

An antisense-based functional genomics approach for identification of genes critical for growth of *Candida albicans*

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Converting the complete genome sequence of *Candida albicans* into meaningful biological information will require comprehensive screens for identifying functional classes of genes. Most systems described so far are not applicable to *C. albicans* because of its difficulty with mating, its diploid nature, and the lack of functional random insertional mutagenesis methods. We examined artificial gene suppression as a means to identify gene products critical for growth of this pathogen; these represent new antifungal drug targets. To achieve gene suppression we combined antisense RNA inhibition and promoter interference. After cloning antisense complementary DNA (cDNA) fragments under control of an inducible GAL1 promoter, we transferred the resulting libraries to *C. albicans*. Over 2,000 transformant colonies were screened for a promoter-induced diminished-growth phenotype. After recovery of the plasmids, sequence determination of their inserts revealed the messenger RNA (mRNA) they inhibited or the gene they disrupted. Eighty-six genes critical for growth were identified, 45 with unknown function. When used in high-throughput screening for antifungals, the crippled *C. albicans* strains generated in this study showed enhanced sensitivity to specific drugs.

Human fungal infections have dramatically increased over the last 15–20 years and have become a significant cause of disease and mortality¹. They are frequently acquired by immunocompromised patients (such as those receiving chemotherapy, undergoing organ transplants, or infected by HIV) and are commonly diagnosed after invasive medical surgery². Currently used antifungal drugs (polyenes such as amphotericin B, triazoles such as itraconazole and fluconazole, and imidazoles such as miconazole) are limited in their use because of their activity spectrum, toxicity, and side effects^{3,4}. The usefulness of the azoles has significantly diminished in recent years as a result of the increasing incidence of resistance, a complex phenomenon that involves several molecular mechanisms and may be difficult to avoid for this reason. The increase in infections, combined with the reduced efficacy of the currently available drugs, highlights the need for new antifungal drugs with distinct modes of action. The development of such drugs would greatly improve the quality of life and life expectancy of infected patients.

In this study we specifically outline an approach to identify new antifungal drug targets of *C. albicans*, the major pathogen causing human fungal infections. Although a vast amount of sequence information from the *C. albicans* genome is now available in both public (<http://www.sequence.stanford.edu/group/candida/>; 79% complete) and private (Incyte Genomics Inc.; 89% complete) databases, “en masse” disruption techniques used to elucidate gene function in other organisms (e.g., the “mass murder” and “genetic footprinting” technology in *Saccharomyces cerevisiae*^{5,6}) are not applicable to *C. albicans*, for several reasons. First, although mating can be induced in *C. albicans* by creating alterations at the mating type locus^{7,8}, it is under normal circumstances not capable of mating, so that genetic crosses remain difficult. Second, no convenient method

is available for random insertional mutagenesis because of a paucity of functional transposable elements⁹. Third, due to the organism's diploid nature, multiple consecutive steps of gene inactivation are required for making genetic knockouts, and this can only be done on a gene-by-gene basis¹⁰. Whereas existing genetic disruption techniques^{11–13} are highly effective for analyzing individual genes, applying them to all of the more than 7,800 predicted *C. albicans* open reading frames (ORFs) would be a tremendous task¹⁴.

Here we describe an approach to alter gene function on a genome-wide scale in *C. albicans* genes by using antisense RNA and promoter interference. Repression of gene expression by promoter interference (i.e., gene of interest flanked by two convergent promoters) can occur by physical collision of elongating RNA polymerases on opposite strands¹⁵. Under these circumstances, complementary antisense RNA may be a by-product of the primary transcriptional-level interaction. Inhibition by antisense RNA has not been used extensively in yeast^{16,17} partly because of the availability of alternative tools for deletion or silencing of genes. We describe a new integrative vector allowing inducible transcription of antisense RNA from a cDNA insert in *C. albicans*. An antisense cDNA library was constructed using this vector and introduced into *C. albicans*. Depending on the site of integration in the *C. albicans* genome, the introduction of such a library plasmid and subsequent induction of the promoter will lead to inhibition of expression of the gene whose cDNA is represented in the library plasmid—the latter either by antisense RNA alone or by a combination of antisense RNA and promoter interference. This approach thus specifically relies on lowering the level of specific *C. albicans* mRNAs by either of the above mechanisms, thereby decreasing the expression level of the corresponding protein. If this *C. albicans* protein is critical for growth, the cell will grow more slow-

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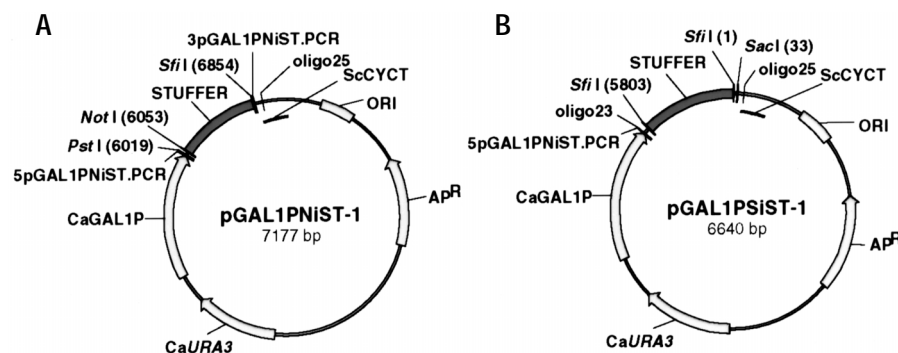


