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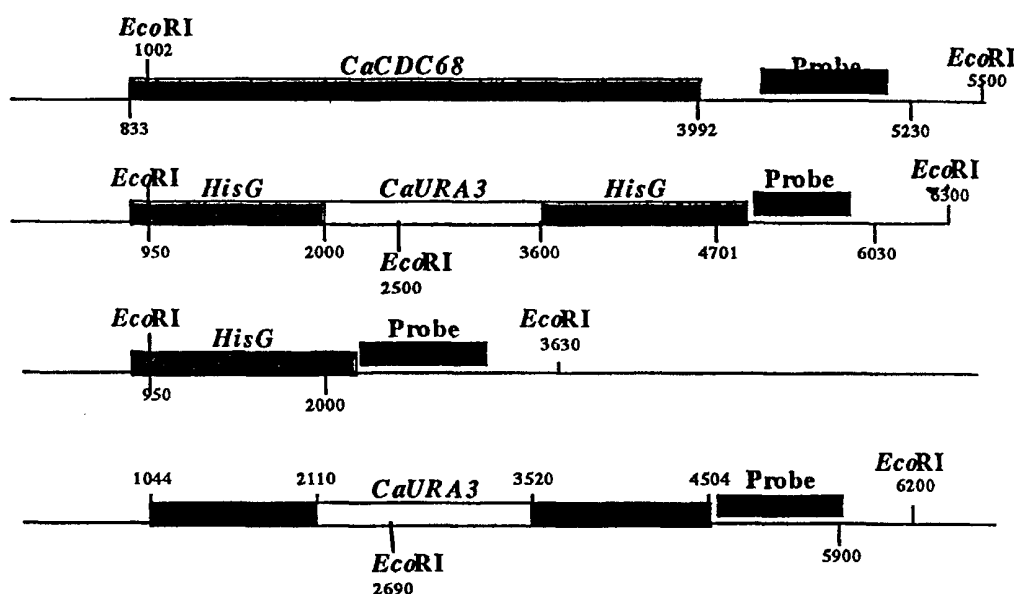
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(54) Title: Cdc68p CHROMATIN REMODELING FACTOR



(57) Abstract: The invention encompasses chromatin remodeling factor from *C. albicans* CaCdc68p, a nucleic acid sequence encoding CaCDC68, and methods of screening for inhibitors of growth of *C. albicans* and other fungi by targeting CaCdc68p.

Cdc68p CHROMATIN REMODELING FACTOR

Field of the Invention

The invention encompasses the chromatin remodeling factor Cdc68p,
5 nucleic acid sequences encoding CDC68, and methods of screening for antifungal agents
and inhibitors of growth of *Candida albicans* and other fungi by targeting Cdc68p.

Background of the Invention

Fungi are a distinct class of microorganisms, of which most are free-living.
10 They are eukaryotic organisms containing a nuclear membrane, mitochondria and
endoplasmic reticulum. In addition, they are non-motile, do not contain chlorophyll and
develop from spores (*i.e.* yeasts, molds, mushrooms and rusts). The cell structure usually
includes a rigid cell wall of mannan, glucan and chitin and a cytoplasmic membrane with a
large percentage of ergosterol. The size and morphology of fungi vary from
15 monomorphic yeasts like *Cryptococcus* and *Saccharomyces* species and dimorphic fungi
like *Candida albicans* to filamentous fungi like *Aspergillus* species.

In contrast to bacteria, which are generally considered mammalian
pathogens, fungi tend to be plant pathogens. However, in addition to the well recognized
group of dermatophytes (*e.g.* cause of “athlete’s foot”), an increasingly large group of
20 fungi turn out to be able to act as opportunistic human pathogens producing disease only in
compromised individuals. As the result of an aging population as well as an increase in
the number of immunocompromised patients, *e.g.*, patients with acquired
immunodeficiency syndrome (AIDS), patients undergoing cancer chemotherapy, or
immunosuppressive therapy (*e.g.* treatment with corticosteroids) and patients undergoing
25 organ transplantation, the incidence of fungal infections is increasing rapidly.

Fungi parasitize many different tissues. Most infections begin by

colonization of the skin, a mucosal membrane or the respiratory epithelium. Superficial fungi and subcutaneous pathogens cause indolent lesions of the skin. Passage through the initial surface barrier is accomplished through a mechanical break in the epithelium.

Although most fungi are readily killed by neutrophils, some species are resistant to phagocytic killing and can infect otherwise healthy individuals. The most virulent fungi cause systemic infections, a progressive disease leading to deep seated visceral infections in otherwise healthy individuals (see *e.g. Sherris Medical Microbiology, Third Edition*, Kenneth J. Ryan, ed., Appleton & Lange, Norwalk, CT, 1994).

The major fungal pathogens in North America are *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Candida* species, such as but not limited to *Candida albicans* and *Aspergillus* species (*Medically Important Fungi, Second Edition*, Davise H. Larone, Ed., American Society for Microbiology, Washington, D.C.). The yeast *C. albicans* (*C. albicans*) is one of the most pervasive fungal pathogens in humans. It is the cause of an increasing financial and logistic burden on the medical care system and its providers due to its ability to opportunistically infect a diverse spectrum of immunocompromised hosts, which are a quickly growing population of patients in today's society. Although *C. albicans* is a member of the normal flora of the mucous membranes in the respiratory, gastrointestinal, and female genital tracts, it may gain dominance (*e.g.* upon treatment with antibacterial antibiotics, in patients with diabetes or in patients using corticosteroids) in such locations and be associated with pathologic conditions. In addition, almost all HIV-positive individuals suffer from a *Candida* infection prior to the onset of developing full-blown AIDS.

Sometimes *C. albicans* produces progressive systemic disease, particularly if cell-mediated immunity is impaired. In 1994, about thirty percent of patients suffering from leukemia or undergoing organ transplants developed a systemic *Candida* infection of which thirty percent have been estimated to have succumbed to the infection.

Only a handful of agents are active against fungi. For life threatening disease caused by any of the pathogenic fungi, amphotericin B is the agent of choice. This drug, however, is associated with numerous severe side effects such as fever, dyspnea and tachycardia, and dosage is limited over the lifetime of the patient because of renal toxicity. An agent frequently used concurrently is flucytosine, a nucleoside analog, which cannot be

used independently of other agents because of the rapid appearance of resistance.

Untoward effects of treatment with flucytosine include leukopenia, thrombocytopenia, rash, nausea, vomiting, diarrhea, and severe enterocolitis.

In conditions where the patient's life is not threatened, ketoconazole can be
5 used as a long-term therapy for blastomycosis, histoplasmosis, or coccidioidomycosis. Fluconazole also has a significant role in the treatment of superficial fungal infections. Both compounds are from the same class, the triazoles, and are cytostatic. The emergence of resistance and hepatic toxicity limits the use of triazoles such as fluconazole and ketoconazole. The newest triazole, itraconazole, has similar pharmacokinetics and
10 spectrum of activity as fluconazole. None of the azoles can be used for life threatening or deep seated fungal infections. They are only effective in reducing colonization of fungi such as *Candida* species and for treating superficial mycoses.

All major antifungal agents function by attacking either directly or indirectly ergosterol, a component of the cell wall. Amphotericin B and other polyene
15 macrolide compounds like nystatin interact with ergosterol in the cell membrane and form pores or channels that increase the permeability of the membrane. Resistance to amphotericin B in mutant strains is accompanied by decreased concentrations of ergosterol in their cell membranes. Imidazoles and triazoles inhibit sterol 14- α -demethylase, a microsomal cytochrome P₄₅₀-dependent enzyme system. Imidazoles and triazoles thus
20 impair the biosynthesis of ergosterol for the cytoplasmic membrane, leading to the accumulation of 14- α -methyl sterols, which impair certain membrane-bound enzyme systems (see, *The Pharmacological Basis of Therapeutics, Eighth Edition*, Goodman and Gilman, Pergamon Press, 1990).

Nystatin, amphotericin B, flucytosine and the various azoles have all been
25 used to treat oral and systemic *Candida* infections. However, orally administered nystatin is limited to treatment within the gut and is not applicable to systemic treatment, and resistance to flucytosine is so widespread that it is only used in combination with other drugs. Some life-threatening systemic infections are susceptible to treatment with the azoles or amphotericin B. Azoles have been the most successful drugs used for treatment
30 of such infections in the last few years but they work relatively slowly, have to be taken for months, and are fungistatic rather than fungicidal. While such azole antifungal agents exhibit significantly lower toxicity compared to amphotericin B, their mechanism of action

and inactivation of cytochrome P₄₅₀ prosthetic groups in certain enzymes preclude their use in patients that are simultaneously receiving other drugs that are metabolized by the body's cytochrome P₄₅₀ enzymes.

Widespread use of azoles has also resulted in an important change in the spectrum of *Candida* infections. Whereas *C. albicans* used to be the common cause of *Candidosis*, 50% of these infections are now caused by non-*albicans* species which tend to be less susceptible to azole treatment. In addition, a quickly rising percentage of *C. albicans* isolates obtained from infected patients have been found to be resistant to azoles.

There is thus an immediate need for an effective treatment of opportunistic infections caused by *C. albicans*. Although the majority of life-threatening fungal infections are caused by *C. albicans*, infections caused by other less common fungi as discussed above, e.g., *Aspergillus fumigatus* have a worse prognosis. In large part this is due to the absence of diagnosis until a very late stage of infection, usually post-mortem. Therefore it is desirable that novel compounds be able to act against all pathogenic fungi, preventing the need for precise, time-consuming diagnosis.

Development of an effective method and composition for treatment of fungal infections is a critical goal of the pharmaceutical industry. The industry has made numerous efforts to identify fungal-specific drugs, with only limited success. It would be of great value to identify a new class of antifungal drugs that block a fungal target other than ergosterol. This target should be fungal-specific and should lead to development of a drug that is effective against the organisms that are resistant to current therapy.

Antifungal drug development often relies on the screening of a large number of compounds before one or more lead compounds are found that are effective against the target fungi. Thus, it is critical for the development of these screens to define proteins essential for survival or growth of the target fungi and to discover means of purifying or producing such proteins. Therefore, there is a need in the art to identify essential fungal structural or functional elements that can serve as targets for drug intervention, and for methods and compositions for identifying useful anti-fungal agents that interact with or inhibit essential fungal elements that can be used to treat fungal infections.

Mutations in a number of genetic loci in *Saccharomyces cerevisiae* result in an inability to proceed through the cell-division cycle required for multiplication and

growth of cells. One of these *CDC* genes (cell-division cycle) is *CDC68*. A temperature sensitive mutation in *CDC68*, *cdc68-1*, was found to render cells incapable of proceeding through the START phase of the cell-division cycle at the restrictive temperature, resulting in enlarged cells (Prendergast *et al.* 1990). Arrest at the START phase was attributed to
5 decreased amounts of G1-cyclins, Cln1p-3p, which are required for the performance of START (Rowley *et al.* 1991). Studies into the nature of the defect showed that transcription of many, and possibly all, genes was strongly decreased after *cdc68-1* mutant cells were incubated at the restrictive temperature.

Independently, another class of *cdc68* mutations was obtained in the yeast
10 *S. cerevisiae*. Insertion of yeast transposable elements like Ty in a promoter stops efficient transcription of a gene under control of that promoter. Mutant cells can be obtained however, in which a defect of this type is overcome by mutations in Spt (suppressor of Ty) loci. One of these loci isolated in *S. cerevisiae*, *SPT16*, has been shown to be identical to *CDC68* (Malone *et al.* 1991). This *S. cerevisiae* *SPT16/CDC68* gene was cloned and the
15 nucleotide sequence determined (Rowley *et al.* 1991). Furthermore, diploid strains of *S. cerevisiae* were constructed in which one copy of the gene *SPT16/CDC68* was disrupted. Sporulation of these strains resulted in only two viable spores which corresponded to those spores carrying the intact copies of *SPT16/CDC68*. Hence, *SPT16/CDC68* is required for sporulation and/or growth of vegetative cells (Malone *et al.* 1991). In addition, the N-
20 terminal third of the *S. cerevisiae* Cdc68p has been shown to be dispensable for gene activation but required for the maintenance of chromatin repression. (Evans *et al.* 1998.)

Biochemical studies have shed light on the nature of the requirement for the Cdc68 protein (Cdc68p) in gene transcription. *In vitro* transcription of class II genes from naked template DNA requires RNA polymerase II (RNAP II) and the general transcription
25 factors IIB, IID, IIE, IIF and IIH. *In vivo* transcription occurs from DNA that is present in nucleosomes. However, when packaged template DNA is used in *in vitro* experiments, RNAP II and the general transcription factors are not sufficient. Transcription elongation has been found to require an additional factor isolated from nuclear extract from HeLa cells, FACT (facilitator of chromatin transcription) (Orphanides *et al.* 1998). FACT is a
30 heterodimer composed of the human homologs of Pob3p and Cdc68p. (Brewster *et al.* 1998.)

Other studies suggest that, in addition to transcription, Cdc68p might be

involved in DNA replication in *S. cerevisiae* (Wittmeyer and Formosa 1997). DNA replication involves a multiprotein complex containing DNA polymerase α . Affinity matrix-linked DNA polymerase α has been shown to be bound to two proteins, Pob3p and Cdc68p, present in *S. cerevisiae* cell extracts. (Wittmeyer and Formosa 1997.)

5

References:

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Prendergast *et al.* (1990) Genetics 124: 81-90.
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Wittmeyer and Formosa (1997) Mol. Cell. Biology 17 (7): 4178-4190.

15 Summary of the Invention

The present invention is based on the isolation of a nucleic acid encoding *Candida albicans* Cdc68 protein (CaCdc68p). In one aspect, the invention provides an isolated nucleic acid having the sequence depicted in Figure 1, SEQ ID NO:1, as well as sequence-conservative and function-conservative variants thereof. In another aspect, the
20 invention provides an isolated nucleic acid which encodes the polypeptide depicted in SEQ ID NO:2. The invention also provides vectors comprising these sequences, and cells comprising the vectors. Methods for producing the polypeptides, which comprise (i) culturing the cells and (ii) recovering the polypeptide from the culture, are also provided.

In another aspect, the invention provides an isolated polypeptide having the
25 amino acid sequence depicted in Figure 1, SEQ ID NO:2, and a fragment or fragments thereof, the fragments or fragments being characterized in that when expressed in *S. cerevisiae* they facilitate rescue of temperature-sensitive knock out mutations in *ScCDC68*, and function-conservative variants of said polypeptide and fragment or fragments thereof.

In a still further aspect, the invention provides a method for producing
30 recombinant *Candida albicans* Cdc68p, comprising culturing a host cell transformed with a nucleic acid encoding *Candida albicans* Cdc68p under conditions sufficient to permit expression of the nucleic acid encoding *Candida albicans* Cdc68p, and isolating the

Candida albicans Cdc68p.

In yet another aspect, the invention provides a screening method for detecting and identifying compounds that bind to Cdc68p, as well as compounds which can inhibit *Candida albicans* growth.

5 Detection may be performed in the presence of a plurality of candidate inhibitor compounds. In carrying out the screening methods of the invention which involve screening a plurality of candidate inhibitor compounds, the plurality of inhibitor compounds may be screened together in a single assay or individually using multiple simultaneous individual detecting steps.

10 In another aspect, the invention provides a method of preventing *Candida albicans* growth in culture, by contacting the culture with an inhibitor compound that selectively inhibits the biological activity of *Candida albicans* Cdc68p.

 In a still further aspect, the invention provides a method of preventing *Candida albicans* growth in a mammal, comprising administering to the mammal an
15 effective amount of an inhibitor compound that selectively inhibits the biological activity of *Candida albicans* Cdc68p.

 In yet another aspect, the invention provides a method of preventing fungal growth, comprising administering to a fungal infection an effective amount of an inhibitor compound that selectively inhibits the biological activity of fungal Cdc68p.

20 Other features and advantages of the invention will be apparent from the description, preferred embodiments thereof, the drawings, and from the claims.

Brief Description of the Drawings

25 Figure 1 depicts the nucleotide sequence encoding *C. albicans* CDC68 (*CaCDC68*), SEQ ID NO: 1 and the predicted amino acid sequence of *C. albicans* Cdc68 protein (*CaCdc68p*), SEQ ID NO: 2.

 Figure 2 depicts a comparison of amino acid sequences of Cdc68p homologous proteins obtained from humans (*H. sapiens*), SEQ ID NO: 3; *D. melanogaster*, SEQ ID NO: 4; *S. cerevisiae*, SEQ ID NO: 5; *C. albicans*, SEQ ID NO: 2
30 and *K. lactis*, SEQ ID NO: 6. The sequences that are shaded with stippling depict those residues that match *H. sapiens* Cdc68 exactly while those that are boxed depict those

residues that match *S. cerevisiae* Cdc68 exactly.

