opportunist must survive *in vivo* (reviewed in REFS 64,69,70). As in *S. cerevisiae*, the cAMP and the mating pheromone response–MAP kinase pathways target transcription factors that promote morphogenesis. Inactivation of the cAMP pathway (by deleting *EFG1*) blocks filamentation in most conditions, whereas inactivation of the MAP-kinase pathway (by deleting *CPH1*) blocks filament formation only in response to a limited set of conditions[35,71,72]. So, it seems that the cAMP pathway has a more prominent role in *C. albicans* morphogenesis than in *S. cerevisiae*.

A *cph1 efg1* mutant, in which both the MAP-kinase and the cAMP pathways are disabled, fails to form filaments in most *in vitro* conditions, and is avirulent in a systemic mouse model of candidiasis[37]. This observation is often cited as evidence that the ability to form hyphae or pseudohyphae is an essential virulence factor. But there are two important caveats to this interpretation. First, these mutations block the expression of hyphal-specific genes (see next section for further discussion), many of which are also required for virulence. Second, *cph1 efg1* mutants are able to produce filaments under some *in vivo* and *in vitro* conditions[73]. This might be due to the action of other pathways of hyphal-growth induction, such as the Rim101 pathway, which is activated by alkaline pH[74,75] and the Czf1 pathway, which is activated by growth in a solid matrix[76] (FIG. 3).

Morphogenesis is repressed by transcriptional inhibitors such as Tup1 (REF. 77), which associates with its DNA-binding partners Nrg1 (REFS 78,79) and Rfg1 (REF. 80). Apart from pH and growth in a matrix, the nature of the environmental signals to which each of these pathways responds is poorly understood.

***Hyphal-specific gene transcription.*** The conditions that induce hyphal growth (BOX 2) also induce the expression of hyphal-specific genes (HSGs). Identifying HSGs is complicated by the fact that the conditions that induce morphogenesis will also induce cell responses that are not necessarily connected with morphogenesis but that are required for physiological adaptation to the new environment. For the most part, induction of hyphae or pseudohyphae requires a combination of two environmental conditions (such as high temperature and serum, or high temperature and neutral pH). So, a gene is only

Box 2 | **Morphology-inducing conditions**

**Yeast cells**
- Cell density >$10^6$ cells $ml^{-1}$
- Growth below 30 °C
- pH 4.0

**Pseudohyphae**
- pH 6.0, 35 °C
- Nitrogen-limited growth on solid medium (SLAD)

**Hyphae**
- Serum, >34 °C
- Lees medium, 37 °C
- pH 7.0, 37 °C

**Other filament-inducing conditions**
- Spider medium
- Engulfment by macrophages
- Mouse kidneys
- Growth in agar matrix
- Iron deprivation
- Anoxia
- *n*-acetyl glucosamine



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Table 2 | **Components of pathways that regulate morphogenesis**

| *C. albicans* protein | *S. cerevisiae* homologue | Protein function* |
|---|---|---|
| Ras1 | Ras2 | GTPase |
| Cst20 | Ste20 | p21-activated kinase (PAK) |
| Hst7 | Ste7 | MAP kinase kinase (MEK) |
| Cek1 | Homologue uncertain | MAP kinase |
| Cph1 | Ste12 | Transcription factor |
| Cyr1 | Cyr1 | Adenylate cyclase |
| Cap1 | Srv2 | Adenylate-cyclase-associated protein |
| Tpk1/Tpk2 | Tpk1/Tpk2 | cAMP-dependent protein kinase catalytic subunits |
| Efg1 | Sok2, Phd1 | Helix–loop–helix transcription factor that binds E-boxes (CANNTG) |
| Tec1 | Tec1 | TEA/ATTS DNA-binding domain family transcription factor |
| Rim20 | *A. niger* PalA | Molecular function unknown |
| Rim8 | YGL046W | Molecular function unknown |
| Rim101 | *A. niger* PacC | Zinc-finger transcription factor activated by proteolytic cleavage |
| Czf1 | No known homologues | Putative zinc-finger transcription factor |
| Cph2 | Hms1 | Helix–loop–helix transcription factor |
| Tup1 | Tup1 | Transcriptional repressor |
| Nrg1 | Nrg1 | DNA-binding partner of Tup1 |
| Rfg1 | Rox1 | DNA-binding partner of Tup1 |
| Rbf1 | Rbf1 | Binds to Rpg box of *S. cerevisiae* and *C. albicans* telomeric repeats |

*A. niger, Aspergillus niger; C. albicans, Candida albicans; S. cerevisiae, Saccharomyces cerevisiae.*
*The function has been confirmed in at least one of the yeast species. In many cases, it is assumed, but not proven in *Candida*.