Figure 1. Integrative *C. albicans* vectors. Vectors used for the preparation of (A) the antisense cDNA library, pGAL1PNiST-1, and (B) a gDNA library, pGAL1PSiST-1. CaURA3, *C. albicans* URA3 gene; CaGAL1P, *C. albicans* galactokinase 1 promoter; ScCYCT, *S. cerevisiae* cytochrome c transcription terminator; ORI, origin of replication for *E. coli*; ApR, ampicillin resistance gene for *E. coli*. STUFFER, stuffer fragment cloned in between *SfiI*/*NotI* (pGAL1PNiST-1) or *SfiI*/*SfiI* (pGAL1PSiST-1) cloning sites. 5pGAL1PNiST.PCR and oligo23 are (clockwise) forward primers; 3pGAL1PNiST.PCR and oligo25 are reverse primers.

To cover the genome as broadly as possible, we also constructed a genomic (gDNA) library containing small genomic fragments in another vector, pGAL1PSiST-1 (Fig. 1B), allowing the targeting of regulatory regions as well as coding regions.

Screening for growth-affected transformants. Libraries were introduced into *C. albicans* strain CAI-4 (ref. 19), and transformants were screened for reduced growth upon activation of the GAL1 promoter in the presence of lithium acetate. Lithium acetate prolongs the G1 phase of the cell cycle, during which antisense is presumed to act most strongly²⁰. More than 2,000 transformants were screened by parallel measurement of growth in noninducing and inducing media. Growth curves of transformants impaired in growth are shown in Figure 3. *Candida albicans* uses galactose (inducer) rather inefficiently as a carbon

ly or die. We used this approach in a genome-wide search for new *C. albicans* genes critical for growth of the pathogen. Among over 2,000 transformant colonies screened, ~10% showed a clear growth defect.

Results and discussion

Experimental design. A *C. albicans* integrative vector, pGAL1PNiST-1, was constructed for directional (antisense orientation) cloning of *C. albicans* cDNA fragments (Fig. 1A). The resulting antisense cDNA library can be introduced into *C. albicans*, and individual library clones can subsequently integrate into the genome by homologous recombination either at the endogenous GAL1 promoter locus (Fig. 2A) or at the locus corresponding to the cDNA insert (i.e., gene-specific; Fig. 2B). In either case GAL1 promoter activation will produce insert-specific antisense RNA. Gene-specific homologous recombination can in addition result in promoter interference (Fig. 2B), and this capacity has been used in mammalian cells to screen for inhibitors of transcriptional activation¹⁸.

source (i.e., the wild-type strain shows reduced growth in antisense induction medium independent of any existing antisense inhibitory effect). To facilitate selection of growth-impaired transformants, maltose was added to both inducing and noninducing media in a later stage of the screening process. Growth of the parental strain CAI-4 was identical in both of these media (data not shown), and the presence of maltose did not significantly influence the induction ratio of the GAL1 promoter (Fig. 4). Approximately 10% (198) of the transformants showed a growth defect and were selected for further analysis. Forty-three percent grew slowly only in inducing medium (consistent with putative promoter interference or antisense effect). Screening with the gDNA library was abandoned because of repetitive isolation of identical DNA inserts (autonomously replicating sequences (ARs) and ribosomal RNAs (rRNAs); see below) in those transformants showing a growth defect.

Isolation of integrated antisense library clones from the *C. albicans* genome. To identify those genes whose inhibition causes a growth