Figure 3 depicts a comparison of predicted amino acid sequences from PCR-amplified nucleotide fragments of Cdc68 obtained from *A. nidulans* [SEQ ID NO: 7] and *N. crassa* [SEQ ID NO: 8] with similar regions of Cdc68p from humans [SEQ ID NO: 9], *D. melanogaster* [SEQ ID NO: 10], *S. cerevisiae* [SEQ ID NO: 11], *K. lactis* [SEQ ID NO: 12], and *C. albicans* [SEQ ID NO: 13]. The sequences that are shaded with stippling depict those residues that match *H. sapiens* Cdc68 exactly while those that are boxed depict those residues that match *S. cerevisiae* Cdc68 exactly. Due to the presence of introns, the exact cDNA sequence, and thus the exact amino acid sequence, around the splice site is uncertain in both *A. nidulans* and *N. crassa*. The probable splice site location is thus indicated by "X".

Figure 4 depicts a graphic representation of viability expressed in colony forming units (CFU)/OD650 versus time (hours) for *S. cerevisiae* *cdc68-1* mutant (circles) and BM390 wild type (squares) strains. Cells were pregrown and incubated at 30°C (open symbols) or 37°C (closed symbols). Optical density (OD650) and CFU/mL were followed from which viability of cultures (CFU/OD650) was calculated.

Figure 5 depicts a graphic representation of viability (CFU/OD650) versus time (hours) upon metal inducible depletion of Cdc68p in *S. cerevisiae* CUY106 wild type (squares) and CUY106::CDC68 mutant (squares) strains. Cells were grown until they reached exponential phase, after which they were incubated with (closed symbols) or without (open symbols) 1mM CuSO₄. Optical density (OD650) and CFU/mL were followed from which viability of cultures (CFU/OD650) was calculated.

Figure 6 is a depiction of the mechanism of gene disruption in *C. albicans*.

Figure 7A is a depiction of *Eco*R1 restriction maps, and fragments obtained following *Eco*R1 digestion, of the *CaCDC68* locus. From top to bottom: before integration (4.5 kb fragment), after integration of the first disruption cassette (3.8 kb fragment), after subsequent 5' fluoro-orotic acid (FOA) selection (2.7 kb fragment), and after integration of the second disruption cassette (3.5 kb fragment).

Figure 7B is a depiction of a Southern blot of genomic DNA digested with *Eco*R1. Lane 1: Control strain CAI4 transformed with second disruption plasmid. Lanes 2 and 4: Independently obtained pre-FOA transformants. Lanes 3 and 5: post-FOA transformants derived from pre-FOA transformants shown in lanes 2 and 4 respectively.

Lane 6: Putative double disruptant. Lane 7: Parental strain CAI4.

Figure 8A is a depiction of an SDS-PAGE gel electrophoresis of various CaCdc68p purification stages. Lane 1: Molecular weight marker. Lane 2: Induced cells. Lane 3: Uninduced cells. Lane 4: Post-hiTrap fraction. Lane 5: Post-MonoQ fraction.
5 Lane 6: purified CaCdc68p.

Figure 8B is a depiction of an SDS-PAGE gel electrophoresis of various ScCdc68p purification stages. Lane 1: Molecular weight marker. Lane 2: Uninduced cells. Lane 3: Induced cells. Lane 4: Post-hiTrap fraction. Lane 5: Purified ScCdc68p in post MonoQ fraction.

10

Detailed Description of the Invention

All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety.

15 This invention is directed to a novel protein, *C. albicans* Cdc68p (CaCdc68p). The protein plays an essential role in cell viability, and is highly conserved among fungi. The invention is also directed to the isolation of recombinant DNA encoding CaCdc68p. Because CaCdc68p is essential for viability of fungal cells, a compound that blocks the biological activity of the protein would be expected to have fungicidal properties. Since amino acid
20 sequences of Cdc68 proteins from fungal sources are more similar to one another than human Cdc68 protein, it is expected that certain compounds that bind to fungal Cdc68 will not bind to human Cdc68, and so will be specific inhibitors of fungal cell growth. Therefore, the invention is also directed to assays to screen for inhibitors of CaCdc68p which are active against other fungi.

25

Definitions:

1. "Inhibition" as used herein refers to a reduction in the parameter being measured, whether it be *C. albicans* growth or viability or *C. albicans* DNA transcription. The amount of such reduction is measured relative to a standard (control).
30 Because of the multiple interactions of *C. albicans* Cdc68p in cell division, growth and cell cycle regulation the target product for detection will vary with respect to the particular screening assay employed. "Reduction" is defined herein as a decrease of at least 25%

relative to a control, preferably of at least 50%, and most preferably of at least 75%.

2. "Growth" as used herein refers to the normal growth pattern of *C. albicans*, *i.e.*, to a cell doubling time of 60-90 minutes during the log phase of growth.

3. "Viability" as used herein refers to the ability of *C. albicans* to multiply, form strings of cells ("hyphae") or increase in size. This can be measured by following the optical density of batches of media inoculated with *C. albicans* cells and spreading samples obtained from these batches on growth plates. An increasing optical density indicates viability; absence of an increase may indicate inviability. Cells that fail to form colonies on growth plates, irrespective of the growth conditions in that plate, are classified as inviable.

4. "Biological activity" as used herein refers to the ability of Cdc68p to promote transcription and cell cycle division through its chromatin remodeling capability.

5. "Nucleic acid" or "polynucleotide" as used herein refers to purine- and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribo-polydeoxyribo nucleotides. This includes single- and double-stranded molecules, *i.e.*, DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases.

6. An "isolated" nucleic acid or polypeptide as used herein refers to a nucleic acid or polypeptide that is removed from its original environment (for example, its natural environment if it is naturally occurring). An isolated nucleic acid or polypeptide contains less than about 50%, preferably less than about 75%, and most preferably less than about 90%, of the cellular components with which it was originally associated.

7. A nucleic acid or polypeptide sequence that is "derived from" a designated sequence refers to a sequence that is related in nucleotide or amino acid sequence to a region of the designated sequence. For nucleic acid sequences, this encompasses sequences that are homologous or complementary to the sequence, as well as "sequence-conservative variants" and "function-conservative variants." For polypeptide sequences, this encompasses "function-conservative variants." Sequence-conservative variants are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Function-conservative variants are those in which a given amino acid residue in a polypeptide has been changed

without altering the overall conformation and function of the native polypeptide, including, but not limited to, replacement of an amino acid with one having similar physico-chemical properties (such as, for example, acidic, basic, hydrophobic, and the like). "Function-conservative" variants of a designated polypeptide also include any polypeptides that have the ability to elicit antibodies specific to the designated polypeptide.

8. Nucleic acids are "hybridizable" to each other when at least one strand of nucleic acid can anneal to another nucleic acid strand under defined stringency conditions. Stringency of hybridization is determined, *e.g.*, by a) the temperature at which hybridization and/or washing is performed, and b) the ionic strength and polarity (*e.g.*, formamide concentration) of the hybridization and washing solutions, as well as other parameters. Hybridization requires that the two nucleic acids contain substantially complementary sequences; depending on the stringency of hybridization, however, mismatches may be tolerated. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementarity, variables well known in the art.

In general, nucleic acid manipulations used in practicing the present invention employ methods that are well known in the art, as disclosed in, *e.g.*, *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor) and *Current Protocols in Molecular Biology* (Eds. Ausubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, NY, 1997).

The present invention is based on the isolation of DNA encoding the protein Cdc 68 from *Candida albicans* (CaCdc68p). Based on results obtained in *S. cerevisiae* and human cells, CaCdc68p is presumed to be a facilitator of DNA transcription elongation through its activity as a chromatin remodeling factor. The discovery and characterization of CaCdc68p, and the elucidation of differences between the fungal and mammalian Cdc68 proteins, implicates this protein as an important target for the development of new methods and compositions for the treatment of fungal infections. As shown in Example 3 below, deletion and rescue analysis in *S. cerevisiae* demonstrated that CaCdc68p is essential for cell viability. Therefore, agents which selectively interfere with

CaCdc68p activity would likely be candidates for anti-*C. albicans* therapeutics. The present invention also encompasses methods for identifying compounds that selectively interfere with CaCdc68p activity and thus may comprise useful antifungal agents.

The gene encoding CaCdc68p was isolated as described in Example 1 below. Briefly, a genomic library from *C. albicans* was used to transform a strain of *S. cerevisiae* containing a temperature sensitive Cdc68p mutation. A *C. albicans*-derived DNA fragment capable of complementing the ScCdc68 temperature sensitive phenotype was isolated. Nucleotide sequencing of the DNA identified by this procedure (Figure 1 and SEQ ID NO: 1) revealed an open reading frame of 3159 bp encoding a protein of 1053 amino acids which showed significant homology to Cdc68p/Spt16p from *S. cerevisiae* and *K. lactis*. However, the inferred amino acid sequence of CaCdc68 diverges from that of *Drosophila melanogaster* and humans. However, despite the homology, the inferred amino acid sequence of CaCdc68 diverges considerably from that of *S. cerevisiae* and humans.

Ideally, an antifungal compound directs its cidal action against a target that is present in fungi but absent in human cells. Cidal targets are important for cell function and tend to be conserved in evolution and, thus, be present in both human and fungal cells. Cdc68p is an example of a protein present in both cell types, as noted above, but the human homolog of Cdc68p has an amino acid sequence that distinguishes it from fungal Cdc68p. This allows for identification of compounds that act against fungal Cdc68p but not human Cdc68p. This is shown in Table 1 which provides a pairwise comparison (% identity) of Cdc68p from various organisms where the human sequence is the preliminary sequence.

TABLE 1 - Pairwise Comparison Expressed in Percent (%) Identity

	<i>H. sapiens</i>	<i>D. melano.</i>	<i>K. lactis</i>	<i>S. cerevisiae</i>	<i>C. albicans</i>
<i>H. sapiens</i>	***	57.0 %	35.0 %	35.0 %	36.0 %
<i>D. melanogaster</i> (" <i>D. melano.</i> ")		***	35.0 %	33.0 %	35.0 %
<i>K. lactis</i>			***	72.0 %	51.0 %
<i>S. cerevisiae</i>				***	51.0 %
<i>C. albicans</i>					***

The numbers indicate the percentage identity resulting from the pairwise alignment according to the algorithm used to obtain the alignments shown in Figure 2. The table shows that fungal species (*K. lactis*, *C. albicans* and *S. cerevisiae*) are 51-72% identical over the full length Cdc68 proteins, while fungal species display approximately 32-36% sequence homology with human Cdc68. A similar result is shown in Figure 2 from which it is clear that there are regions within CaCdc68p that are conserved among fungi but are absent from human Cdc68p.

The present invention provides isolated nucleic acids encoding CaCdc68 such as, *e.g.*, the nucleic acid sequence depicted in Figure 1, SEQ ID NO: 1. The invention also encompasses isolated nucleic acids encoding enzymatically active fragments derived therefrom, and related sequences. For example, function-conservative variants of CaCdc68-encoding nucleic acids are nucleic acids that encode polypeptides that retain one or more of the chromatin remodeling and transcriptional regulatory functions of CaCdc68. These functions of CaCdc68 as used herein include the gene transcription and elongation and/or rescue of temperature sensitive mutations of *Cdc68*. Fragments of CaCdc68 that retain one or more of these functions can be identified according to the methods described herein, such as *e.g.*, transcription assays and rescue experiments.

Also encompassed by the invention are nucleic acids that are hybridizable to, or derived from, the CaCdc68 sequences described above. In one embodiment, the invention relates to isolated nucleic acids capable of hybridizing with the CaCdc68 sequences or with their complements under the hybridization conditions defined below.

-- Prehybridization treatment of the support (nitrocellulose filter or nylon membrane), to which is bound the nucleic acid capable of hybridizing with nucleic acid encoding CaCdc68 at 65°C for 6 hours with a solution having the following composition: 4 x SSC, 10 x Denhardt (1X Denhardt is 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA (bovine serum albumin)); 1 x SSC consists of 0.15M of NaCl and 0.015M of sodium citrate, pH 7);

-- Replacement of the pre-hybridization solution in contact with the support by a buffer solution having the following composition: 4 x SSC, 1 x Denhardt, 25 mM NaPO₄, pH 7, 2 mM EDTA, 0.5% SDS, 100 µg/mL of sonicated salmon sperm DNA containing a nucleic acid derived from the CaCdc68 sequence as probe, in particular as radioactive probe, and previously denatured by a treatment at 100°C for 3 minutes;

- Incubation for 12 hours at 65°C;
- Successive washings with the following solutions: (i) four washings with 2 x SSC, 1 x Denhardt, 0.5% SDS for 45 minutes at 65°C; (ii) two washings with 0.2 x SSC, 0.1 x SSC for 45 minutes at 65°C; and (iii) 0.1 x SSC, 0.1% SDS for 45 minutes at 5 65°C.

The invention also encompasses any nucleic acid exhibiting the property of hybridizing specifically with the above-described CaCdc68-encoding DNA under the conditions described above, but at 40°C, including successive washings in 2X SSC at 45°C for 15 minutes.

- 10 It will be understood that the conditions of hybridization defined above constitute preferred conditions for hybridization, but are in no way limiting and may be modified in ways known in the art which do not affect the overall properties of recognition and hybridization of the probes and nucleic acids mentioned above.

- 15 The salt conditions and temperature during the hybridization and the washing of the membranes can be modified without the detection of the hybridization being affected. For example, it is possible to add formamide in order to lower the temperature during hybridization.

- The invention also encompasses vectors comprising CaCdc68-encoding sequences, cells comprising the vectors, and methods for producing CaCdc68 that involve 20 culturing the cells.

- A large number of vectors, including plasmid and fungal vectors, have been described for expression in a variety of eukaryotic and prokaryotic hosts. Such vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, *e.g.* antibiotic resistance, and one or more expression 25 cassettes. The inserted CaCdc68 coding sequences may be synthesized, isolated from natural sources, prepared as hybrids, etc. Ligation of the coding sequences to the transcriptional regulatory sequences may be achieved by known methods. Suitable host cells may be transformed/transfected/infected by any suitable method including electroporation, CaCl₂ mediated DNA uptake, fungal infection, microinjection, 30 microprojectile, or other established methods.

A wide variety of host/expression vector combinations may be employed in expressing DNA sequences encoding CaCdc68. Useful expression vectors, for example,

may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, *e.g.*, *E. coli* plasmids col E1, pCR1, pBR322, pMal-C2, pET, pGEX (Smith *et al.*, Gene 67:31-40, 1988), pMB9 and their derivatives, plasmids such as RP4; phage DNAs, *e.g.*, the numerous derivatives of phage λ , *e.g.*, NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 micron plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

Appropriate host cells for expressing protein include bacteria, Archaeobacteria, fungi, especially yeast, and plant and animal cells, especially mammalian cells. Of particular interest are *E. coli*, *B. subtilis*, *S. cerevisiae*, Sf9 cells, C129 cells, 293 cells, *Neurospora*, and CHO cells, COS cells, HeLa cells, and immortalized mammalian myeloid and lymphoid cell lines. Preferred replication systems include M13, ColE1, SV40, baculovirus, lambda, adenovirus, and the like. A large number of transcription initiation and termination regulatory regions have been isolated and shown to be effective in the transcription and translation of heterologous proteins in the various hosts. Examples of these regions, methods of isolation, manner of manipulation, etc. are known in the art. Under the appropriate expression conditions, host cells can be used as a source of recombinantly produced CaCdc68. Advantageously, vectors may also include a promoter sequence operably linked to the CaCdc68 encoding portion. The encoded CaCdc68 may be expressed by using any suitable vectors and host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. The particular choice of vector/host is not critical to the invention.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with

nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

Expression of CaCdc68 protein may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control CaCdc68 gene expression include, but are not limited to, Cytomegalovirus immediate early promoter (CMV promoter; US Patent Nos. 5,385,839 and 5,168,062) the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296:39-42); prokaryotic expression vectors such as the b-lactamase promoter (Villa-Kamaroff, *et al.*, 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer, *et al.*, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adames *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogam *et al.*, 1985, Nature 315:338-340; Kollias

et al., 1986, Cell 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378).

Nucleic acids encoding wild-type or variant CaCdc68 polypeptides may also be introduced into cells by recombination events. For example, such a sequence can be introduced into a cell, and thereby effect homologous recombination at the site of an endogenous gene or a sequence with substantial identity to the gene. Other recombination-based methods, such as non-homologous recombinations or deletion of endogenous genes by homologous recombination, may also be used.

The invention also encompasses isolated and purified CaCdc68 polypeptides, including, *e.g.*, a polypeptide having the amino acid sequence depicted in Figure 2, as well as function-conservative variants of this polypeptide, including fragments that retain transcriptional regulatory activity as described above.