ISOTROPIC
Growth in all directions (opposite of polarized growth).

considered to be an HSG when it is induced during hyphal development, but not when only one of these conditions applies[69]. Many of the HSGs that have been isolated so far encode known or putative virulence factors[69,81]. These include genes that encode secreted aspartyl proteases (*SAP4,5,6*), cell-wall proteins (*HWP1*), adhesins (*ALS3* and *ALS8*) and proteins that are required for invasive growth (*RBT1*). The Hwp1 cell-wall protein is particularly interesting because it has been shown to be the substrate for a transglutaminase in the host epithelium that forms covalent bonds that anchor the *C. albicans* cell onto the surface of the epithelium[82].

None of the HSGs that have been isolated so far are actually required for hyphal or pseudohyphal morphogenesis. Rather, they are coordinately induced by the signals that also induce morphogenesis. Their expression is blocked in an *efg1 efg1* mutant and is induced in *tup1 tup1*, *nrg1 nrg1* or *rfg1 rfg1* mutants, indicating that they might be among the targets of the morphogenesis signalling pathways[79].

A European consortium led by Alistair Brown used filters with 2002 *C. albicans* genes to examine the complex regulation of gene expression that is mediated by Tup1 together with Nrg1 and Mig1 (REFS 79,83). The analysis showed that an association of Tup1 with Nrg1 targets it to a specific subset of Tup1-regulated genes, including known HSGs and known virulence factors. Other subsets of genes are repressed by Tup1 when it is associated with other partners, such as Mig1. Moreover, both Nrg1 and Mig1 can repress further subsets of genes independently of Tup1. Haoping Liu and colleagues prepared filter arrays that were printed with 700 different *C. albicans* ORFs to study genes that regulate yeast and hyphal growth by the Cph1, Cph2 and Efg1 transcription factors[84]. The results indicated that several distinct signalling pathways convergently regulate a common set of genes that encode cell-wall proteins and proteases. In addition, both Efg1 and Cph2 regulate Tec1, a transcription factor that is important for morphogenesis. Because many of the known HSGs are virulence factors, some of these newly discovered genes might also turn out to have a role in virulence.

Both of these studies used only a subset of the genes in the *C. albicans* genome. However, their success offers a realistic prospect of understanding how environmental cues are translated into cellular responses, and how the necessary complex changes in the pattern of gene expression are orchestrated. Now that whole-genome microarrays on glass slides are being used to compare the time courses of hyphal induction under different conditions, as well as in different mutant strains[41], this understanding is certain to become more complete. For example, several genes that are important for hyphal growth have now been identified in *C. albicans* that are unique and that do not have homologues in *S. cerevisiae* or other related fungi[41]. Such genes might be especially important for *C. albicans* pathogenicity.

***Role of the cell cycle in morphogenesis.*** In *S. cerevisiae*, morphogenesis is regulated during the cell cycle by the association of cyclins with the Cdc28 cyclin-dependent kinase (CDK). Association of the CDK with G1 cyclins (Cln1 and Cln2) promotes polarized growth; its association with the B-cyclins promotes ISOTROPIC growth[85] (reviewed in REF. 86). The *C. albicans* Cln1 G1 cyclin is not required for filamentous growth, although it seems to promote the maintenance of filamentous growth in wild-type cells[87].