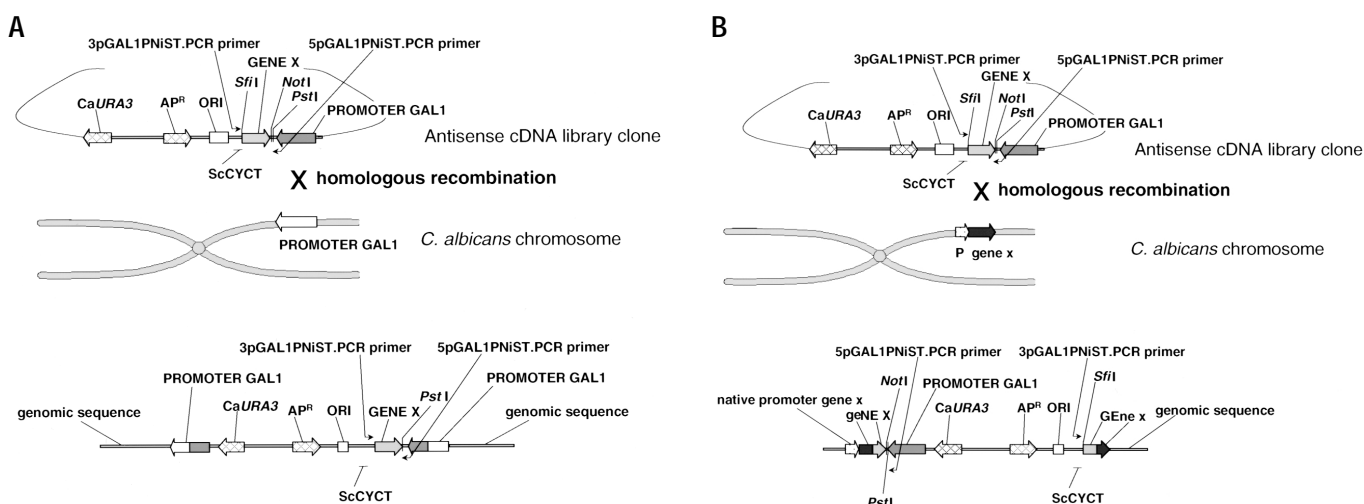


Figure 2. Integration of library plasmids into the *C. albicans* genome. (A) Integration of the antisense cDNA library plasmid at the GAL1 promoter region, resulting in a duplication of the GAL1p region. Antisense RNA can be produced from GENE X upon induction of the GAL1 promoter. (B) Integration due to homologous recombination between the cDNA insert (GENE X) of an antisense library clone and the homologous gene (gene x) within the *Candida* genome. As a result the gene is duplicated: the first copy of the gene, GENE X, is flanked by its endogenous promoter and divergently oriented, the GAL1 promoter resulting in a so-called "collision construct". If RNA polymerase II complexes start from both the upstream and downstream promoters, they may collide, thereby preventing the formation of a full-length mRNA transcript. Antisense RNA can be produced from GENE X upon induction of the GAL1 promoter. The second copy of the gene, GENE x, is devoid of a promoter and will not be transcribed. PCR primers 5pGAL1PNiST.PCR and 3pGAL1PNiST.PCR used to amplify the cDNA library insert from either uncut gDNA (A) or by inverse PCR from cut and re-ligated gDNA (B) are indicated on the map.

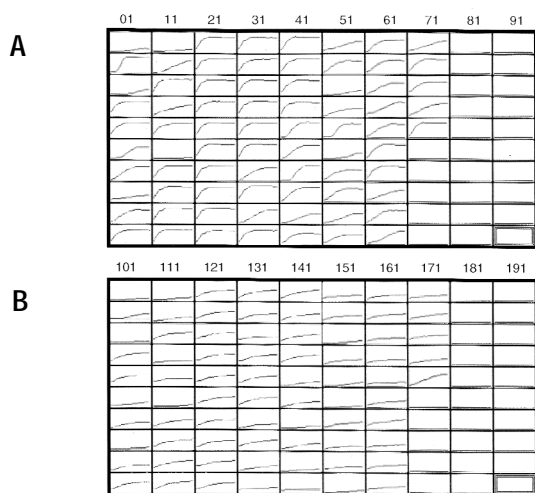


Figure 3. Growth curves of 74 transformants (wells 1–74 and 101–174, respectively) and from CAI-4 (wells 75 and 175, respectively) grown for three days in antisense (A) noninducing (S medium pH 6, containing 50 mM lithium acetate and 2% glucose; wells 1–74) and (B) inducing (S medium pH 6, containing 50 mM lithium acetate and 2% galactose; wells 101–174) conditions in Honeywell 100-well plates (Labsystems). Growth of clone 36 is shown in wells 02 and 102, whereas growth of clone 38 is shown in wells 03 and 103. Optical densities were measured every hour using a Bioscreen Analyzer (Labsystems), which allows simultaneous monitoring of growth of 200 cultures and subsequent automated generation of growth curves. Inserts isolated from these strains are described in Table 1. Wells 76–100 and 176–200 are empty.

defect, we needed to investigate which library clones (cDNA inserts) had integrated at which location in the genome of those transformants showing reduced growth. cDNA inserts from the integrated antisense library were isolated from the disruptants by PCR. For integration at the GAL1 promoter (Fig 2A; 11% of all transformants), PCR with 5pGAL1PNI:ST.PCR and 3pGAL1PNI:ST.PCR primers amplified the cDNA insert. Because these primers are divergently oriented if integration occurs at the cDNA insert (Fig. 2B), we used inverse PCR (see Experimental Protocol for details).

Some transformants yielded PCR products of different lengths implying multiple integrations. In that case, each of the disrupted genes had to be inactivated separately to find the one causing the growth defect.

Identification of genes critical for growth of *C. albicans*. Comparison of the sequences of the PCR products with public and proprietary sequence databases identified 86 different genes (all sequences were submitted to European Molecular Biology Laboratory (EMBL); see Table 1). Surprisingly, 45 of these were of unknown function and in 33 no homologs could be found in other organisms (as judged based on an E-value cutoff of 10^{-9} ($P < 1e^{-9}$) for BLAST search hits²¹). In other completely sequenced microbial genomes approximately 20% of the ORFs are species-specific^{22,23}. We found 38% (33/86) putative *Candida*-specific sequences, perhaps because we included noncoding (i.e., less conserved) gDNA sequences.

From the selected transformants obtained with the gDNA library, ARS2 and 18S or 25 rRNA genes were repeatedly isolated (43, 13, and 8 times, respectively; Table 1), so that we abandoned this approach. The *Candida* genome contains multiple copies of both ARS2 and rRNA sequences. This would account for their over-representation in the gDNA library but not for their growth-inhibitory effect. However, extra-

chromosomal rDNA circles (ERCs) accumulate in old yeast cells and actually cause ageing²⁴. Whether there is a relation between this observation and ours is subject to further study.

Many of the genes we identified using the cDNA library are known to be essential in *S. cerevisiae* or in other organisms (e.g., ribosomal proteins, *RPS7*, *RPL37*, *RPL27*, *RPS21*, *RPL16*; translation elongation factors, *EFB1*, *TEF3*, *TEF4*, *TUF1*; others such as *ABP1*, *RHO1*, *RNR1*, *YAE1*, *TRAI*, *MEG1*; Table 1). Genes involved in carbon source metabolism and nutrient uptake (e.g., a galactose permease, *HXT6*) were identified as well.