CaCdc68-derived polypeptides according to the present invention, including function-conservative variants, may be isolated from wild-type or mutant *C. albicans* cells, or from heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) into which a CaCdc68-derived protein-coding sequence has been introduced and expressed. Furthermore, the polypeptides may be part of recombinant fusion proteins. Alternatively, polypeptides may be chemically synthesized by commercially available automated procedures, including, without limitation, exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis.

"Purification" of a CaCdc68 polypeptide refers to the isolation of the polypeptide in a form that allows its transcription-activating activity to be measured without interference by other components of the cell in which the polypeptide is expressed. Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that

facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against CaCdc68 or against peptides derived therefrom can be used as purification reagents. Other purification
5 methods are possible.

The isolated polypeptides may be modified by, for example, phosphorylation, sulfation, acylation, or other protein modifications. They may also be modified with a label capable of providing a detectable signal, either directly or indirectly, including, but not limited to, radioisotopes and fluorescent compounds.

10 The present invention encompasses antibodies that are specific for CaCdc68 or fragment identified as described above. As used herein, antibodies "specific" for CaCdc68 include without limitation antibodies that: bind to CaCdc68 but do not bind to other nuclear proteins, bind Cdc68 proteins from non-*Candida* species with a lower affinity share to CaCdc68, identify associational or other functional domains present in
15 CaCdc68 but not in other species, and the like. The antibodies may be polyclonal or monoclonal. The antibodies may be elicited in an animal host by immunization with CaCdc68 or fragments derived therefrom or may be formed by *in vitro* immunization of immune cells. The immunogens used to elicit the antibodies may be isolated from *C. albicans* cells or produced in recombinant systems. The antibodies may also be produced
20 in recombinant systems programmed with appropriate antibody-encoding DNA. Alternatively, the antibodies may be constructed by biochemical reconstitution of purified heavy and light chains. The antibodies include hybrid antibodies (*i.e.*, containing two sets of heavy chain/light chain combinations, each of which recognizes a different antigen), chimeric antibodies (*i.e.*, in which either the heavy chains, light chains, or both, are fusion
25 proteins), and univalent antibodies (*i.e.*, comprised of a heavy chain/light chain complex bound to the constant region of a second heavy chain). Also included are Fab fragments, including Fab' and F(ab)₂ fragments of antibodies.

Methods for the production of all of the above types of antibodies and derivatives are well-known in the art and are discussed in more detail below. For example,
30 techniques for producing and processing polyclonal antisera are disclosed in Mayer and Walker, 1987, *Immunochemical Methods in Cell and Molecular Biology*, (Academic Press, London). Such antibodies are conveniently made using the methods and compositions

disclosed in Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988, as well as immunological and hybridoma technologies known to those of ordinary skill in the art. Where natural or synthetic CaCdc68-derived peptides are used to induce an CaCdc68-specific immune response, the peptides may be conveniently
5 coupled to a suitable carrier such as KLH and administered in a suitable adjuvant such as Freund's. Preferably, selected peptides are coupled to a lysine core carrier substantially according to the methods of Tam (*Proc. Natl. Acad. Sci. USA* **85**:5409, 1988).

In one embodiment, purified recombinant CaCdc68 is used to immunize mice, after which their spleens are removed, and splenocytes used to form cell hybrids
10 with myeloma cells and obtain clones of antibody-secreting cells according to techniques that are standard in the art. The resulting monoclonal antibodies are screened using *in vitro* assays such as those described above for binding to CaCdc68 or inhibition of the incorporation of CaCdc68 into the transcriptional machinery for use as a chromatin remodeling factor.

15 Anti-CaCdc68 antibodies may be used to quantify CaCdc68, using immunoassays such as, but not limited to ELISA. Anti-CaCdc68 antibodies may also be used to block the transcriptional function CaCdc68 by inhibiting the formation of complexes between CaCdc68 subunits or between assembled RNA polymerase II complexes and other transcription components, or by immunodepleting cell extracts or
20 transcription reactions of CaCdc68. In addition, these antibodies can be used to identify, isolate, and purify CaCdc68 from different sources, and to perform subcellular and histochemical localization studies.

Methods for Modifying Transcription

25 The present invention provides methods of modifying gene transcription by contacting the CaCdc68 protein with substances that bind to, or interact with, the CaCdc68 protein or the DNA/RNA encoding the CaCdc68 protein. These substances modify the influence of the CaCdc68 protein on transcription, chromatin remodeling or other processes essential to gene transcription. Substances that bind to, or interact with, the
30 CaCdc68 protein or the DNA/RNA encoding the CaCdc68 protein can prevent or enhance the remodeling of chromatin required for elongation of RNA during transcription thus inhibiting or enhancing gene transcription. For example, antisense or nonsense nucleotide

sequences that hybridize with the CaCdc68 DNA or RNA and either completely inhibit or decrease their translation or transcription can prevent and inhibit the transcription of other fungal genes. Alternatively, compounds that can bind to or interact with the CaCdc68 protein can prevent or enhance the function of the protein in the transcription process.

- 5 These substances include antibodies that are reactive with and bind to the CaCdc68 protein.

Candidate Inhibitors

- A “candidate inhibitor,” as used herein, is any compound with a potential to
10 inhibit in *Candida albicans* the CaCdc68-mediated elongation of RNA during transcriptions, chromatin remodeling, or cell division cycle maintenance. A candidate inhibitor is tested in a concentration range that depends upon the molecular weight of the molecule and the type of assay. For example, for inhibition of protein/protein or protein/DNA complex formation or transcription elongation, small molecules (as defined
15 below) may be tested in a concentration range of 1pg - 100 ug/mL, preferably at about 100 pg - 20 ug/mL; large molecules, *e.g.*, peptides, may be tested in the range of 10 ng - 100 ug/mL, preferably 100 ng - 10 ug/mL.

- Inhibitors of *Candida albicans* growth or viability may target the novel protein described herein, CaCdc68, or it may target a protein or nucleic acid that interacts
20 with CaCdc68 to prevent the natural biological interaction that occurs *in vivo*. An inhibitor identified as described herein must possess the property that at some concentration it will inhibit *Candida albicans* growth or viability, most preferably at the same concentration it will not significantly affect the growth of mammalian, particularly human, cells.

- 25 Candidate inhibitors include peptide and polypeptide inhibitors having an amino acid sequence based upon the novel CaCdc68 sequences described herein. For example, a fragment of CaCdc68 may act to prevent the growth of wild type *Candida albicans* cells because it acts as a competitive inhibitor with respect to CaCdc68 binding to other proteins involved in *Candida* chromatin binding, cell division or transcription.

- 30 Test inhibitory compounds are screened from large libraries of synthetic or natural compounds. Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based compounds. Synthetic compound libraries

are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich (Milwaukee, WI).

Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from *e.g.* Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means.

Useful compounds may be found within numerous chemical classes, though typically they are organic compounds, and preferably small organic compounds. Small organic compounds have a molecular weight of more than 50 yet less than about 2,500 daltons, preferably less than about 750, more preferably less than about 350 daltons. Exemplary classes include heterocycles, peptides, saccharides, steroids, and the like. The compounds may be modified to enhance efficacy, stability, pharmaceutical compatibility, and the like. Structural identification of an agent may be used to identify, generate, or screen additional agents. For example, where peptide agents are identified, they may be modified in a variety of ways to enhance their stability, such as using an unnatural amino acid, such as a D-amino acid, particularly D-alanine, by functionalizing the amino or carboxylic terminus, *e.g.* for the amino group, acylation or alkylation, and for the carboxyl group, esterification or amidification, or the like. Other methods of stabilization may include encapsulation, for example, in liposomes, etc.

Inhibitor Screening

The present invention encompasses the identification of agents useful in modulating fungal gene transcription, particularly the transcription of genes by RNA polymerase II in a CaCdc68-dependent manner

High-Throughput Methods For Screening Inhibitors:

In a preferred embodiment, a high-throughput screening protocol is used to survey a large number of test compounds for their ability to bind with CaCdc68. High-throughput screening methods are described in U.S. Patent Nos. 5,585,277 and 5,679,582, in U.S.S.N. 08/547,889, and in PCT published application PCT/US96/19698 and may be used for identifying a ligand that binds the CaCdc68 protein. According to these methods,

a ligand, or a plurality of ligands for CaCdc68 target protein is identified by its ability to influence the extent of folding or the rate of folding or unfolding of the target protein. Experimental conditions are chosen so that the target protein unfolds to a measurable extent, whether reversible or irreversible. If the test ligand binds to the target protein

5 under these conditions, the relative amount of folded:unfolded target protein or the rate of folding or unfolding of the target protein in the presence of the test ligand will be different, *i.e.* higher or lower, than that observed in the absence of the test ligand. Thus, the method encompasses incubating CaCdc68 in the presence and absence of a plurality of test ligands under conditions in which (in the absence of ligand) CaCdc68 would partially or totally

10 unfold. This is followed by analysis of the absolute or relative amounts of folded vs. unfolded target protein or of the rate of folding or unfolding of the target protein.

An important feature of this method is that it will detect any compound that binds to any sequence or domain of CaCdc68, and not only to sequences or domains that are intimately involved in a biological activity or function. The binding sequence, region,

15 or domain may be present on the surface of CaCdc68 when it is in its folded state, or may be buried in the interior of the protein. Some binding sites may only become accessible to ligand binding when the protein is partially or totally unfolded.

Briefly, to carry out this method, the test ligand or ligands are combined with CaCdc68, and the mixture is maintained under appropriate conditions and for a

20 sufficient time to allow binding of the test ligand. Experimental conditions are determined empirically. When testing test ligands, incubation conditions are chosen so that most ligand:CaCdc68 protein interactions would be expected to proceed to completion. The test ligand is present in molar excess relative to CaCdc68. The target protein can be in a soluble form, or, alternatively, can be bound to a solid phase matrix. The matrix may

25 comprise without limitation beads, membrane filters, plastic surfaces, or other suitable solid supports.

In a preferred embodiment, binding of test ligand or ligands to CaCdc68 is detected through the use of proteolysis. This assay is based on the increased susceptibility of unfolded, denatured polypeptides to protease digestion relative to that of folded

30 proteins. In this case, the test ligand-CaCdc68 protein combination, and a control combination lacking the test ligand, are treated with one or more proteases that act preferentially upon unfolded target protein. After an appropriate period of incubation, the

level of intact *i.e.* unproteolysed target protein is assessed using one of the methods described below *e.g.* gel electrophoresis and/or immunoassay.

There are two possible outcomes that indicate that the test ligand has bound the target protein. Either 1) a significantly higher, or 2) a significantly lower absolute
5 amount of intact or degraded protein may be observed in the presence of ligand than in its absence.

Proteases useful in practicing the present invention include without limitation trypsin, chymotrypsin, V8 protease, elastase, carboxypeptidase, proteinase K, thermolysin, papain and subtilisin (all of which can be obtained from Sigma Chemical Co.,
10 St. Louis, MO). The most important criterion in selecting a protease or proteases for use in practicing the present invention is that the protease(s) must be capable of digesting the CaCdc68 protein under the chosen incubation conditions, and that this activity be preferentially directed towards the unfolded form of the protein. To avoid "false positive" results caused by test ligands that directly inhibit the protease, more than one protease,
15 particularly proteases with different enzymatic mechanisms of action, can be used simultaneously or in parallel assays. In addition, co-factors that are required for the activity of the protease(s) are provided in excess, to avoid false positive results due to test ligands that may sequester these factors.

In a typical embodiment of this method, purified CaCdc68 protein is first
20 taken up to a final concentration of 1-100 µg/mL in a buffer containing 50 mM Tris-HCl, pH 7.5, 10% DMSO, 50 mM NaCl, 10% glycerol, and 1.0 mM DTT. Proteases, such as, for example, proteinase K or thermolysin (proteases with distinct mechanisms of action), are then added individually to a final concentration of 0.2-10.0 µg/mL. Parallel incubations are performed for different time periods ranging from 5 minutes to one hour,
25 preferably 30 minutes, at 4°C, 15°C, 25°C, and 35°C. Reactions are terminated by addition of an appropriate protease inhibitor, such as, for example, phenylmethylsulfonyl chloride (PMSF) to a final concentration of 1mM (for serine proteases), ethylenediaminetetraacetic acid (EDTA) to a final concentration of 20 mM (for metalloproteases), or iodoacetamide (for cysteine proteases). The amount of intact protein
30 remaining in the reaction mixture at the end of the incubation period may then be assessed by any method, including without limitation polyacrylamide gel electrophoresis, ELISA, or binding to nitrocellulose filters. It will be understood that additional experiments

employing a narrower range of temperatures can be performed to establish appropriate conditions. This protocol allows the selection of appropriate conditions (*e.g.*, protease concentration and digestion temperature) that result in digestion of approximately 70% of the target protein within a 30 minute incubation period, indicating that a significant degree of unfolding has occurred.

In another embodiment, the relative amount of folded and unfolded CaCdc68 protein in the presence and absence of test ligand is assessed by measuring the relative amount of the protein that binds to an appropriate surface. This method takes advantage of the increased propensity of unfolded proteins to adhere to surfaces, which is due to the increased surface area, and decrease in masking of hydrophobic residues, that results from unfolding. If a test ligand binds the CaCdc68 (*i.e.*, is a ligand), it may stabilize the folded form of the target protein and decrease its binding to a solid surface. Alternatively, a ligand may stabilize the unfolded form of the protein and increase its binding to a solid surface.

Surfaces suitable for this purpose include without limitation microtiter plates constructed from a variety of treated or untreated plastics, plates treated for tissue culture or for high protein binding, nitrocellulose filters and PVDF filters.

In another embodiment, the extent to which folded and unfolded target protein are present in the test combination is assessed through the use of antibodies specific for either the unfolded state or the folded state of the protein *i.e.* denatured-specific ("DS"), or native-specific ("NS") antibodies, respectively. (Breyer, 1989, *J. Biol. Chem.*, 264 (5):13348-13354). Polyclonal or monoclonal antibodies are prepared as described above. The resulting antibodies are screened for preferential binding to CaCdc68 in its denatured state. These antibodies are used to screen for inhibitors of these interactions.

In another embodiment, molecular chaperones are used to assess the relative levels of folded and unfolded protein in a test combination. Chaperones encompass known proteins that bind unfolded proteins as part of their normal physiological function. In this embodiment, a test combination containing the test ligand and CaCdc68 is exposed to a solid support *e.g.* microtiter plate or other suitable surface coated with a molecular chaperone, under conditions appropriate for binding CaCdc68 with its ligand and binding of the molecular chaperone to unfolded target protein. The

unfolded target protein in the solution will have a greater tendency to bind to the molecular chaperone-covered surface relative to the ligand-stabilized folded target protein. Thus, the ability of the test ligand to bind target protein can be determined by determining the amount of target protein remaining unbound, or the amount bound to the chaperone-coated surface. Alternatively, a competition assay for binding to molecular chaperones can be utilized.

Once conditions are established for high-throughput screening as described above, the protocol is repeated simultaneously with a large number of test ligands at concentrations ranging from 20 to 200 μ M. Observation of at least a two-fold increase or decrease in the extent of digestion of the target protein signifies a "hit" compound, *i.e.*, a ligand that binds the target protein. Preferred conditions are those in which between 0.1% and 1% of test ligands are identified as "hit" compounds using this procedure.

In yet another embodiment, the test and control combinations described above can be contacted with a conformation-sensitive fluorescence probe, *i.e.*, a probe that binds preferentially to the folded, unfolded, or molten globule state of CaCdc68 or whose fluorescence properties are in any way affected by the folding status of CaCdc68 protein.

Phage Display Technology Screening:

In addition to the high-throughput screening techniques described above, technologies for molecular identification can be employed in the identification of inhibitor molecules. One of these technologies is phage display technology (U.S. Patent No. 5,403,484. Viruses Expressing Chimeric Binding Proteins). Phage display permits identification of a binding protein against a chosen target. Phage display is a protocol of molecular screening which utilizes recombinant bacteriophage. The technology involves transforming bacteriophage with a gene that encodes an appropriate ligand (in this case, a candidate inhibitor) capable of binding to the target molecule of interest. For the purposes of this disclosure, the target molecule may be CaCdc68. The transformed bacteriophage (which preferably is tethered to a solid support) express the candidate inhibitor and display it on their phage coat. The cells or viruses bearing the candidate inhibitor which recognize the target molecule are isolated and amplified. The successful inhibitors are then characterized.

Phage display technology has advantages over standard affinity ligand

screening technologies. The phage surface displays the microprotein ligand in a three dimensional conformation, more closely resembling its naturally occurring conformation. This allows for more specific and higher affinity binding for screening purposes.