Many lines of evidence indicate that pseudohyphal growth in *S. cerevisiae* might involve regulation of the Clb2 Cdc28 kinase, but the mechanism by which this comes about is still unclear. In *S. cerevisiae*, transcription of *CLB2*, the main mitotic B-cyclin, is regulated by the forkhead transcription family members Fkh1 and Fkh2. Cells that lack these transcription factors have reduced periodicity of *CLB2* transcription and grow constitutively as pseudohyphae. Only one homologue of Fkh1/2, Fkh2, is present in *C. albicans*, and its deletion results in a constitutive pseudohyphal phenotype under both yeast and hyphal growth conditions. Cells that lack Fkh2 in *C. albicans* have increased levels of a B-cyclin transcript, but have reduced levels of hyphal cell-wall proteins and of enzymes that dissolve the connections between mother and daughter yeast



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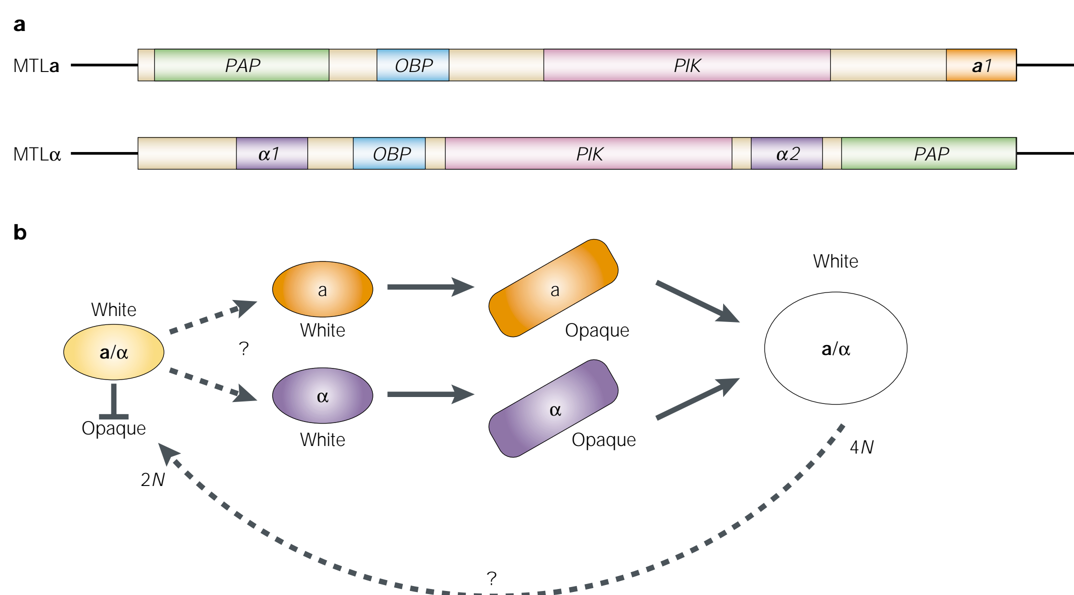


Figure 4 | **Relationships between mating and white–opaque phenotypic switching. a** | Organization of the *Candida albicans* mating-type-like (MTL) loci. The MTL loci encode the homeodomain proteins MTL**a**1 (**a**1) and MTLα2 (α2), and the transcription regulator MTLα1 (α1). Each MTL locus also encodes a poly(A) polymerase (PAP), a phosphatidyl inositol kinase (PIK) and a protein with sequence similarity to oxysterol binding proteins (OBP)[6]. **b** | Cells that are homozygous for *MTL**a*** or *MTLα* (orange or purple, respectively) can switch to the opaque state because the **a**1/α2 transcriptional regulator inhibits the expression of opaque-specific genes. Opaque cells mate with good efficiency to yield tetraploid cells that express both *MTL**a*** and *MTLα* alleles. How cells initially become homozygous at MTL and how tetraploid cells reduce their chromosome number to 2*N* is not known.

SPINDLE POLE BODY
The microtubule organizing centre in fungi. In *Candida albicans*, as in *Saccharomyces cerevisiae*, the spindle pole body is embedded in the nuclear membrane, and this membrane remains intact throughout the cell cycle.