We chose as examples one transformant with an inducible (clone 36; see growth curves in Fig. 3, wells 02 and 102, respectively) and one with a noninducible (clone 38; see Fig. 3 wells 03 and 103, respectively) growth defect for further discussion.

In clone 36, recombination occurred at the cDNA insert as shown by amplification of a ~600 base pair gene fragment by inverse PCR. The sequence of this fragment was 74% identical to the *S. cerevisiae* S-adenosyl methionine synthetase 2 (*SAM2*) gene. Growth on 5-fluoroorotic acid (i.e., excision of the integrated plasmid from the genome by homologous recombination between the duplicated genes; Fig. 2B) completely restored the wild-type growth phenotype (data not shown), supporting the specificity of the observed growth defect. Northern blot analysis revealed a 0.9 kilobase *SAM2* antisense transcript in clone 36 that was absent in the wild-type strain (Fig. 5A). The presence of low amounts of antisense transcript in clone 36 under noninducing conditions is in agreement with the observed leakiness of the GAL1 promoter. Northern blot analysis (Fig. 5B) further revealed a reduced amount of a 1.3 kilobase *SAM2* sense transcript (*SAM2* mRNA) in clone 36 compared to the wild-type strain when grown in antisense-inducing medium. This is to be expected when sense and antisense transcripts interact to form RNA hybrids (which might subsequently be degraded). To verify the length of *SAM2* mRNA, a clone containing the complete ORF (1,155 base pairs) of the *SAM2* gene, including 5'- and 3'-flanking regions, was isolated by hybridization screening of a *C. albicans* gDNA library. Based on a putative TATA box at -27 base pairs and a T-rich (>10 base pairs) region (element described in yeast as necessary for transcript release²⁵) downstream of the ORF, a total transcript length

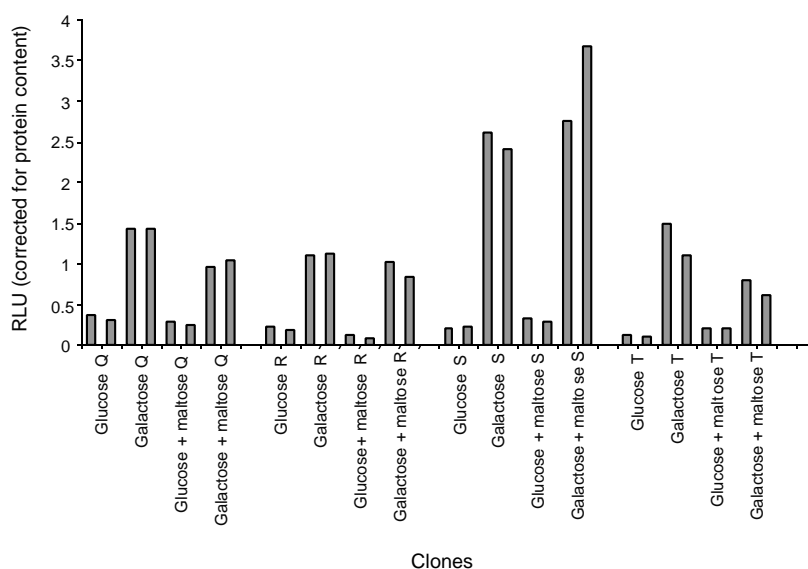


Figure 4. Activity of the *C. albicans* GAL1 promoter in the absence and presence of maltose as a carbon source. CAI-4 was transformed with LAC4/ pGAL1PNI:ST, and β -galactosidase reporter gene expression was measured for four transformants (Q, R, S, T) upon growth in glucose, galactose, glucose + maltose, and galactose + maltose-containing S medium. All experiments were performed in duplicate and corrected for protein content. RLU, Relative light units.