5 *Biospecific Interaction Analysis Screening:*

Another relatively new screening technology which may be applied to the inhibitor screening assays of this invention is biospecific interaction analysis (BIAcore, Pharmacia Biosensor AB, Uppsala, Sweden). This technology is described in detail by Jonsson *et al.* (Biotechniques 11:5, 620-627 (1991)). Biospecific interaction analysis
10 utilizes surface plasmon resonance (SPR) to monitor the adsorption of biomolecular complexes on a sensor chip. SPR measures the changes in refractive index of a polarized light directed at the surface of the sensor chip.

Specific ligands (*i.e.*, candidate inhibitors) capable of binding to the target molecule of interest (*i.e.*, CaCdc68 or a protein-protein or protein-DNA complex
15 containing CaCdc68) are immobilized to the sensor chip. In the presence of the target molecule, specific binding to the immobilized ligand occurs. The nascent immobilized ligand-target molecule complex causes a change in the refractive index of the polarized light and is detected on a diode array. Biospecific interaction analysis provides the advantages of; 1) allowing for label-free studies of molecular complex formation; 2)
20 studying molecular interactions in real time as the assay is passed over the sensor chip; 3) detecting surface concentrations down to 10 pg/mm²; detecting interactions between two or more molecules; and 4) being fully automated (Biotechniques 11:5, 620-627 (1991)).

Screening Through Use Of A Transcription Assay:

25 Because CaCdc68 is essential for transcription elongation, inhibitors of *Candida albicans* growth and viability may also be screened either by measuring inhibition of transcription, transcription elongation, or cell cycle division regulation or by assaying formation of a protein/DNA complex or inhibition of sporulation when cells are contacted with *Candida* inhibitors.

30

In Vitro Transcription Assay

Once a particular test compound has been identified according to any of the

screening methods described above, its activity can be confirmed by adding it to an *in vitro* transcription reaction, and measuring its effect on CaCdc68-mediated activated transcription, using an *in vitro* transcription assay. For example, DNA of interest (*i.e.*, DNA to be transcribed) can be admixed with (i) purified RNA polymerase II, (ii) the SRB proteins, (iii) transcription factors b, e, g or a, (iv) CaCdc68 and (v) the substance (ligand) to be tested. The mixture is maintained under conditions sufficient for transcription to occur. The resulting combination is referred to as a test mixture. DNA transcription can be assessed by determining the quantity of mRNA produced. Transcription is determined in the presence of the substance being tested and compared to DNA transcription in the absence of the test substance taking place under identical conditions (*e.g.*, a control mixture). If transcription occurs to a lesser extent in the test mixture, (*i.e.*, in the presence of the substance being evaluated) than in the control mixture, the substance may have interacted with one or more SRB proteins, or with CaCdc68, preferably in such a manner as to inhibit transcription. If transcription occurs to a greater extent in the test mixture than in the control mixture, the substance has interacted in such a manner as to stimulate transcription.

Transcription of DNA sequences, or translation of mRNA sequences encoding the CaCdc68 protein can also be inhibited or decreased by inhibitor compounds, resulting in decreased production of, or the complete absence of CaCdc68. Gene transcription can be modified by introducing an effective amount of a substance into a cell that inhibits transcription of the CaCdc68 gene, or that inhibits translation of mRNA encoding CaCdc68. For example, antisense nucleotide sequences can be introduced into the cell that will hybridize with the gene encoding the CaCdc68 protein and inhibit transcription of the gene. (*See, Current Protocols in Molecular Biology*, Eds. Ausubel *et al.* Greene Publ. Assoc., Wiley-Interscience, NY, NY, 1997). Alternatively, an antisense sequence can be introduced into the cell that will interfere with translation of the mRNA encoding a CaCdc68 protein.

Other Methods For Testing Activity of Potential Candidate Inhibitors

1. Using *S. cerevisiae* conditional *Cdc68* knock out mutants, expression of Cdc68p (either *S. cerevisiae* or *C. albicans*) can be varied utilizing metal-inducible repression of transcription of *Cdc68* combined with a simultaneous, metal-inducible

degradation of Cdc68p already present in the cells. Cells are grown to exponential phase, after which they are incubated with CuSO₄. Within one hour of initiation of incubation in the presence of CuSO₄, viability starts to drop. Lower Cdc68p expression in the cell will make the strain more sensitive to compounds that interfere with Cdc68p.

5 2. Temperature-sensitive *Cdc68* mutant strains have been found to stop growing shortly before Start Phase, resulting in enlarged cells. Treatment of wild type cells (either *S. cerevisiae* or *C. albicans*) with candidate inhibitor compounds can be followed by examination of the percentage of budded/unbudded cells, average cell size and DNA content. Potential inhibitors are expected to stop growth shortly before START
10 phase.

 3. ScCdc68p has been found as a heterodimer complexed with Pob3p. Some potential inhibitors would be expected to interfere with heterodimer formation. Cell extracts of either *S. cerevisiae* or *C. albicans* can be loaded onto size selective columns. Eluted fractions are separated in SDS-PAGE and subjected to Western blot analysis. Any
15 decrease in the quantity of heterodimer and increase in the amount of monomer would be observed. It also possible that monomer is quickly degraded in the cell and Cdc68p would disappear from the cell extract derived from treated cells.

 4. Overexpression of Cdc68p in *S. cerevisiae* can overcome changes in transcription patterns caused by insertion of δ elements in promoters. Pob3p is apparently
20 not involved in this change in transcription. Some potential inhibitors could be tested at sublethal concentrations for their ability to lower expression of genes fused to such promoters. Any potential inhibitors could then be tested for an effect on Cdc68p.

 5. The Swi-Snf complex has chromatin remodeling activity, which is required for efficient expression of a number of genes like *SUC2*, *IN01* and *HO*. When
25 one of the genes encoding a subunit of the Swi-Snf complex, *snf5*, is deleted, expression of the genes is strongly reduced. The deletion can be compensated by overexpression of Cdc68p or Pob3p. Promoters of genes like *SUC2*, *IN01* and *HO* can be fused to any reporter gene. These constructs can be put in strains lacking *SNF5* but overexpressing Cdc68p (or Pob3p). Despite the absence of *SNF5*, this overexpression leads to an increase
30 in reporter signal. Some potential inhibitors could be tested at sublethal concentrations for their ability to lower expression of genes fused to such promoters. Any potential inhibitors could then be tested for an effect on Cdc68p.

Measurement of Inhibition of *Candida albicans* Growth in Culture

Once a putative inhibitor has been identified, it may be desirable to determine the effect of the inhibitor on the growth and/or viability of *Candida albicans* in culture. Methods for performing tests on growth inhibition of *Candida* in culture are well-known in the art. Once such procedure is based on the NCCLS M27P method (The National Committee for Clinical Laboratory Standards, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; proposed standard, 1992), as follows. Serial dilutions (two- or three-fold steps starting from a maximum concentration of 100 - 200 µg/mL) of candidate inhibitor are prepared using RPMI-1640 medium as diluent and an aliquot of 100 µl of each dilution is added to the wells of a 96-well polystyrene microtiter plate. Five *Candida albicans* colonies, picked from a Sabouraud Dextrose Agar plate inoculated 14-20 hr previously with the test *Candida albicans* strain (Catalog number 10231 from the American Type Culture Collection Yeast Catalog), are suspended with RPMI-1640 medium such that the density of cells is 10,000 - 30,000 cells/mL. 100 µl of the cell suspension is added to each of the wells of the 96-well microtiter plate containing diluted candidate inhibitor and medium control. Cultures are mixed by agitation and incubated at 35°C for 48 hr without agitation, after which cell growth is monitored by visual inspection for the formation of turbidity and/or mycelial colonies. The minimum concentration of candidate inhibitor at which no cell growth is detected by this method is defined as the minimum inhibitory concentration (MIC) for that compound. Examples of MICs for known antifungal compounds obtained using this technique are 0.125 - 0.5 µg/mL for fluconazole and 0.25 - 1.0 µg/mL for amphotericin B (The National Committee for Clinical Laboratory Standards, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; proposed standard, 1992). An inhibitor identified by the methods described herein, will have MIC which is equivalent to or less than the MICs for fluconazole or amphotericin B.

Transcription Inhibition Counterscreen Using Human Cdc68

A compound identified as an inhibitor of *Candida albicans* according to one or more of the assays described herein may be tested further in order to determine its effect on the host organism. In the development of useful antifungal compounds for human therapeutics, it is desirable that such compounds act as effective agents in

inhibiting the viability of the fungal pathogen while not significantly inhibiting human cell systems. Specifically, inhibitors of *Candida albicans* identified in any one of the above described assays may be counterscreened for inhibition of human Cdc68.

The human Cdc68 gene is available. Human Cdc68p can be expressed and
5 purified utilizing published methods and its homology to yeast Cdc68 homologues.
Human Cdc68p can be contacted with candidate inhibitor in assays such as those described above using a human cell culture system. The effectiveness of a CaCdc68 inhibitor as a human therapeutic is determined as one which exhibits a low level of inhibition against human Cdc68 relative to the level of inhibition with respect to CaCdc68. For example, it
10 is preferred that the amount of inhibition by a given inhibitor of human Cdc68 in a human system be no more than 20% with respect to the amount of inhibition of CaCdc68. Such inhibitors are "selective inhibitors" of CaCdc68P which "selective inhibit" CaCdc68. The lack of effect of a test compound on mammalian transcription is tested by replacing yeast components with an analogous human *in vitro* transcription system as in *e.g.* Manley *et al.*
15 *Proc.Natl.Acad.Sci.USA* 77:3855, 1980.

Dosage and Pharmaceutical Formulations

For therapeutic uses, inhibitors identified as described herein may be administered in a pharmaceutically acceptable/biologically compatible formulations. The
20 compositions of the present invention can be administered in dosages and by techniques well known to those skilled in the medical, veterinary, and agricultural arts taking into consideration such factors as the age, sex, weight, species and condition of the particular patient, and the route of administration. The compositions of the present invention can be administered alone or in combination, or can be co-administered or sequentially
25 administered with additional antifungal agents, such as, *e.g.*, nystatin, amphotericin B, flucytosine and the various antifungal azoles.

Moreover, the formulations of the present invention can be administered in a formulation suitable for the manner of administration, including but not limited to liquid preparations for mucosal administration, *e.g.*, oral, nasal, anal, vaginal, peroral; intragastric
30 administration and the like, such as solutions, suspensions, syrups, elixirs; and topical administration *e.g.*, in the form of a cream, ointment, lotion or spray. Further, liquid preparations for administration of the compositions of the present invention for parenteral,

subcutaneous, intradermal, intramuscular, intravenous administrations, and the like, such as sterile solutions, suspensions or emulsions, e.g. for administration by injection, can be formulated without undue experimentation.

In order for a composition to be administered to an animal or human, and
5 for any particular method of administration, it is preferred to determine the toxicity, such as by determining the lethal dose (LD) and LD₅₀ in a suitable animal model, e.g., mouse; the dosage of the composition(s), and the concentration of components in the composition; and the timing of administration in order to maximize the antiviral and/or antimicrobial response. Such factors can be determined without undue experimentation by such methods
10 as titrations and analysis of sera for antibodies or antigens, e.g., by ELISA and/or EFFIT analysis. Such determinations do not require undue experimentation from the knowledge of the skilled artisan, the present disclosure and the documents cited herein.

The formulations can be administered in a pharmaceutically effective amount and/or an antifungal effective amount, taking into account such factors as the
15 relative activity and toxicity for the target indication, e.g., antifungal activity, as well as the route of administration, and the age, sex, weight, species and condition of the particular patient.

The compositions of the present invention can be solutions, suspensions, emulsions, syrups, elixirs, capsules, tablets, creams, lotions and the like. The
20 compositions may contain a suitable carrier, diluent, or excipient, such as sterile water, physiological saline, glucose, or the like. Moreover, the compositions can also be lyophilized, and/or may contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, adjuvants, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of
25 administration and the preparation desired. Standard texts, such as "Remington's Pharmaceutical Science", 17th Ed., 1985, incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation.

The amount of inhibitor administered will be determined according to the degree of pathogenic infection and whether the infection is systemic or localized, and will
30 typically be in the range of about 1ug - 100 mg/kg body weight. Where the inhibitor is a peptide or polypeptide, it will be administered in the range of about 100 - 500 ug/mL per dose. A single dose of inhibitor or multiple doses, daily, weekly, or intermittently, is

contemplated according to the invention.

The route of administration will be chosen by the physician, and may be topical, oral, transdermal, nasal, rectal, intravenous, intramuscular, or subcutaneous.

In a further embodiment, the compounds of the present invention can be used as lead compounds to improve the antifungal activity of the compounds. This can be done by modifying certain functional groups of the compounds of the present invention based upon a recognition of the structure/activity relationship between a particular functional group in a compound and its biological activity. Such modifications include synthetic manipulation of the size, hydrophilicity, hydrophobicity, acidity and basicity of a functional group, which may inhibit or enhance the activity of a compound.

The following examples are intended as non-limiting illustration of the present invention.

15 **EXAMPLES**

Plasmids and Strains Used

	<u>Strain</u>	<u>Genotype</u>	
	ART68-1	<i>S. cerevisiae cdc68-1 leu2-3,112 ura3-52 ade</i>	
20	BL21(DE3)pLysS	<i>E. coli F - ompT hsdSB(r-Bm-B) gal dcm (DE3) pLysS</i>	(Novagen)
	BM390	<i>S. cerevisiae leu2-3,112 ura3-52 ade</i>	
	BL21(DE3)pLysS	<i>E. coli ompT hsdSB(r-Bm-B) gal dcm (DE3) pLysS</i>	(Novagen)
	CAI-4	<i>C. albicans ura3::limm434 ura3::limm434</i>	
	CUY106	<i>S. cerevisiae Ace::ROX1 Ace::</i>	
25		<i>UBR1 DSLF1 his3D200 leu2-3,112 ura3-52</i>	
	CUY106::CDC68	<i>CUY106 HIS3::ABN1::UBR1::CDC68</i>	
	YEB101	<i>CAI-4 CDC68 cdc68::hisG-CaURA3-hisG</i>	
	YEB103	<i>CAI-4 CDC68 cdc68::CaURA3</i>	
	YEB104	<i>CAI-4 CDC68 cdc68::hisG</i>	
30			
	<u>Plasmid</u>	<u>Description</u>	
	pET14	<i>E. coli</i> expression vector for N-His6 tagged proteins	(Novagen)
	pET23	<i>E. coli</i> expression vector for C-His6 tagged proteins	(Novagen)
	pEB201	pET14 with <i>ScCDC68</i>	
35	pEB203	pET23 with <i>ScCDC68</i>	
	pEB207	pBCSK with <i>CaCDC68::HisG-CaURA3-HisG</i>	
	pEB209	pET23 with <i>CaCDC68</i>	
	pEB211	pKS with <i>CaCDC68::CaURA3</i>	
	pEB214	pET14 with <i>CaCDC68</i>	
40	pEB225	pKS + 2 PCR amplified products, one of which is <i>NcCDC68</i>	

pEB227 pKS + PCR amplified *AnCDC68*

pKS, pSK, pBCSK Standard cloning vectors Standard cloning vector (Stratagene)

pWJ68-1 pRS202 + *C. albicans* genomic fragment containing *CaCDC68*

5 EXAMPLE 1

Isolation and Characterization of the *C. albicans* *CDC68* Gene

The approach used to clone the *C. albicans* homolog of *CDC68* involved genetic complementation of a *S. cerevisiae* strain, ART68-1, containing a *cdc68-1* temperature sensitive mutation (Rowley *et al.* 1991). This strain carries a temperature sensitive mutation that is unable to grow at 37°C and grows normally at 30°C. A library of genomic *C. albicans* sequences was introduced into this strain and transformants were selected by growing at the restrictive 37°C temperature. Plasmids were isolated from transformants and transformed again into the temperature sensitive *S. cerevisiae* ART68-1 strain to confirm their ability to rescue this at 37°C.

The nucleotide sequence of the insert of one of the clones, pWJ68-1, was determined, SEQ ID NO: 1 (see also Figure 1) and found to contain a single ORF of which the predicted amino acid sequence, SEQ ID NO: 2 (see also Figure 1) showed homology to Cdc68 proteins from various organisms, some of which can be found in Genebank. These include: *H. sapiens*, [SEQ ID NO: 3]; *Drosophila melanogaster*, Genebank accession number AF023270, [SEQ ID NO: 4]; *Saccharomyces cerevisiae*, Genebank accession number M73533, [SEQ ID NO: 5]; and *Kluyveromyces lactis*, Genebank accession number U48701, [SEQ ID NO: 6] (see also Figure 2). This indicated that the isolated gene encodes CaCdc68p.