CHECKPOINT PROTEIN
A protein that is involved in one of the pathways that monitor aspects of cellular function (such as replication or spindle formation) that are required for proper cell-cycle progression. If a defect is detected, the checkpoint pathway delays the cell cycle so that the defect can be corrected.

ASCOMYCETE
The class of fungi in which the meitoic progeny (ascospores) are found in sac-like structures (asci).

cells[88]. These observations have led to a model in which Fkh2 regulates the cell-cycle processes that are necessary for the morphogenesis of true hyphal and yeast cells.

During hyphal induction in serum, germ tubes evaginate rapidly, much earlier than events such as SPINDLE POLE BODY duplication that normally signal the start of the cell cycle. This indicates that hyphal evagination can occur independently of other cell-cycle events[89] and that the initial polarized evagination of a hyphal germ tube is distinct from budding[66]. It also raises a possibility that evaginating hyphal germ tubes might have features that are analogous to *S. cerevisiae* mating projections — they both initiate polarized growth in non-cycling cells in response to external signals and both form a disorganized septin band at their base[66,89].

Interestingly, *MAD2* in *C. albicans*, which encodes a homologue of the *S. cerevisiae* spindle assembly CHECKPOINT PROTEIN, is required for virulence in mice and for survival of *C. albicans* in the presence of macrophages[90]. However, Mad2 is not required for growth in liquid or solid media, which implies that cell-cycle checkpoints, and in particular the checkpoint that monitors spindle assembly, are important for the survival of *C. albicans* in the host. Perhaps host-defence mechanisms damage crucial cellular components, such as the mitotic spindle, which the pathogen can only repair if it triggers the Mad2 checkpoints and delays cell-cycle progression[90].

### Phenotypic switching

Several properties of growth in *C. albicans* seem to be epigenetically controlled. One of them is reversible colony switching, which was first characterized in 1985 (REFS 91,92). Yeast cells normally form smooth, white dome-shaped colonies. However, at low frequency, *C. albicans* strain 3153A can spontaneously and reversibly convert to a variant colony shape (such as star, ring, irregular wrinkle, hat, stipple and fuzzy). A simpler, biphasic 'white–opaque' switching system, found in the *C. albicans* strain WO-1, involves a switch between white, domed colonies that contain typical yeast cells and opaque, flat colonies that contain characteristically oblong cells[93] (FIG. 2). White and opaque cultures have different virulence properties, and several white-specific and opaque-specific gene products have been identified[93]. For example, high levels of the Efg1 transcription factor induce and maintain the white cell-state, whereas low Efg1 levels induce and maintain the opaque cell-state[94]. Tup1, a transcriptional repressor, promotes the conversion from the white to the opaque phase in cells, although it is not required for the maintenance of either phase[95]. It is also known that a MADS-box consensus binding site, that is most closely related to the Mcm1 binding site of *S. cerevisiae*, is necessary for expression of the opaque-specific *OP4* gene[96]. The frequency of switching between white and opaque states is affected by histone deacetylases[97,98], indicating that this phenotypic switch might be controlled, at least in part, through a regulation of chromatin structure.



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***Mating in* Candida albicans.** *C. albicans* is classified as an asexual, obligate diploid yeast, which is related to ASCOMYCETES. Studies of genetic diversity in *C. albicans* indicate that meiotic recombination, if it occurs at all, does so at a low frequency[99]. Nonetheless, the genome sequencing project has revealed that the *C. albicans* genome contains ORFs that are similar to the *S. cerevisiae* mating-type genes, *MATa1*, *MATα1* and *MATα2* (REF. 7). These genes are organized into two non-homologous mating-type-like (MTL) loci, *MTL**a*** and *MTLα*, on chromosome 5, and include the MTL genes *MTL**a**1*, *MTLα1* and *MTLα2*, as well as ORFs with no obvious mating function[7] (FIG. 4). Strains in which either the *MTL**a*** or *MTLα* genes of one MTL locus have been deleted, or one copy of chromosome 5 has been lost, can mate with strains that carry only the MTL locus of the opposite mating type[8,9]. However, strains that have been engineered to carry such deletions mate only infrequently, either *in vivo* or on plates held at room temperature, and produce apparently tetraploid strains. The physiological significance of these 'mating' reactions, therefore, remains unclear[8,9].