**Table 1. Overview of identified molecular targets^a**

Identified <i>C. albicans</i> genes critical for growth	EMBL Acc. No.	Insert size (bp)	Library source	Integration site	Growth
rRNAs					
<i>C. albicans</i> 18S rRNA	AJ249486	440 ^b	gDNA	GAL1p/insert	6/106 ^c
<i>C. albicans</i> 25S rRNA	AJ249486	486 ^d	gDNA	GAL1p/insert	7/107 ^c
<i>C. albicans</i> 5S rRNA	AJ249486	530	gDNA	Insert	67/167
Similar to <i>S. cerevisiae</i> mitochondrial 15S rRNA	AJ390486	508	gDNA	Insert	9/109
Protein synthesis					
<i>C. albicans</i> ribosomal protein RPS7	U37009	1,143	cDNA	GAL1p	19/119
<i>C. albicans</i> large-subunit ribosomal protein RPL37	AJ390504	468	cDNA	Insert	34/134
Similar to <i>S. cerevisiae</i> ribosomal protein RPL27	AJ390496	564	cDNA	Insert	14/114
Similar to <i>S. cerevisiae</i> ribosomal protein RPS21	AJ390495	415	cDNA	GAL1p	4/104
Similar to <i>S. cerevisiae</i> ribosomal protein RPL16	AJ390506	823	cDNA	Insert	17/117
Translation factors					
<i>C. albicans</i> translation elongation factor EFB1	X96517	712	cDNA	GAL1p	72/172
<i>C. albicans</i> translation elongation factor TEF3	Z11484	3,671	cDNA	Insert	73/173
Similar to <i>S. cerevisiae</i> translation elongation factor TEF4	AJ390494	712	gDNA	Insert	52/152
Similar to <i>S. cerevisiae</i> mitochondrial translation factor TUF1	AJ390502	262	cDNA	Insert	32/132
Structural proteins and their regulators					
Similar to <i>S. cerevisiae</i> actin-binding protein ABP1	AJ390503	1,218	cDNA	Insert	15/115
Similar to <i>S. cerevisiae</i> YDR388w/RVS167 gene; affects actin binding and bipolar budding in <i>S. cerevisiae</i>	AJ390509	616	gDNA	Insert	53/153
<i>C. albicans</i> Rho-type GTP-ase; essential protein RHO1	D86430	933	cDNA	Insert	15/115
Nonrelated essential functions					
<i>C. albicans</i> ribonucleotide reductase, large subunit; essential protein RNR1	AJ390500	340	cDNA	Insert	3/103
<i>C. albicans</i> tRNA ^{Ser} ; essential	D13706	869	gDNA	Insert	10/110
<i>C. albicans</i> essential protein of unknown function; YAE1	AJ390499	543	gDNA	Insert	39/139
Similar to of <i>S. cerevisiae</i> 5'-untranslated region of essential protein TRA1	AJ390489	667	gDNA	Insert	54/154
Similar to <i>Homo sapiens</i> spliceosome-associated essential protein SAP45	AJ390490	899	cDNA	GAL1p	27/127
Similar to <i>S. cerevisiae</i> ATP-dependent RNA helicase; essential protein FAL1	AJ390488	771	gDNA	Insert	12/112
Similar to <i>S. cerevisiae</i> nuclear essential protein MEG1	AJ390507	725	gDNA	Insert	44/144
Metabolism					
<i>C. albicans</i> enolase 1	L04943	1,446	cDNA	GAL1p	55/155
Similar to <i>S. cerevisiae</i> serine/threonine protein kinase SHA3	AJ390510	1,015	cDNA	Insert	56/156
Similar to <i>S. cerevisiae</i> succinate dehydrogenase FE/S subunit SDH2	AJ390505	402	gDNA	Insert	41/141
Similar to <i>S. cerevisiae</i> galactose and/or glucose permease; member of hexose transporter family HXT6	AJ390498	1,350	cDNA	Insert	49/149
Similar to <i>S. cerevisiae</i> triosephosphate isomerase TPI1	AJ390491	857	cDNA	Insert	48/148
Similar to <i>S. cerevisiae</i> 2-oxoglutarate dehydrogenase complex E2 component KGD2	AJ390511	651	cDNA	GAL1p	26/126
Other					
<i>C. albicans</i> autonomous replicating sequence (ARS1/2)	X16634	434 ^e	gDNA	Insert	11/111 ^c
<i>C. albicans</i> S-adenosylmethionine synthetase 2	AJ390497	600	cDNA	Insert	2/102
<i>C. albicans</i> repeat element RPS102/RPS1	S71769	670	gDNA	Insert	50/150
Similar to <i>S. cerevisiae</i> RAD18	AL033391	550	gDNA	GAL1p	45/145
Similar to <i>S. cerevisiae</i> HOL1 protein; member of major facilitator superfamily (MFS-MDR)	AL033497	597	cDNA	Insert	15/115
Similar to <i>S. cerevisiae</i> mitochondrial NADH dehydrogenase NDE1	AJ390487	1,834	cDNA	GAL1p	25/125
Similar to <i>S. cerevisiae</i> oligosaccharyltransferase subunit OST4	AJ390501	478	cDNA	Insert	31/131
Similar to <i>S. cerevisiae</i> high-copy suppressor of temperature-sensitive mutations in DNA polymerase alpha; mitochondrial regulator of splicing PSP2	AJ390508	362	gDNA	Insert	46/146
Similar to <i>S. cerevisiae</i> ECM21; protein possibly involved in cell wall structure or biosynthesis	AJ390493	515	gDNA	Insert	51/151
Similar to <i>E. coli</i> maltose acetyltransferase MAA	AJ390492	864	cDNA	GAL1p	23/123
Similar to <i>Schizosaccharomyces pombe</i> / <i>S. cerevisiae</i> putative cytochrome b ₅ reductase CBR1	AJ251827	1,063	cDNA	Insert	25/125
Similar to <i>C. tropicalis</i> mitochondrial citrate synthase precursor CIT	AJ390512	642	gDNA	Insert	8/108
Hypothetical proteins (24)	AJ390513-AJ390557	Variable	13 gDNA, 11 cDNA	GAL1p/insert	
Unknowns (21)	AJ390513-AJ390557	Variable	19 gDNA, 2 cDNA	GAL1p/insert	

^aFor each identified clone, the DDBJ/EMBL/GenBank database accession number is given, followed by the length (bp) of the obtained (inverse) PCR fragment, the library source (gDNA or cDNA library), the site of integration (GAL1 promoter or insert-specific locus), and the location of growth curves (–/+ induced/well numbers as in Fig. 3). ^bFound 13 times with inserts of different lengths. ^cOne representative growth curve is given. ^dFound 8 times with inserts of different lengths. ^eFound 43 times with inserts of different lengths.