25 EXAMPLE 2

Presence of *CDC68* Genes in Other Fungi: Potential of Cdc68p as a Broad Range Antifungal Target

Based on sequence alignments of the predicted amino acid sequences of Cdc68p of *C. albicans*, SEQ ID NO: 2; *S. cerevisiae* SEQ ID NO: 5; and *K. lactis*, SEQ ID NO: 6, degenerate oligonucleotides were designed with 5'*Bam*HI restriction sites and used for amplification of similar sequences from genomic DNA from two widely different fungi, *Aspergillus nidulans* and *Neurospora crassa*. In both cases, products of the expected size were cloned in pKS using the *Bam*HI sites, resulting in plasmids pEB227

and pEB225 respectively, and sequenced. The predicted amino acid sequences encoded by these products *A. nidulans*, SEQ ID NO: 7 and *N. crassa*, SEQ ID NO: 8 were compared to similar regions in Cdc68 proteins from the fungi and higher eukaryotes, namely, human, SEQ ID NO: 9; *Drosophila melanogaster*, SEQ ID NO: 10; *S. cerevisiae*, SEQ ID NO: 11; 5 *Kluyveromyces lactis*, SEQ ID NO: 12 and *C. albicans* SEQ ID NO: 13 (see Figure 3). From this comparison it is clear that in this part of the proteins, and very likely therefore also in other parts of the proteins, there are sequence features that are present in fungi but absent from higher eukaryotes. Compounds active against such parts of the protein could therefore act as broad range antifungal compounds.

10

EXAMPLE 3

Cdc68p of *S. cerevisiae* is required for cell viability

Although Cdc68p has been shown to be required for vegetative growth of spores, it was not clear whether Cdc68p was required for the sporulation process itself and 15 whether absence of Cdc68p results in lack of growth in otherwise viable cells. To this end, cells of both *S. cerevisiae cdc68-1* mutant strain (ART68-1) and wild type strain BM390 (control) were pregrown at the permissive temperature (30°C) and incubated at either the permissive temperature (30°C) or the restrictive temperature (37°C). Optical density (OD650) and CFU/mL were followed and the viability of the cultures (CFU/OD650) was 20 calculated. Figure 4 shows that very quickly after the temperature shift to 37°C, viability of the ART68-1 cells dropped and reached a 10 million-fold decrease after 52 hours. There was no temperature related growth effect on the wild type BM390 strain.

A similar experiment as that carried out above was performed using metal-inducible repression of transcription of *CDC68* combined with a simultaneous, metal- 25 inducible degradation of Cdc68p already present in the cells. Figure 5 depicts a graphic representation of viability of a *S. cerevisiae* CUY106::CDC68 mutant and a *S. cerevisiae* CUY106 wild type strains upon metal inducible depletion of Cdc68p present in the cells. The cells were grown until exponential phase, after which they were incubated with 1mM CuSO₄. Optical density (OD650) and CFU/mL were followed from which viability of 30 cultures (CFU/OD650) was calculated. One hour after incubation in the presence of CuSO₄, viability started to drop and reached a 100,000-fold reduction after 24 hours (Figure 5). This decrease parallels the reduction in viability found with the temperature

sensitive ART68-1 strain after 24 hours (Figure 4).

EXAMPLE 4

Cdc68p of *C. albicans* is required for cell viability.

5 *C. albicans* is a diploid fungus which, largely due to the absence of a sexual phase in its life cycle, is resistant to a considerable number of genetic techniques that are applicable to *S. cerevisiae*. In order to assess whether Cdc68p is essential in *C. albicans*, we used plasmids pEB207 and pEB211, to construct a double *CaCDC68* disruptant strain according to the rationale shown in Figure 6. This is a common technique used in genetic manipulation and screening in *C. albicans*. In this approach, a uridine auxotrophic strain of *C. albicans* was transformed with linearized DNA fragments containing the *CaURA3* gene (able to confer uridine prototrophy upon transformants) flanked by identical *HisG* sequences. This *HisG-CaURA3-HisG* cassette is flanked by sequences upstream of the gene of interest on one site and downstream of it on the other site. Prototrophic transformants have undergone replacement of one copy of the gene of interest with the *HisG-CaURA3-HisG* cassette. Auxotrophic, uridine requiring derivatives can be isolated by selecting for 5' fluoro-orotic acid (FOA) resistance in the presence of uridine. The *URA3* gene product converts FOA into fluorouracil which is toxic. FOA selection therefore allows one to select cells that have lost the *URA3* gene upon *cis*-recombination of the two identical *hisG* flanking regions. A second disruption plasmid was used to attempt to inactivate the second copy of the gene. The *CaURA3* gene is flanked by sequences removed from the first copy. Generation of prototrophic transformants can only occur by integration of the cassette in the non-disrupted allele and is therefore proof that the gene of interest is not essential.

25 The generation of viable transformants in the final step would thus indicate that the remaining allele of *CaCDC68* is not essential for growth.

Following the procedure outlined above and in Figure 6, the only viable transformants obtained turned out to have a second disruption plasmid inserted into the previously inactivated locus (Figures 7A and 7B). A total of 13 transformants obtained in 4 independent transformations were analyzed. None of the transformants had both *Cdc68* alleles inactivated. Since the second disruption plasmid had been designed to integrate into the intact allele, integration in the already disrupted locus can be considered an extremely

rare event. The results indicate that *CaCDC68* is essential for growth.

EXAMPLE 5

Standard Techniques for Overproduction and Isolation of Recombinant ScCdc68p and

5 CaCdc68p

CDC68 genes from *S. cerevisiae*, SEQ ID NO: 5 and *C. albicans*, SEQ ID NO: 1 were cloned into both pET14 and pET23 vectors (Novagen) and the resulting plasmids (pEB201, pEB203, pEB209, pEB214) were transformed into *E. coli* BL21(DE3) pLysS (Novagen). Expression was induced by adding IPTG (0.25 mM final concentration) to exponentially growing cultures in LB media supplemented with added antibiotics (70 ug/mL ampicillin and 34 ug/mL chloramphenicol) at room temperature. After 4-16 hours cells were harvested by centrifugation (10 min., 10000 rpm, 4°C) and resuspended in 0.1 volume of buffer (20 mM NaPi pH 7.2, 1 mM DTT, 1 mM PMSF). This was followed by sonication for 6 min on ice with pulses of 1 second after which the extract was twice centrifuged at 17000 rpm for 20 min. at 4°C. The soluble fraction was collected, brought to 0.5 M NaCl, 25 mM imidazole pH 7.2 and loaded onto a hiTrap column (Pharmacia).

Various fractions were eluted with increasing imidazole concentrations. Fractions containing Cdc68p or a truncated form of it were eluted between 100-250 mM imidazole, and the 25 mL fraction was dialyzed overnight at 4°C in 20 mM Tris HCl pH 8.0. This was loaded on to a HiTrap Q column and various fractions were eluted with increasing NaCl concentration. Cdc68p eluted between 350-500 mM NaCl. These fractions were combined and concentrated in a Centriprep-50 spin column. In order to dilute low molecular weight contaminants the concentrated Cdc68p can be diluted in 10 mM NaPi pH7.2, 5% (v/v) glycerol and concentrated again. Finally, the preparation was dialyzed into 10 mM NaPi pH 7.2, 5% (v/v) glycerol. This procedure yielded 2-3 mg Cdc68p per liter of culture with a purity of more than 90% (Figures 8A and 8B).

OTHER EMBODIMENTS

The foregoing examples demonstrate experiments performed and contemplated by the present inventors in making and carrying out the invention. It is believed that these examples include a disclosure of techniques which serve to both apprise the art of the practice of the invention and to demonstrate its usefulness. It will be

appreciated by those of skill in the art that the techniques and embodiments disclosed herein are preferred embodiments only that in general numerous equivalent methods and techniques may be employed to achieve the same result.

- All of the references identified hereinabove, are hereby expressly
- 5 incorporated herein by reference to the extent that they describe, set forth, provide a basis for or enable compositions and/or methods which may be important to the practice of one or more embodiments of the present inventions.

WHAT IS CLAIMED IS:

- 1 1. An isolated nucleic acid having the sequence depicted in Figure 1,
2 SEQ ID NO: 1.
- 1 2. An isolated nucleic acid comprising a nucleotide sequence that
2 encodes the polypeptide with SEQ ID NO:2.
- 1 3. An isolated nucleic acid that hybridizes to a nucleic acid as defined
2 in claim 1 under stringent hybridization conditions.
- 1 4. A nucleic acid vector comprising a nucleic acid as defined in claim
2 1 operably linked to a transcription regulatory element.
- 1 5. A cell comprising a vector as defined in claim 3.
- 1 6. A cell as defined in claim 4, wherein said cell is selected from a
2 group consisting of bacterial, fungal, insect, and mammalian cells.
- 1 7. A method for producing a polypeptide, which comprises:
2 (i) culturing a cell as defined in claim 5 under conditions
3 suitable for the expression of CaCdc68 polypeptide; and
4 (ii) recovering said polypeptide from said culture.
- 1 8. An isolated polypeptide having the amino acid sequence depicted in
2 Figure 1, SEQ ID NO:2.
- 1 9. An antibody that specifically recognizes CaCdc68 polypeptide.
- 1 10. A fragment of CaCdc68 polypeptide or function-conservative
2 variants of said polypeptide, said fragment or function-conservative variant being

characterized in that when expressed in a yeast cell, it rescues a temperature sensitive or knock out mutation of *Cdc68*, and fragment or fragments thereof.

11. A fragment of CaCdc68, said fragment being characterized in that when expressed in a wild type *Candida albicans* cell it prevents the growth of said cell.

12. A method for rapid, large-scale screening to identify a ligand that binds to a folded CaCdc68 protein from a plurality of test ligands not known to bind folded CaCdc68 protein, said CaCdc68 protein having been incubated in the presence and absence of a plurality of test ligands and subsequently subjected to unfolding conditions, which comprises:

detecting an increase or a decrease in the amount of CaCdc68 protein in the folded state, wherein said increase or said decrease identifies a test ligand that binds to said CaCdc68 protein.

13. A method for rapid, large-scale screening to identify a ligand that binds to a folded CaCdc68 protein from a plurality of test ligands not known to bind to said folded CaCdc68 protein, said CaCdc68 protein having been incubated in the presence and absence of a plurality of test ligands which comprises:

subjecting said CaCdc68 protein to unfolding conditions; and
detecting an increase or a decrease in the amount of CaCdc68 protein in the folded state, wherein said increase or said decrease identifies a test ligand that binds to said CaCdc68 protein.

14. A method for rapid, large-scale screening to identify a ligand that binds to a folded CaCdc68 protein from a plurality of test ligands not known to bind to a folded CaCdc68 protein which comprises:
incubating said CaCdc68 protein in the presence and absence of a plurality of test ligands;
subjecting said CaCdc68 protein to unfolding conditions; and
detecting an increase or a decrease in the amount of CaCdc68 protein in the folded state, wherein said increase or said decrease identifies a test ligand that binds to said

1 CaCdc68 protein.

1 15. A method of preventing *Candida albicans* growth in culture,
2 comprising contacting said culture with an inhibitor compound that inhibits the biological
3 activity of CaCdc68.

1 16. A method of preventing *Candida albicans* growth in a mammal,
2 comprising exposing said mammal to an inhibitor that selectively inhibits the biological
3 activity of CaCdc68.

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1 ATG TCG GAA GTT AAC ATT GAT GCA GGA TTG TTT TAT AAA CGA TTA TCA ATT TTT CAA AAA 60
1 M S E V N I D A G L F Y K R L S I F Q K 20

61 CAA TTA ACT GCC AAC AAT ATT CCT CAA GCA TTA ATT ATT GTT GGT GCT CGT AGT GAT GAT 120
21 Q L T A N N I P Q A L I I V G A R S D D 40

121 AAC ACT TAT AAA AAA TCA ACA GTT TTA CAA AAT TGG TTA TTA GGT TAT GAA TTC ATT CAT 180
41 N T Y K K S T V L Q N W L L G Y E F I H 60

181 ACA GCA ATT TAT ATC ACT GAT AAG AAA TGT ATT TTC ATT ACT TCG GAA GGG AAA TCC AAA 240
61 T A I Y I T D K K C I F I T S E G K S K 80

241 CAT TTA AAA CAT TTG ACT AAT CAG AAA CCT GAT TTG GTT GAA TTA TGG ATT AGA ACT AAA 300
81 H L K H L T N Q K P D L V E L W I R T K 100

301 GAT GTT GAA CAT AAT AAA CAA TTG TTT ATT AAA TTA TTG GAA ACA ATG ACA AAA TTA GAT 360
101 D V E H N K Q L F I K L L E T M T K L D 120

361 TCT AAA TAT GGG AAA ATT TTA AAA GAT AAA TAT GAT GGG AAA TTT ATT GAT GAA TGG AAT 420
121 S K Y G K I L K D K Y D G K F I D E W N 140

421 CAA ATT TTG AAT GAT AAC AAC AAC AAT ACC ACC AAC GAC CAC GCG TTG AGT GCT GTT GAT 480
141 Q I L N D N N N N T T N D H A L S A V D 160

481 TTG GCC GTT ACG GTT TCT CAA GCA TTA GCA GTT AAA GAT TCT GAA GAG TTT AAT AAT ACC 540
161 L A V T V S Q A L A V K D S E E F N N T 180

541 AAG ATT GCA TCT AAT GCC AGT GTA GTT ATG ATG GAT ACA TTT GTT AAT GAT ATG ATG ATT 600
181 K I A S N A S V V M M D T F V N D M M I 200

601 ATT GTT GAT GAT GAG AAA AAA ATC ACC AAT AGT CAA TTG ACT GAT CAA ATT GAA GAT AAA 660
201 I V D D E K K I T N S Q L T D Q I E D K 220

661 ATT GAA AAT AAT AAA TGG TAT TTA AAA ACT AAA TTA GGG AAA AAT TTA TTA CAA TCA ATT 720
221 I E N N K W Y L K T K L G K N L L Q S I 240

721 AAA GAT TTT GAT CCA GAA TAT CTT GAA TAT TGT TAT TCA CCG ATT ATT CAA AGT GGT GGT 780
241 K D F D P E Y L E Y C Y S P I I Q S G G 260

781 GAT TAT GAT TTA AAA CCA AGT GCT GTT TCC ACT GAT AAA CCA TTA ATT GGT GAA GGG GTC 840
261 D Y D L K P S A V S T D K P L I G E G V 280

841 ATT TTA TCA TCA ATT GGT TTA AGA TAT AAA TCT TAT TGT TCT AAT ATT GCC AGA ACA TTT 900
281 I L S S I G L R Y K S Y C S N I A R T F 300

901 TTA ATT GAT CCA ACT AGT GAA ATG GAA ACT AAT TAT GAT TTC TTA TTA CAA TTA CAA AAA 960
301 L I D P T S E M E T N Y D F L L Q L Q K 320

961 TAT ATT GTT GAT AAT TTA TTG AAA GAT GGT GTC CCG GCT AGT AAA GTT TAT CAA GAC ACG 1020
321 Y I V D N L L K D G V P A S K V Y Q D T 340

1021 ATA GAC TAT ATT AAA AAG GAA AGA CCA GAT TTA GTT AAT CAT TTC ACG AAA AAC TGT GGT 1080
341 I D Y I K K E R P D L V N H F T K N C G 360

1081 TGG TTA TTA GGT ATG GAA TTT AGA GAT CCG ACA TTT ATT TTA AAT GCT AAA ACT ACT GAT 1140
361 W L L G M E F R D S T F I L N A K T T D 380

Fig. 1 (1/3)

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1141 AGA AAA TTG ACT ACT GGA CAA ATC ATT TCT TTA ACT ATT GGA TTC AAT AAT TTA TCA AAT 1200
381 R K L T T G Q I I S L T I G F N N L S N 400

1201 GAT AAA AAT GAT AAA AAT GAT AAT AAG ACA AAT CAT CAA AAA AAT AAA CAA ACT TAT GCT 1260
401 D K N D K N D N K T N H Q K N K Q T Y A 420

1261 TTA TTA TTG ACT GAC ACA ATT AAA ATT ACT GAT GAT TCA TCA ATC TTA TTA ACC AAT TAT 1320
421 L L L T D T I K I T D D S S I L L T N Y 440

1321 TCA AAA GAT AGA GCT GCC ATT AGT TTC TCC TTC AAT GAT GAT AAT GAG ACC CAG AAG GAA 1380
441 S K D R A A I S F S F N D D N E T Q K E 460

1381 AAT AAT AAT AAC AAT AAT AAA CGA CCA GGA TTA TCC CAG ACA TCA AAC ACC ACA GGC TTG 1440
461 N N N N N N N K R P G L S Q T S N T T G L 480

1441 AAG TTA GAG TCT ACA GAA AAT ACT GCT ATT CTT AAA TCG AAA TTA CGT CAT GAA AAT ACT 1500
481 K L E S T E N T A I L K S K L R H E N T 500