Interestingly, a proportion of clinical *C. albicans* isolates, including some that are resistant to the fungicide fluconozole, are homozygous at the MTL locus and are therefore, at least theoretically, mating competent[100]. Although the loss of one copy of chromosome 5 can occur under certain stress conditions[56] and generates mating-competent strains, the fluconozole-resistant isolates that carry only *MTL**a*** or *MTLα* seem to have retained both copies of chromosome 5 and therefore seem to retain their diploid state[100]. An intriguing, open question is how these, as well as other wild-type strains, become homozygous for MTL. In *S. cerevisiae*, as in other fungi, such as *S. pombe*, the sequence in the active mating-type locus is replaced by homologous recombination with donor sequence from the silent mating loci. However, unlike *S. cerevisiae*, the *C. albicans* genome sequence does not seem to include extra copies of *MTL**a**1*, *MTLα1* or *MTLα2* that could function as silent donor mating loci.

***Mating and phenotypic switching connections.*** Alexander Johnson and colleagues recently showed that strains that are engineered to express only *MTL**a*** or *MTLα* have an increased tendency to switch to the opaque state. Once in the opaque state, these cells mate with an efficiency that is similar to that seen in the laboratory strains of *S. cerevisiae*[101]. This intriguing result indicates that *C. albicans* mating might be more elaborate than that of *S. cerevisiae* — heterozygosity at MTL must be lost and a switch to the opaque state must occur as a prerequisite to mating. It is thought that the presence of 'pimples' in the walls of opaque, but not white, cells facilitates the cell–cell interactions that occur during mating[101].

Mtl**a**1 and Mtlα2, which are products of the *MTL**a*** and *MTLα* loci, respectively, transcriptionally repress opaque-specific genes[101], presumably to limit mating interactions between diploid cells (FIG. 4). So, the *C. albicans* MTL loci regulate the white–opaque transition, and the white–opaque transition in turn regulates mating efficiency.

No evidence for meiosis in *C. albicans* has yet been found and, if studies of recombination are correct, it should be much less frequent than clonal reproduction[99]. It will be important to determine whether the diversity of *C. albicans* strains, with their changing karyotypes, is generated by a non-meiotic chromosome loss mechanism (which was exploited in parasexual studies before the availability of molecular-genetic tools[102]) or by true, albeit rare, meiotic segregation events.

## Conclusions

In the past several years, *C. albicans* research has moved from awkward parasexual manipulations to studies driven by genomic information. Genome-sequence information has already greatly accelerated the ability to carry out genetic manipulations in this organism. We can now disrupt genes, tag them, express them from conditional promoters and follow cellular localization of their products in living cells. But the number of selectable markers, especially those that are useful for the transformation of clinical isolates, remains limited, especially in the light of the general need to alter both copies of a gene being studied. In addition, although desired transformants make up a workable proportion (4–60%) of the total transformant population, undesired transformants (that result from non-homologous recombination or from other poorly understood events) occur frequently. So, although the manipulation of *C. albicans* is feasible, it remains less facile than in *S. cerevisiae*. It is clear that more direct analysis of *C. albicans* will follow. However, it is likely that biological processes that were analysed first in the model yeast, will continue, where applicable, to inform many studies of *C. albicans* biology.

Still to be elucidated are the mechanisms by which mating, genome rearrangements, cell-cycle processes, signal-transduction pathways and morphogenesis contribute to pathogenesis. Essential genes might become targets for new fungicides. Cell-surface gene products (such as hyphal-wall proteins) might provide more-accessible targets for drugs that can interfere with fungal–host interactions. The discovery of mating has generated much excitement about the biology of *C. albicans*, especially because the relationship between mating and white–opaque switching indicates unique life-cycle strategies. The discovery of mating also raises the tantalizing possibility of carrying out classical genetic experiments in this organism. A combination of expression studies, mutant phenotypes and sequence comparisons, all in the context of a complete and annotated genome sequence, will provide a much deeper understanding of the pathways and functions of many *C. albicans* genes, including those that are important for pathogenesis.



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