Table 2. Crippled *C. albicans* strains show enhanced drug sensitivity^a

Compound	Growth of various strains (% of normal)							
	CAI-4	tef3/TEF3	pfy1/PFY1	rnr1/RNR1	fal1/FAL1	meg1/MEG1	rpl27/RPL27	tpi1/TPI1
R076831	99.4	106.1	102.6	115.1	111.1	109.5	105.7	
R057438	94.9	97.4	47.3	73.2	106.2	98.8	104.4	99.6
R103822	103.6	96.0	75.3	55.5	101.2	102.3	94.9	108.5
R079341	96.1	92.6	99.4	96.2	49.8	97.9	70.4	68.1
R061112	99.6	91.2	100.4	98.8	55.6	52.6	105.1	74.5
R053353	97.9	98.1	99.1	97.4	97.3	97.6	45	99.9
R172663	104.9	NT	NT	NT	84.2	84.0	78.4	54.4

^aParental strain CAI-4 and strains crippled in *TEF3*, *PFY1*, *RNR1*, *FAL1*, *MEG1*, *RPL27* or *TPI1* (Table 1) were grown in the presence of 10 μ M drug (R compounds, see Experimental Protocol). Growth is expressed as percentage of control growth (growth in the absence of any compound is set at 100%). One representative compound is shown for every crippled strain. NT, not tested.

of 1.3 kilobases could be predicted, which is in agreement with what we found. Both the presence of *SAM2* antisense RNA and the reduced *SAM2* mRNA level upon promoter activation in clone 36 clearly suggest inhibition of *SAM2* expression by interference at RNA level.

Clone 38 showed a noninducible growth defect, which is expected when the integration event by itself leads to inhibition of gene expression (e.g., if mutations were present in the original library clone, the protein encoded by the gene after homologous recombination would be nonfunctional; gene suppression would then occur independently of *GAL1* promoter activation). PCR analysis revealed the presence of a cDNA library insert (340 base pairs) encoding ribonucleotide reductase 1 (*RNR1*). Northern blot analysis (Fig. 5C) showed a reduced level of *RNR1* mRNA in clone 38 compared to wild-type strain, which was confirmed by real-time quantitative PCR using an *RNR1* fluorogenic probe (Fig. 5D). This clearly suggested inhibition of *RNR1* expression by interference at the RNA level. To further support the specificity of the observed growth defect in clone 38, revertants were generated on 5-fluoroorotic acid (excision of the integrated plasmid from the genome by homologous recombination

between the duplicated *GAL1* promoter regions; see Fig. 2A) and showed restoration of the wild-type growth phenotype (data not shown).

Many of the genes that were identified using the screening approach outlined here have an essential function (Table 1), suggesting that the approach succeeds in the identification of genes critical for growth of the pathogen. To support this conclusion, heterozygous knockouts were made in six randomly chosen genes (*TEF3*, *TUF1*, *RPL27*, *RHO1*, *FAL1*, and one hypothetical protein (*HYP*); all identified in the screening outlined here) using the *URA*-blaster disruption method¹¹. Four disruptants (*rho1/RHO1*, *tuf1/TUF1*, *rpl27/RPL27* and *hyp/HYP*) showed a clear growth defect indicating that a gene critical for growth had been targeted. Such a growth defect is seldom observed in heterozygous knockouts of genes randomly chosen from the *C. albicans* sequence database (unpublished results). The *tef3/TEF3* heterozygote strain showed a very clear growth defect when grown on solid growth medium; however, this effect was less apparent in liquid culture. The *fal1/FAL1* (ref. 13) heterozygote strain, however, did not show a pronounced growth defect. A possible explanation is that a more pronounced inhibitory effect can be expected with antisense RNA (both copies are targeted) as compared to single-copy inactivation. Therefore, experiments to regulate expression of the second allele of *FAL1* (by promoter replacement) are ongoing.

Use of crippled *C. albicans* strains in drug screening. The genetically crippled *C. albicans* strains that were generated in this study were used in high-throughput screening for antifungal drugs. Such a high-throughput screening assay typically involves measurement of growth of a genetically crippled strain relative to a wild-type strain in the presence of various compounds, and is based on observations in bacteria and in yeast suggesting that underexpression of any component of a process leads to increased sensitivity to an inhibitor of a relevant step in that process^{26,27,28}. Lowering the dosage of a specific gene in *C. albicans* results in a heterozygote that is sensitized to a drug that acts on the product of the targeted gene. This method thus provides a more sensitive means to identify test compounds with antifungal activity and gives an indication of the site or pathway at which the compounds exert their effect.

Previously Jensen-Pergakes *et al.*²⁹ showed that a *C. albicans* sterol methyltransferase (*ERG6*) mutant was hypersusceptible to a number of sterol synthesis and metabolic inhibitors, including terbinafine, tridemorph, fenpropimorph, fluphenazine, cycloheximide, cerulenin, and brefeldin A. Similarly, an *ERG3* mutant was found to be hypersensitive to certain azole antifungals (Frank Odds and Bart Van Den Hazel, personal communication).

Several crippled *C. albicans* strains generated in this study (a representative data set is shown in Table 2) showed enhanced sensitivity to specific drugs. Besides strongly inhibiting one crippled strain, some compounds also caused a weak to modest inhibition of other crippled strains. Apart from random variation in growth, this could be due to