1501 AAT GCT GAT GAT GCC AAT TCT GAA AAA TTA AGA CAA GAA ATT CAA ATA AAA TTA CAT GAA 1560
501 N A D D A N S E K L R Q E I Q I K L H E 520

1561 AAA AGA TTA CAA GAA GGA TTA GCA AGA TTT TCA AAA GCT GAT GCC ACT GAT GCT GAT GAT 1620
521 K R L Q E G L A R F S K A D A T D A D D 540

1621 TTT AAA CCA ATT TTT AAA AAA TAT GAA TCT TAT GTT AGA GAA TCA CAA ATC CCT AAT AGT 1680
541 F K P I F K K Y E S Y V R E S Q I P N S 560

1681 GTT AAT GAT TTA AAA ATT CAT ATT GAT TAT AAA AAT CAA ACA ATA ATT TTA CCA ATT TCT 1740
561 V N D L K I H I D Y K N Q T I I L P I S 580

1741 GGT CGT CCC GTT CCA TTT CAT ATC AAT TCT TAT AAG AGT GGA TCA CAA AAT GAA GAA GGA 1800
581 G R P V P F H I N S Y K S G S Q N E E G 600

1801 GAT TTC ACT TAT CTT CGA TTA AAT TTT AAT TCA CCA GGT GCT GGT GGG AAT GTC ACC AAA 1860
601 D F T Y L R L N F N S P G A G G N V T K 620

1861 AAA CAA GAA TTA CCT TAT GAA GAT TCC CCT GAT AAT TCA TTT TTA AGA TCA ATT ACT ATT 1920
621 K Q E L P Y E D S P D N S F L R S I T I 640

1921 CGG TCA AGA GAT CGT CAA AGA ATG GTT GAT GTT TAT AAA GCC ATT CAA GAT TTG AAA AAA 1980
641 R S R D R Q R M V D V Y K A I Q D L K K 660

1981 GAT TCT GTT AAA CGA GAA CAA GAA AAG AAA CAA ATG GCT GAT GTT ATC ACT CAA GCT AAT 2040
661 D S V K R E Q E K K Q M A D V I T Q A N 680

2041 TTA ATT GAA TTA AAA GGT TCA AGA GTT AAA AAA TTA AAT AAT GTA TTT ATT AGA CCA ACA 2100
681 L I E L K G S R V K K L N N V F I R P T 700

2101 CCT GAT ACG AAA AAA ATT GGA GGA GTT TTA CAA ATT CAT GAA AAT GGG TTA AGA TAT CAA 2160
701 P D T K K I G G V L Q I H E N G L R Y Q 720

2161 TCA CAA CCA CAA TCA CAA TCA CAA TCA AAT TTT AAA AAT GAT CAA AGA GTT GAT GTA TTA 2220
721 S Q P Q S Q S Q S N F K N D Q R V D V L 740

2221 TTT TCC AAT ATT AAA CAT TTA TTT TTC CAA CCT TGT AAA GAT GAA TTA ATT GTT TTA ATT 2280
741 F S N I K H L F F Q P C K D E L I V L I 760

Fig. 1 cont. (2/3)

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2281 CAT TGT CAT TTG AAA AAT CCA ATC ATG ATT GGT AAA AGG AAA ACT TTT GAT GTT CAA TTT 2340
761 H C H L K N P I M I G K R K T F D V Q F 780

2341 TAT CGT GAA GCT AGT GAT ATG GCA TTT GAT GAA ACT GGT GGT CGT AAA CGG AAA TAT CGT 2400
781 Y R E A S D M A F D E T G G R K R K Y R 800

2401 TAT GGA GAT GAA GAT GAA TTA CAA CAA GAA CAA GAA CGA AGA AGG AAA GCC CTC TTG 2460
801 Y G D E D E L Q Q E Q E E R R R K A L L 820

2461 GAT AAA GAA TTT AAA GGA TTT GCT GAA TTA ATT GCT GAT TCT TCT CAT GGT ATG GTT GAT 2520
821 D K E F K G F A E L I A D S S H G M V D 840

2521 TTA GAT ATT CCA TTC CGA GAA TTA GGA TTC CAA GGG GTT CCA TTT AGA TCC TCA GTA TTA 2580
841 L D I P F R E L G F Q G V P F R S S V L 860

2581 TGT GTT CCT ACA AGA GAT TGT CTT GTT CAA TTA ATT GAT CCA CCT TAT TTA GTC GTC ACA 2640
861 C V P T R D C L V Q L I D P P Y L V V T 880

2641 CTA GAA GAA ATT GAA ATT GCT CAT TTA GAA AGA GTT CAA TTT GGG TTA AAA AAT TTC GAT 2700
881 L E E I E I A H L E R V Q F G L K N F D 900

2701 TTA GTG TTT GTT TTC AAA GAT TTT AAT AAA CCC GTG GTT CAT ATT AAT ACT ATC CCC GTG 2760
901 L V F V F K D F N K P V V H I N T I P V 920

2761 GAA TTA TTA GAA GAT GTT AAA TCT TGG TTA ACT GAT GTT GAT ATT CCT ATA AGT GAA GGA 2820
921 E L L E D V K S W L T D V D I P I S E G 940

2821 CAA ATG AAT TTA AAT TGG GTA CAA ATT ATG AAA ACT GTA TTG GCA GAT CCT TAT CAA TTT 2880
941 Q M N L N W V Q I M K T V L A D P Y Q F 960

2881 TTC ATT GAT GGT GGT TGG GCA TTT TTA ACT GGT CAA GGT GAA AGT GAT GAA GAA GAA GAA 2940
961 F I D G G W A F L T G Q G E S D E E E E 980

2941 AGT GAT GAA GAA AGT GAT TTC CGT GTT AGT GAT GAA GAT CCT CAA GAT GAA GAT GAA GAA 3000
981 S D E E S D F R V S D E D P Q D E D E E 1000

3001 AGT GAT GAT TAT GCT AGT GAA GAA GAA AGT GAT GAT TAT TCT GGA AGT GAT GAT GAT GGT 3060
1001 S D D Y A S E E E S D D Y S G S D D D G 1020

3061 AGT GGT GGT GGT GAT GAT GAT GAT TCA GAA AGT GGA GAA GAT TGG GAT GCA TTA GAA AGA 3120
1021 S G G G D D D D S E S G E D W D A L E R 1040

3121 AAA GCT GCT AAA GCC GAT AGA AAT TCT GGT TTT GAT TAA 3159
1041 K A A K A D R N S G F D * 1053

Fig. 1 cont. (3/3)

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MSE--VNIDFDAFYRXXXL-----XKXYXE-EGSPN L- Majority

Fig. 2 (1/6)

Alignment Report of Cdc68p alignment, using the Hein method with PAM250 residue weight table.
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I E N K I D D K K - Y L K S L S - D L - - - - - K F D P X L L L A Y S Majority									
250 260 270 280									
221	V E K A A E E K K	- Y L A - G A - - - - - D P E T Y E M C Y P	Cdc68-H.sapiens						
183	C A A A G E K K	- Y T S - G L - - - - - E P R L L D M A Y P	Cdc68-D.melanogaster						
221	I E N K I D D V K	- F E K - Q L S P D L S A L C P P N Y K F N F D L L D W T Y S	Cdc68-S.cerevisiae						
217	I E D K I E N N K	W Y L K T K L G K N L L Q - - - S I K D F D E E Y L E Y C Y S	Cdc68-C.albicans						
221	I E N K I D D S K	- F E K - K L S S D L N P L C P T D E K F E V N F L D W A Y S	Cdc68-K.lactis						
P I I Q S G G K Y D L K V S A V S T N D P L H G F G V I L A S C G I R Y K S Y C Majority									
290 300 310 320									
245	P I I Q S G G N Y N I K F S	V Y E D K N H M H - F G A I T C A M E I R F E S Y C	Cdc68-H.sapiens						
207	P I I Q S G G A Y S I K F S	A V A D K N P L H - F G V I V C S L G I A R Y K S Y C	Cdc68-D.melanogaster						
259	P I I Q S G G K F D L R V S	A R S T N D Q L Y G N G C I L A S C G I E Y N N Y C	Cdc68-S.cerevisiae						
254	P I I Q S G G D Y D I K F S	A M S T D K P L I G E G V I L S S I S I R Y K S Y C	Cdc68-C.albicans						
259	P E V D S G S K F D L R V S	A R S N N D S L F I G K E S I L A S C G I E Y K N Y C	Cdc68-K.lactis						
S N I T R T F L I D P T E E M Q E N Y D F L L Q L Q E E I V D N L L K P G V K P Majority									
330 340 350 360									
284	S N I V R T L N W D P S Q E Y	G E N Y N F L L Q L Q E E I L - K E L R H G V K I	Cdc68-H.sapiens						
246	S N I S E T F L V N F T E A M	Q E N Y T F L V S V Q E E I L - K L L V P G T K L	Cdc68-D.melanogaster						
299	S N I T R T F L I D P S E E M	A N N Y D F L L T L Q E I V T N I I K P E R T P	Cdc68-S.cerevisiae						
294	S N I A R T F L I D P T S E M	E T N Y D F L L Q L Q E I V D N L K D E Y P A	Cdc68-C.albicans						
299	S N I T R T F L I D P T D E M	T D N Y D F L L I L Q E K I I D D L K V E A D P	Cdc68-K.lactis						
C D V Y E K T L D F I K K E K P E L V N N F T K N X G S L M G L E F R D S S F V Majority									
370 380 390 400									
323	C D V Y E K T L D F V F K K E	K P S M V D N V P K S F G F A M G L E F R E N S I V	Cdc68-H.sapiens						
285	C D V Y E K T L D F V F K K E	K P S M V D N V P K S F G F A M G L E F R E N S I V	Cdc68-D.melanogaster						
339	K E V Y E S W I E Y I E K T K	P E L V P N F T K N I G S L I G L E F F D S N F I	Cdc68-S.cerevisiae						
334	S K V Y Q D T I D Y I K K E R	P D L V T H F T K N C Q W L L G M E F F D L T F I	Cdc68-C.albicans						
339	T S I Y E K T L E F I K E K K	P E L L S H F T K N V G S L M G L E F F D S A G M	Cdc68-K.lactis						
I N A K N T A R K L K K G M V F N I S L G F S N L T N - - - S K E G K N - - - Majority									
410 420 430 440									
363	I N E K N O Y - Y L K K G M V F S	I N I G E S L L T N K E G K K P E E K T - - -	Cdc68-H.sapiens						
325	I G P K C O A - L I K K N M Y F	N L H V C I S N L T N P E A T D K E G K N - - -	Cdc68-D.melanogaster						
379	L N V K N D Y R K I O R E D C E	N S F G F N N L K D - - - S O S A N N - - -	Cdc68-S.cerevisiae						
374	L N A K T T D R K I T T G Q I I	S L T I G F N N L S N - - - D K N D K N D N K	Cdc68-C.albicans						
379	I N A K P T A H K I S E N C C Y N	T S L G F G N L K D - - - S K T G Q V - - -	Cdc68-K.lactis						
- - - - - Y A L Q L G D T V L - - V D E D G P A S V L T N Y T K A R S N Majority									
450 460 470 480									
399	- - - - - Y A L F I G D T V L - -	V D E D G P A T V L T S V K K K V K N	Cdc68-H.sapiens						
361	- - - - - Y A L F I G D T V L - -	V G E Q S P A S V M T P S K K K I K N	Cdc68-D.melanogaster						
412	- - - - - Y A L Q L A D T V O I P L E	T E P R F L T N Y T K A K S O	Cdc68-S.cerevisiae						
410	T N H Q K N K Q T Y A L L L T D	T I K - - I T D D S - S I L L T N Y S K D R A A	Cdc68-C.albicans						
412	- - - - - Y A V O L A R T V O - -	L S S D G K F S T L T K Y T K A R S O	Cdc68-K.lactis						

Fig. 2 (2/6)
continued

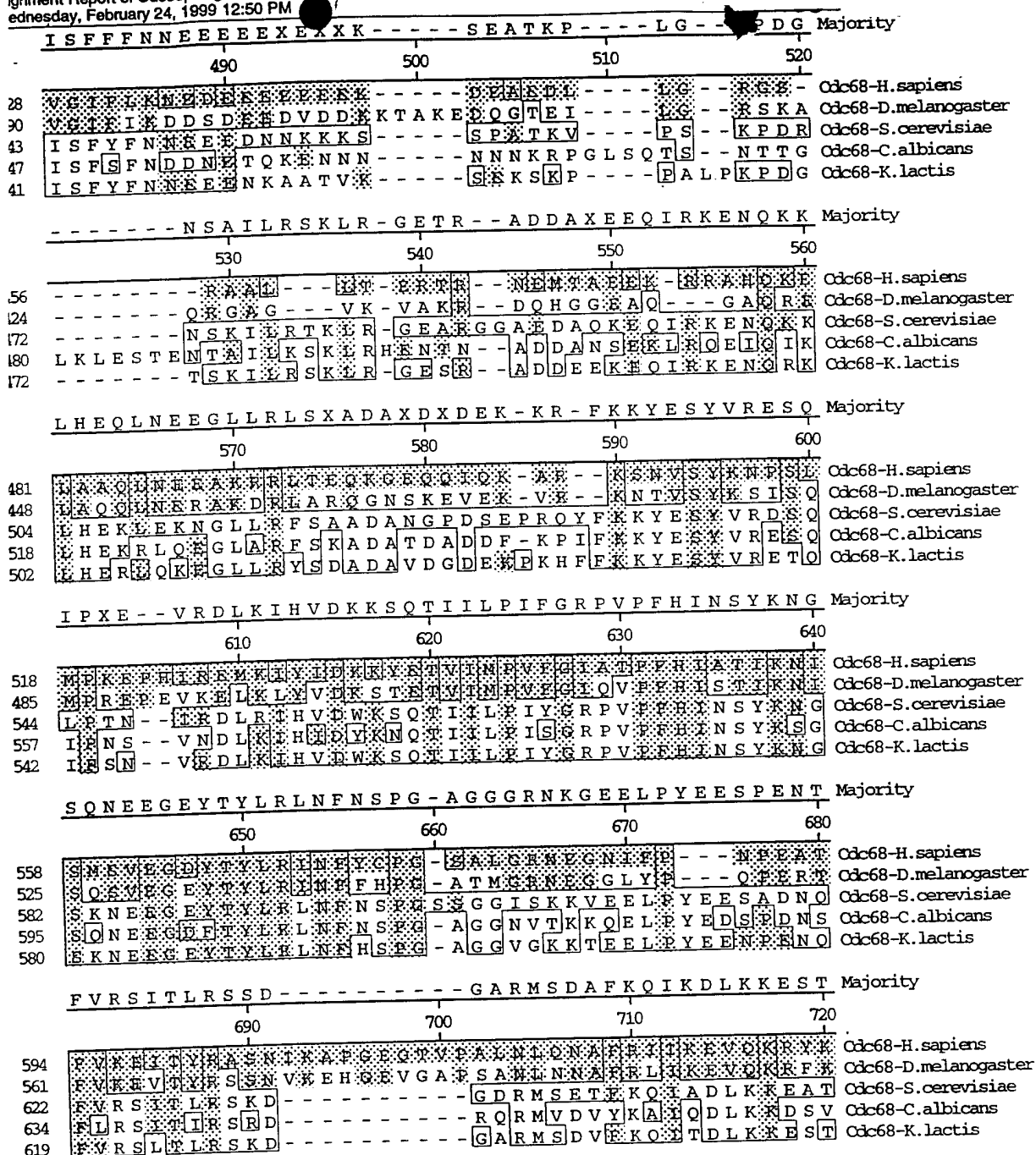


Fig. 2 (3/6)
continued

ignment Report of Cdc68p alignment, using the Hejn method with PAM250 residue weight table.
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ednesday, February 24, 1999 12:50 PM