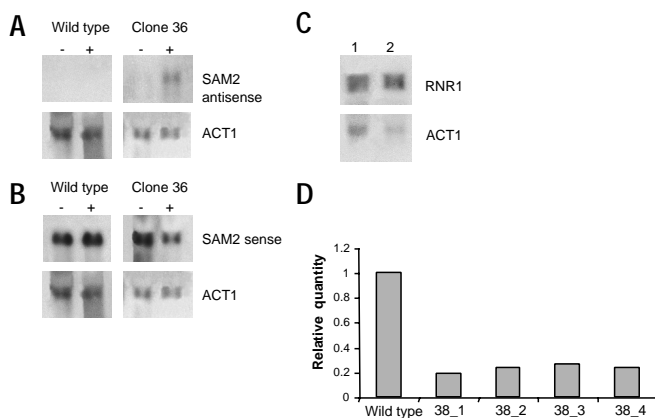


Figure 5. Expression analysis of genes targeted in clones 36 and 38. (A) Northern blot analysis of wild-type and clone 36 grown in antisense noninducing (–) or inducing (+) conditions. Hybridization to a sense *SAM2* *in vitro*-synthesized RNA probe allows for the detection of *SAM2* antisense transcript. A 0.9 kb *SAM2* antisense transcript was detected in clone 36. (B) Hybridization of identical blot to an antisense *SAM2* *in vitro*-synthesized RNA probe allows for the detection of *SAM2* mRNA. A 1.3 kb *SAM2* sense transcript was detected in both wild-type and clone 36. Levels of *SAM2* mRNA were lower in clone 36 when grown in antisense-inducing (+) conditions. (C) Northern blot analysis of (1) clone 38 and (2) wild type. Hybridization to *RNR1* and *ACT1* DNA probes revealed higher levels of *RNR1* mRNA in the wild-type strain compared to clone 38 (*ACT1* levels were lower in wild type). (D) Real-time quantitative PCR on cDNA from wild type and clone 38. Relative levels of *RNR1* mRNA in clone 38 compared to wild type are shown for four independent quantitation experiments using two different cDNA preparations (38_1-2 and 38_3-4, respectively).



nonspecific inhibitory effects or some cross-reactivity (virtually no pharmacological compound is 100% specific, especially at high concentrations). Analogs of the compounds we identified are now being synthesized and screened for activity against wild-type *Candida* strains.

In conclusion, the strategy presented in this study demonstrates a successful approach to alter gene function on a genome-wide scale in an important human pathogenic fungus. Genes critical for growth of *C. albicans* were successfully identified, and the specificity of the observed growth defects was supported by both rescue experiments and the creation of heterozygous knockouts. This approach, which requires no prior sequence information, provides a tool for functional analysis in some organisms for which existing techniques for "en masse" gene disruption are not applicable. Although the outlined strategy may be adaptable to any diploid organism, imperfect fungi might prove ideal candidates. In-depth investigation (including exploration in animal models) of the targets and drugs outlined in this study is now ongoing.

Experimental protocol

Preparation of DNA libraries. *cDNA library:* pGAL1PNiST-1 (antisense *cDNA SfiI*-*NotI* vector; Fig. 1A) was constructed as described¹⁹. First-strand *cDNA* was synthesized from mRNA (strain B2630 (ref. 30), Invitrogen Fast Track; Invitrogen, Carlsbad, CA) using an oligo dT-*NotI* primer adapter and Superscript RT (Bio-Rad Laboratories Inc., Hercules, CA). Phosphorylated *SfiI* adapters were ligated to double-stranded *cDNA*, which was subsequently cut with *NotI*. The *SfiI/NotI* *cDNA* was size-fractionated on a Biogel column A150M and ligated in the *NotI/SfiI* cut vector. Approximately 38,720 clones (average insert size 1,500 bp) were obtained upon electroporation into *Escherichia coli*. *gDNA library:* The vector pGAL1PSiST-1 (integrative *SfiI*-*SfiI* vector) was created for cloning small *gDNA* fragments behind the GAL1 promoter (Fig. 1B). *gDNA* of B2630 (ATCC No. 44858) was isolated³¹, partially digested with *AluI*, and size-fractionated; then *DNA* fragments (0.5–1.25 kb) were eluted from gel by centrifugal filtration³². *SfiI* adapters (5'-GTTGGC-CTTTT-3') were attached to the *DNA* ends (blunt; 400–1000 bp), and fragments were cloned in a *SfiI*-cut pGAL1PSiST-1 vector. Approximately 400,000 clones (average insert size 600 bp) were obtained.

Transformation and screening for growth-affected transformants. Strains, media, and growth conditions were as described³⁰. CAI-4 was transformed with *cDNA* and *gDNA* libraries using a transformation protocol typically used for *Pichia pastoris* (http://www.invitrogen.com/pdf_manuals/pichspher_man.pdf). Transformants were screened by parallel measurement of growth in noninducing and inducing media on a Bioscreen Analyzer (Labsystems, Helsinki, Finland).

In vitro drug susceptibility testing. Yeast cultures were grown at 30°C while shaking at 250 r.p.m. until a final OD of 0.2 (±0.1) was reached. At this point, 200 µl of yeast suspension were added to MW96 plates containing R-compounds in a total volume of 50 µl, and plates were incubated (static) at 30°C for 48 h. Growth in positive control (compound-free) and test wells was determined using OD₆₂₀ turbidity readings. (For the purpose of duplicating experiments described in this paper, compounds are available from the Department of Collaboration and Technology Transfer, Janssen Pharmaceutica, Beerse, Belgium.)

Measurement of GAL1 promoter activity. CAI-4 was transformed with plasmid LAC4/pGAL1PNiST-1, which contains a *Kluyveromyces lactis* β-galactosidase gene (obtained from Dr. J. Ernst, Germany) under control of the *C. albicans* GAL1 promoter. Resulting transformants were grown in 5 ml of both noninduction (S+ glucose +/- maltose) and induction (S+ galactose +/- maltose) medium and processed as described³³.

Isolation of gDNA or cDNA inserts. *gDNA* was isolated from transformants using the Nucleon MI Yeast kit (Amersham, Little Chalfont, UK), and 20 ng were digested for 3 h with an enzyme that cuts uniquely in the library vector (*SacI* for the *gDNA* library; *PstI* for the *cDNA* library). Samples were phenol/chloroform extracted, precipitated with NaOAc/ethanol, resuspended in 500 µl ligation mixture (1× ligation buffer and T4 DNA ligase; both from Boehringer Mannheim, Indianapolis, IN) and incubated overnight at 16°C. After denaturation (10 min 65°C), purification (phenol/chloroform extraction), and precipitation (NaOAc/ethanol), the pellet was resuspended in 10 µl MilliQ (Millipore, Bedford, MA) water. Inverse PCR was done on 1 µl of the precipitated ligation reaction using library vector-specific primers (Fig. 1A,

B) (3pGAL1PSiST.PCR: 5'-GAG-GGC-GTG-AAT-GTA-AGC-GTG-3' and 5pGAL1PNiST.PCR: 5'-GAG-TTA-TAC-CCT-GCA-GCT-CGA-C-3' for the *gDNA* library; 3pGAL1PNiST.PCR: 5'-TGA-GCA-GCT-CGC-CGT-CGC-GC-3' and 5pGAL1PNiST.PCR for the *cDNA* library; all primers from Eurogentec, Seraing, Belgium).

PCR conditions. PCR was done for 30 cycles each consisting of (a) 1 min at 95°C, (b) 1 min at 61°C, (or 57°C for the *cDNA* library primers), and (c) 3 min at 72°C. In the reaction mixture 2.5 units of *Taq* polymerase (Boehringer) with *TaqStart* antibody (Clontech, Palo Alto, CA) (1:1) were used, and the final concentrations were 0.2 µM of each primer, 3 mM MgCl₂ (Perkin Elmer Cetus, Foster City, CA) and 200 µM dNTPs (Perkin Elmer Cetus). All PCR reactions were performed in a Robocycler (Stratagene, La Jolla, CA). PCR analysis was also performed on *gDNA* isolated from the transformants using primers 3pGAL1PSiST.PCR and 5pGAL1PNiST.PCR for the *gDNA* library transformants and using primers oligo23': 5'-TGC-AGC-TCG-ACC-TCG-AGG-3' and oligo25': 5'-GCG-TGA-ATG-TAA-GCG-TGA-C-3' (Thybr. = 53°C) for the *cDNA* library transformants. Resulting PCR products were purified using the PCR purification kit (Qiagen, Basel, Switzerland).

Sequence determination and analysis. *DNA* sequencing was performed as described³⁰. Sequence similarity searches against public (EMBL, SWISS-PROT, TrEMBL, and ALCEs (Stanford University, University of Minnesota)) and commercial (LifeSeq and PathoSeq (Incyte Genomics Inc., Palo Alto, CA), and GENESeq (Derwent, London, UK)) sequence databases were performed using BLAST. The 5' untranslated region of the *SAM2* gene was analyzed using the "Findpatterns" algorithm (GCG, University of Wisconsin, Madison).

mRNA quantitation. DIG-labeled RNA probes were prepared by *in vitro* RNA transcription from 1 µg RNase-free, *NsiI*-linearized template *DNA* (SAM2part/pDPI9) using the Maxi script kit (Ambion, Austin, TX). DIG-labeled *DNA* probes were prepared by PCR³⁰. Five micrograms of total RNA from each sample was loaded and probed as described³⁰. Normalization was done by hybridization to an *ACT1*-specific *DNA* probe. PCR quantitations using specific primers and probes were performed according to the *TaqMan* procedure^{34,35} as described³⁰. Fluorogenic probe for *RNR1*: 5'-TGA-TCT-CAA-AAA-GTG-CTG-GAG-GAA-TCG-GT-3'; forward primer for *RNR1*: 5'-CGA-CAC-TTT-GAA-ATC-GTG-TGC-T-3'; and reverse primer for *RNR1*: 5'-GCA-CCG-GTA-GAA-CGA-ATG-TTG-3'. Data were normalized according to *ACT1* C_T values.

Isolation of a full-length SAM2 gene. Amplification of 600 bp of *SAM2* gene was by PCR from SAM2/pGAL1PNiST-1 (isolated from clone 36) using primers 5- and 3-pGAL1PNiST.PCR. A *C. albicans* *gDNA* library (10–23 kb insert size) in YCp50 (S. Dewaele, University of Gent, Belgium) was probed with radiolabeled *SAM2* fragment, and positive colonies were selected for plasmid preparation^{36,37}. Subsequent restriction enzyme analysis identified a 1.1 kb *HpaI* fragment from clone 36.13.1 covering the entire hybridizing segment.

Construction of gene disruption cassettes for generation of heterozygous mutants. All disruption cassettes for the generation of single-allele knockouts were generated basically as described^{11,30}. Fragments of *TEF3*, *TUF1*, *RPL27*, *RHO1*, and *HYP* (hypothetical protein; clone 80g3) genes were PCR-amplified from CAI-4 *gDNA* and subcloned into the pCR2.1-TOPO vector (Invitrogen). Intermediate constructs were cleaved so as to release (for each gene) part of the ORF and served as recipients for the ~4 kb URA-blaster cassette (released from pMB7 using *PvuI* (ref. 11) resulting in six gene-specific disruption constructs. Correct integration of the disruption cassettes was confirmed by Southern blot analysis³⁰. The *fal1/FAL1* heterozygous knockout was constructed as described earlier¹³.

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