K R E Q E E K A L A D V V R Q D K L I E N K T G R T K K L D D L F I R K P D T Majority									
730 740 750 760									
34	T T E A E S K E E F C F M K O E L I V I N I M R S N P K L K D L Y I R P N I A C Odc68-H.sapiens								
01	T T E A E S K E E F C F M K O E L I V I N I M R S N P K L K D L Y I R P N I A C Odc68-D.melanogaster								
52	K R E Q E E K A L A D V V R Q D K L I E N K T G R T K K L D D L F I R K P D T Odc68-S.cerevisiae								
64	K R E Q E E K A L A D V V R Q D K L I E N K T G R T K K L D D L F I R K P D T Odc68-C.albicans								
49	K R E Q E E K A L A D V V R Q D K L I E N K T G R T K K L D D L F I R K P D T Odc68-K.lactis								
K R V P G S L E I H E N G I R Y Q S - - - - - V L R T D S R V D I L F S N Majority									
770 780 790 800									
574	K R M O G E L E A H V N G R F F T S - - - - - V - - R G D K V D I L Y N N Odc68-H.sapiens								
341	K R M T G S L E A H S N G R F Y I S - - - - - V - - R G D K V D I L Y N N Odc68-D.melanogaster								
592	K R V P S T V F I H E N G I R Y Q S - - - - - P L R T D S R I D I L F S N Odc68-S.cerevisiae								
704	K K I G G V L Q I H E N G L E Y Q S Q P Q S Q S Q S N F K N D Q R V D I L F S N Odc68-C.albicans								
689	K R V P S T V F I H E N G I R Y Q S - - - - - P L R T D S R I D I L F S N Odc68-K.lactis								
I K N L F F O P C K G E L I V V I H F H L K N P I M F G K K K I O D V Q F Y R E Majority									
810 820 830 840									
704	T K H A L F O P C D G E M I L V I H F H L K N P I M F G K K K I O D V Q F Y R E Odc68-H.sapiens								
671	T K S A F F O P C D G E M I L V I H F H L K N P I M F G K K K I O D V Q F Y R E Odc68-D.melanogaster								
724	T K N L I F O S C K G E L I V V I H F H L K N P I M F G K K K I O D V Q F Y R E Odc68-S.cerevisiae								
744	T K N L F F O P C K D E L I V L I H C H L K N P I M I G K R K T E D V Q F Y R E Odc68-C.albicans								
721	T K N L F F O S S K G E L I V I I H V H L K N P I L M G E K K I O D V Q F Y R E Odc68-K.lactis								
A S D M A V D E T G G Q H R H D - - - - R Y G D E D E L E Q E Q E E R R K R A A Majority									
850 860 870 880									
744	V G E I T T D L G K H Q H M H D - - - - D D L Y A E Q M E R E M R H K Odc68-H.sapiens								
711	V G E I T T D L G K H Q H M H D - - - - D D L A A E Q A E E L R H K Odc68-D.melanogaster								
764	A S D M S V D E T G G R G Q S R F R Y G D E D E L E Q E Q E E R R K R A A Odc68-S.cerevisiae								
784	A S D M A F D E T G G R K R K Y - - - - R Y G D E D E L O Q E Q E E R R K R A L Odc68-C.albicans								
761	A S D M A V D E T G N S R R N N M K F R R Y G D E D E L E Q E Q E E R R K R A A Odc68-K.lactis								
L D K E F K Y F A E A I A E A S K G L L D V D X P F R D L G F Q G V P F R S A V Majority									
890 900 910 920									
776	L K T A F K N F I E K V E A L T K E N I E F F V P F R D L G F Q G V P F R S A V Odc68-H.sapiens								
743	L K T A F K S F C E K V E T M T K S V V F E D T F F R E L G F Q G V P F R S T V Odc68-D.melanogaster								
804	L D K E F K Y F A D A I A E A S N G L I T V E N T F R D L G F Q G V P F R S A V Odc68-S.cerevisiae								
820	L D K E F K G F A E L I A D S S H G M V D L D I F F R E L G F Q G V P F R S S V Odc68-C.albicans								
801	L D K E F R Y E A E A I A E A S D G L L D V D S P F R D L G F Q G V P S R E A V Odc68-K.lactis								
L C Q P T S D C L V Q L I E - P P F L V I T L D E V E I C H L E R V Q F G L K N Majority									
930 940 950 960									
816	L L Q P T S S A L V N A T E W P P F - V Y T L D E V E L L H F E R V D P H L K M Odc68-H.sapiens								
783	T L Q P T S G S L V N L T E W P P F - V I T L D E V L V H F E R V D P H L R N Odc68-D.melanogaster								
844	F C M P T T D C L V Q L I E - P P E L V I N I E E V E I C I L E R V Q F G L K N Odc68-S.cerevisiae								
860	F C V P T R D C L V Q L I D - P P Y L V Y T L E E I E I A H L E R V Q F G L K N Odc68-C.albicans								
841	F C M P T R D C L I O L V E - P P E L V I N I N E V E I C I L E R V Q F G L K N Odc68-K.lactis								

Fig. 2 (4/6)
Continued

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alignment Report of Cdc68p alignment, using
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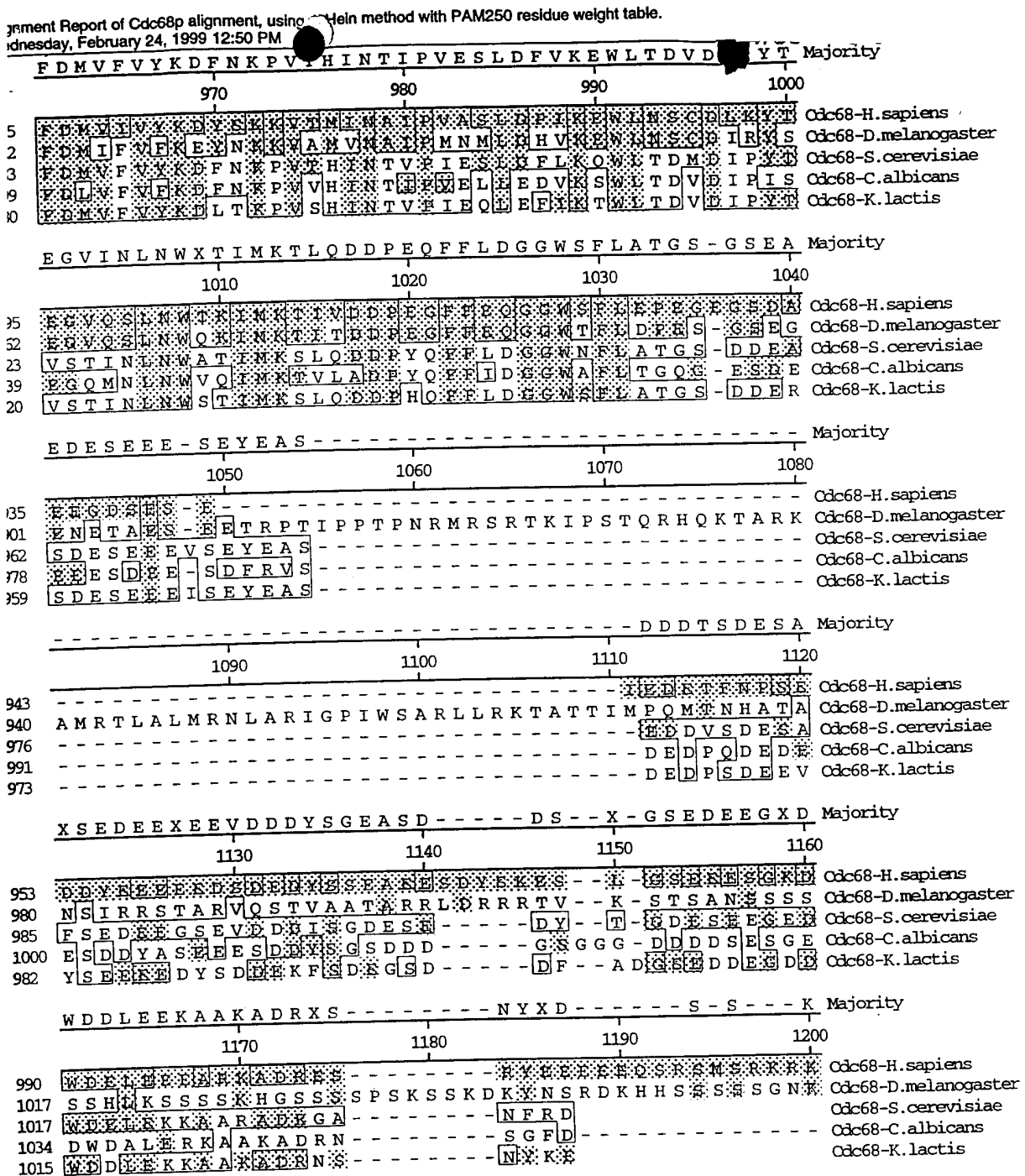


Fig. 2 (5/6)
continued

Alignment Report of Cdc68p alignment, using the Hein method with PAM250 residue weight table,
Wednesday, February 24, 1999 12:50 PM

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- S - - - - R - - - R - - - S R - - - - K - - R - -
      1210              1220
022 A E V H S E G R G S N P - G S F H S S A P P K K K K K
057 I E S K D K D R K R S E D D S E D N G H K S K N A R H
.035
.053 - - - - -
.033

```

Majority

Cdc68-H.sapiens
Cdc68-D.melanogaster
Cdc68-S.cerevisiae
Cdc68-C.albicans
Cdc68-K.lactis

Decoration 'Decoration #1': Shade (with black at 25% fill) residues that match Cdc68-H.sapiens exactly.

Decoration 'Decoration #2': Box residues that match Cdc68-S.cerevisiae exactly.

Fig. 2 (8/6)
continued

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V - PEHINTIKNGSKS EGEYTYLRINFLSPG - AGGGK 6 Majority

		10	20	30	40	
T	-	P F H T A T I K N L S M E V E G D I T Y L R L N F P Y C P G	-	S A L G G N E E G	Cdc68-PCR-H.sapiens	
V	-	P F H I S T K K L Q S V E G E X T Y L R L N F F H P G	-	A T M E R N E E G	Cdc68-PCR-D.melanogaster	
V	-	P F H I N S Y K N G S K N E E G E X T Y L R L N F N S P G	S S	G G I S K K V	Cdc68-PCR-S.cerevisiae	
V	-	P F H I N S Y K N G S K N E E G E X T Y L R L N F H S P G	-	A G G V G K K T	Cdc68-PCR-K.lactis	
V	-	P F H I N S Y K S G S Q N E E G D F T Y L R L N F N S P G	-	A G G N V T K K	Cdc68-PCR-C.albicans	
V	-	P F H I V N T I K N A S K S D E G E W A L L R I N F L S P G	-	Q G V G R K D D	Cdc68-PCR-A.nidulans	
P	V	E F H I N T I K N A S K S D E G E W S F L R I N F L S P G	-	Q G V G R K D E	Cdc68-PCR-N.crassa	
Q E L P - - - S P E A T F V R S L T L R S K D - - - - - G D R M A D V					Majority	
		50	60	70	80	
9	N	I F P - - - N P E A T F V K E L I T Y R A S M T K A P G E Q T V F A L N L Q N A	Cdc68-PCR-H.sapiens			
9	G	L Y P - - - Q P E R T F V K K V I T Y R S S N V K E H G E V G A P S A N I N N A	Cdc68-PCR-D.melanogaster			
0	E	E L P Y E E S A D N Q F V R S I T L R S K D - - - - - G D R M S E T	Cdc68-PCR-S.cerevisiae			
9	E	E L P Y E E N P E E N O F V R S I T L R S K D - - - - - G A R M S D V	Cdc68-PCR-K.lactis			
9	Q	E L P Y E D S P D N S F L R S I T L R S K D - - - - - R Q R M V D V	Cdc68-PCR-C.albicans			
9	Q	P F E - - - D L S A H F L R N L T L R S K D - - - - - N D R F A Q V	Cdc68-PCR-A.nidulans			
0	Q	P F E - - - D A S A H F V R S L M F K S T D - - - - - G D R Y A D I	Cdc68-PCR-N.crassa			
F K Q I T D L K K D A V K R E Q E K K E M E D V V E Q D K L V E N K N R R T K K					Majority	
		90	100	110	120	
76	F	R L I K E V Q R E Y K T R E A E R K E E E G I V K Q D S L V I A S N E E N P K	Cdc68-PCR-H.sapiens			
76	F	R L I K E V Q R E R F K T R E A E R E E E E D L V K Q D T L I L S Q N K G N P K	Cdc68-PCR-D.melanogaster			
70	F	K Q I A D L K K E A T K R E Q E R K A L A D V V Q Q K I E N K T G R T K R	Cdc68-PCR-S.cerevisiae			
69	F	K Q I T D L K K E S T K R E Q E R K A L A D V V V O A K L V E N K T G R T K R	Cdc68-PCR-K.lactis			
69	Y	K A Q Q D L K K D S V K R E Q E K K O M A D V I T O A N L I E L K G S R V K K	Cdc68-PCR-C.albicans			
66	A	Q D I T E L R K N A L R R E Q E K K E M E D V V E Q D K L V E I R N R R P V K	Cdc68-PCR-A.nidulans			
67	A	N Q S N L K R D A V K K E Q E K K D M E D V V E Q D K L V E I R N R R P A V	Cdc68-PCR-N.crassa			
L D D V F I R P N P D T K R V P G S V E I H E N G I R Y Q S - - - - - P L					Majority	
		130	140	150	160	
116	L	D N V F I R P N I A Q E H M O G S L E A R Y M E F R F Y S - - - - - V -	Cdc68-PCR-H.sapiens			
116	L	D N V F I R P N I V T E R M T G S L E A R S M G F R Y I - - - - - V -	Cdc68-PCR-D.melanogaster			
110	L	D Q I F V E E N P D T E R V P S T V F I H E N G I R Y Q S - - - - - P L	Cdc68-PCR-S.cerevisiae			
109	L	D Q I F V E E S P D T E R V P S T V F I H E N G I R Y Q S - - - - - P L	Cdc68-PCR-K.lactis			
109	I	N N V F I R P T P D T K K I G G V E Q I H E N G L R Y Q S Q P Q S Q S Q S N F	Cdc68-PCR-C.albicans			
106	L	P D V F L R P L D G K R V P G E V E I H Q N G L R Y V S - - - - - P F	Cdc68-PCR-A.nidulans			
107	L	D N V F I R P A M E G K K V P G K V E I H Q N G L R Y Q S - - - - - P L	Cdc68-PCR-N.crassa			
R T D S R V D I L F S N I K H L F F Q P C K G E L I V L I H V H L K N P I M I G					Majority	
		170	180	190	200	
147	-	H G D K V D I L Y N N I K H A L F O P C D G E M L I V L I H F H L K N A I M F G	Cdc68-PCR-H.sapiens			
147	-	H G D K V D I L Y N N I K S A F T O P C D G E M L I L L H F H L K Y A I M F G	Cdc68-PCR-D.melanogaster			
142	R	T D S R I D I L F S N I K N L I F O S S K G E L I V I I H I H L K N P I L M G	Cdc68-PCR-S.cerevisiae			
141	R	T D S R I D I L F S N I K N L F F O S S K G E L I V I I H I H L K N P I L M G	Cdc68-PCR-K.lactis			
149	K	N D Q R V D I L F S N I K N L F F O C K G E L I V L I H C H L K N P I M I G	Cdc68-PCR-C.albicans			
138	R	N E H - V D I L F S N V K H L F F O C A H E L V L I H V H L T P I M I G	Cdc68-PCR-A.nidulans			
139	S	T T Q R V D I L F S N V R H L F F O C Q H E L V I I H I H L T P I - - - -	Cdc68-PCR-N.crassa			

Fig 3. $C(1/2)$

Alignment Report of Cdc68-PCR, using J. P. method with PAM250 residue weight table.
Wednesday, February 24, 1999 12:57 PM

K K K I Q D V Q F Y R E A S P M A V D E T G G Q R R K D - - - - R Y G D E Majority																			
210					220					230									
186	K	K	K	H	T	D	V	Q	F	Y	R	E	A	S	P	M	A	V	D
186	K	K	K	H	V	D	V	Q	F	Y	R	E	A	S	P	M	A	V	D
182	K	K	K	I	Q	D	V	Q	F	Y	R	E	A	S	D	M	S	V	E
181	K	K	K	I	Q	D	V	Q	F	Y	R	E	A	S	D	M	A	V	D
189	K	K	K	T	F	D	V	Q	F	Y	R	E	A	S	D	M	A	F	D
177	K	R	K	T	R	D	I	Q	F	Y	R	E	A	T	E	M	Q	F	D
175	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	S	P

Cdc68-PCR-H.sapiens
Cdc68-PCR-D.melanogaster
Cdc68-PCR-S.cerevisiae
Cdc68-PCR-K.lactis
Cdc68-PCR-C.albicans
Cdc68-PCR-A.nidulans
Cdc68-PCR-N.crassa

Decoration 'Decoration #1': Shade (with black at 25% fill) residues that match Cdc68-PCR-H.sapiens exactly.

Decoration 'Decoration #2': Box residues that match Cdc68-PCR-S.cerevisiae exactly.

Fig 3 cont. (2/2)

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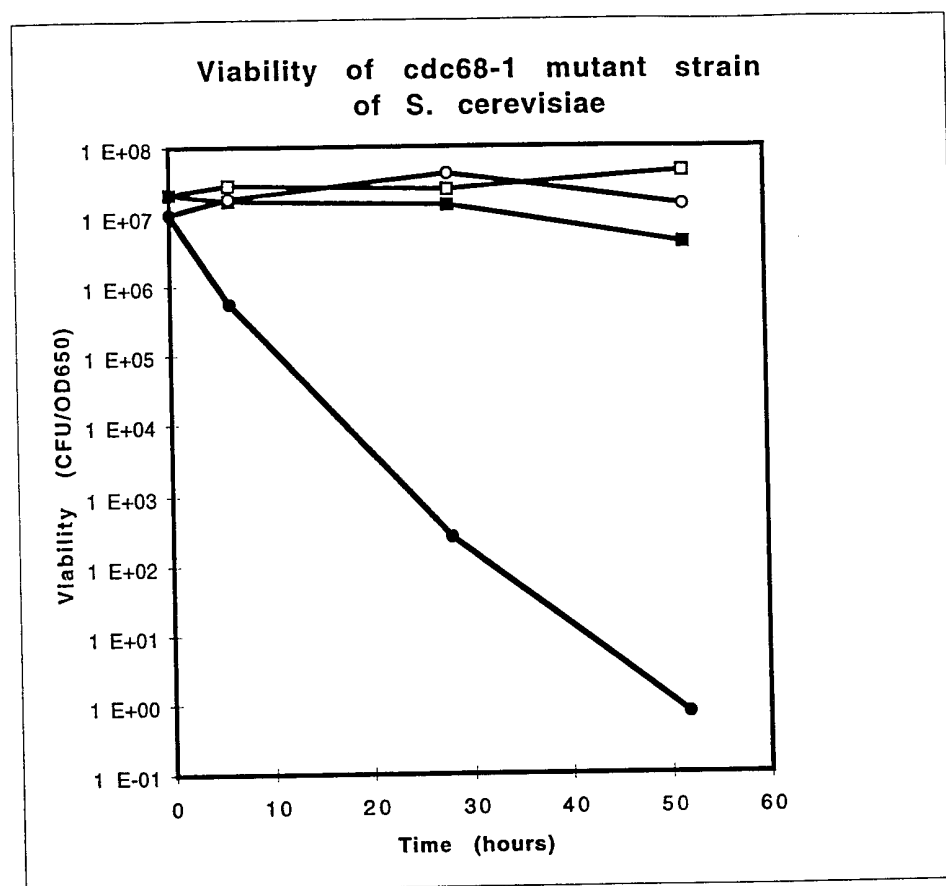


Fig. 4

**Viability of *S. cerevisiae* upon
metal-inducible depletion of Cdc68p**

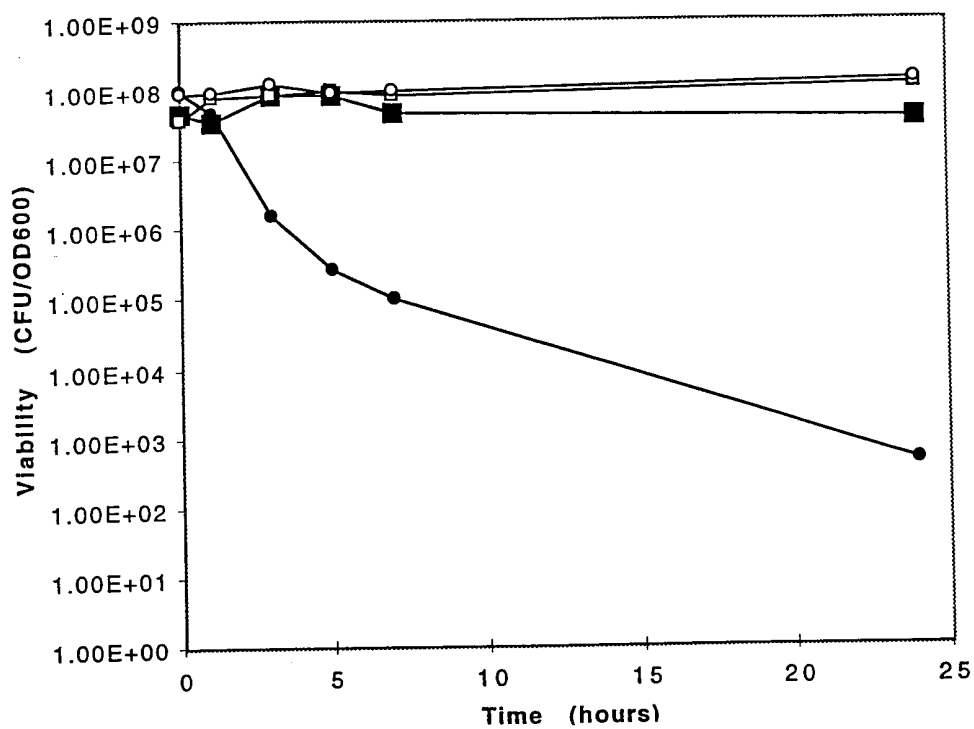


Fig. 5

Gene disruption in *C. albicans*

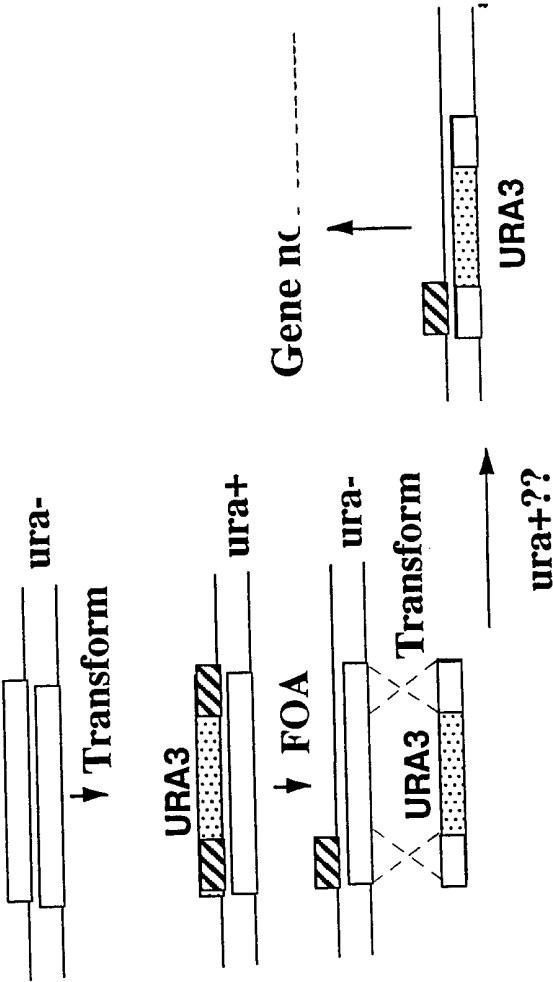


Fig. 6

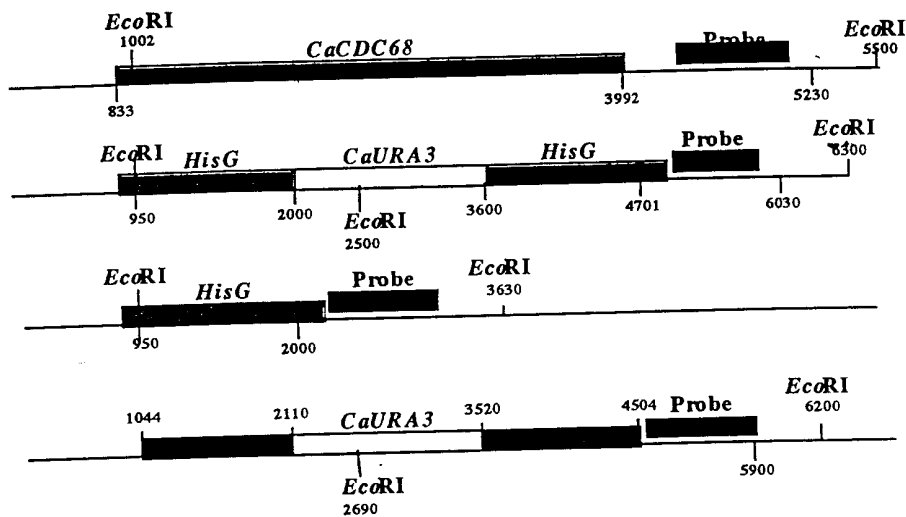


Fig. 7A

Disruption of *CaCDC68*

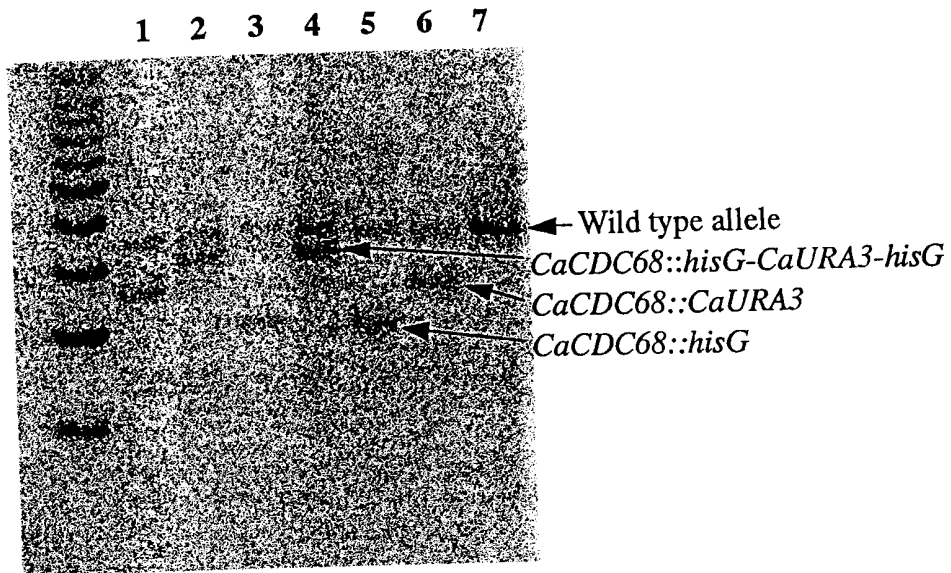


Fig. 7B

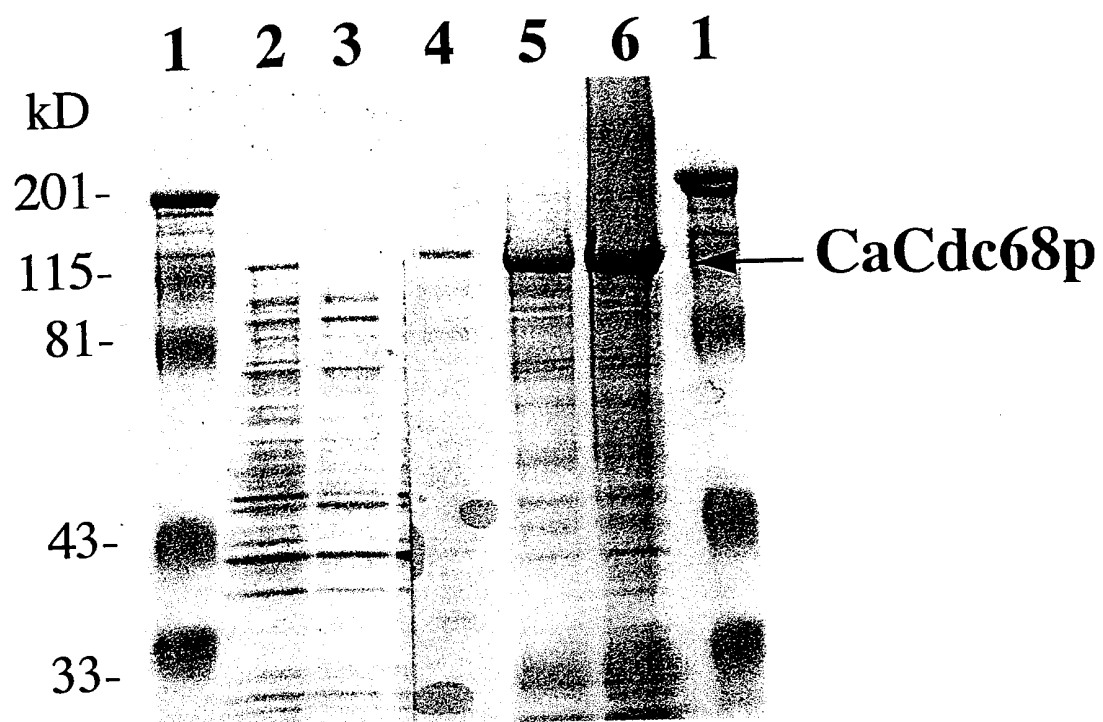


Fig. 8A

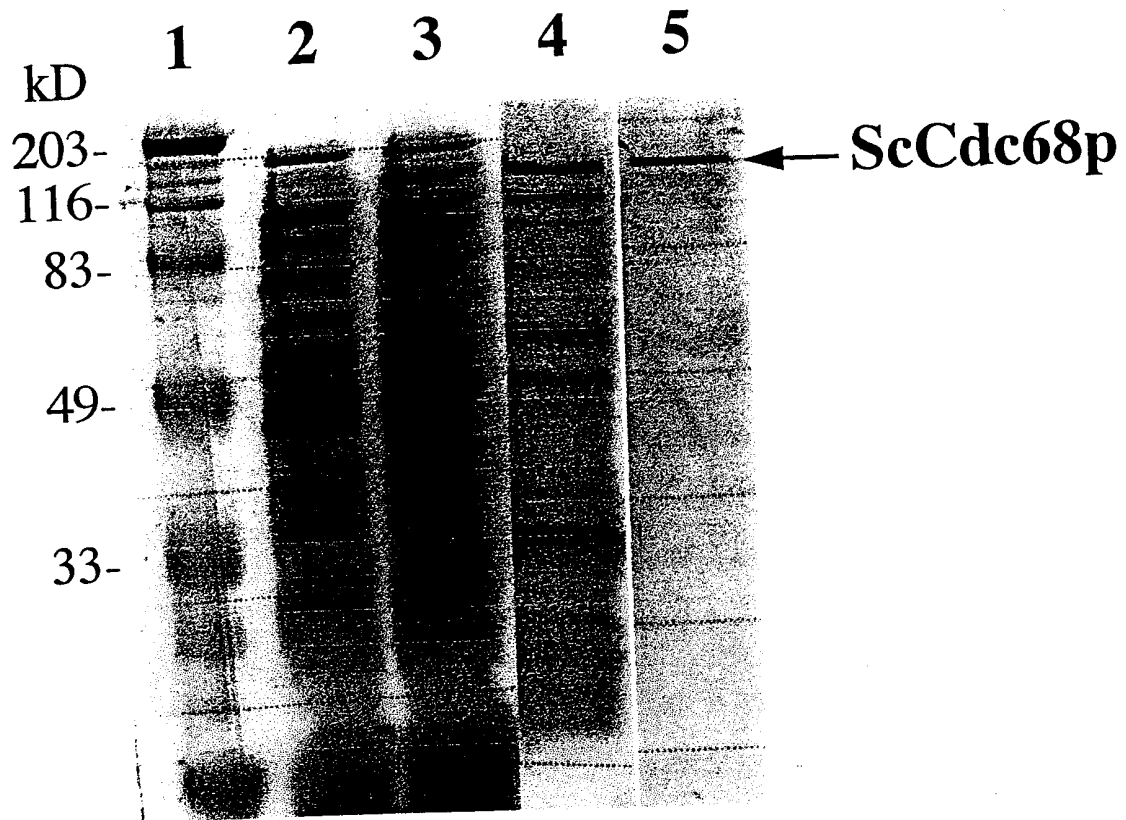


Fig. 8B

INTERNATIONAL SEARCH REPORT

National Application No

PCT/US 00/16560

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/31 C07K14/40 C12N5/10 C07K16/14 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

STRAND, EPO-Internal, PAJ, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROWLEY A ET AL: "CDC68 A YEAST GENE THAT AFFECTS REGULATION OF CELL PROLIFERATION AND TRANSCRIPTION ENCODES A PROTEIN WITH A HIGHLY ACIDIC CARBOXYL TERMINUS" MOLECULAR AND CELLULAR BIOLOGY, vol. 11, no. 11, 1991, pages 5718-5726, XP000956286 ISSN: 0270-7306 cited in the application the whole document	3
X	DATABASE EMBL SEQUENCE DATABASE 'Online! 23 March 1996 (1996-03-23) ALTHEIM, B., ET AL. : "untitled" XP002150215 accession no. U48701 ----- -/-	3

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☒ Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

23 October 2000

Date of mailing of the international search report

07/11/2000

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Holtorf, S

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 00/16560

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 37230 A (SCRIPTGEN PHARM INC ;HARVARD COLLEGE (US)) 9 October 1997 (1997-10-09) the whole document ---	
A	BREWSTER NEIL K ET AL: "Characterization of the CP complex, an abundant dimer of Cdc68 and Pob3 proteins that regulates yeast transcriptional activation and chromatin repression." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 34, 21 August 1998 (1998-08-21), pages 21972-21979, XP002150213 ISSN: 0021-9258 cited in the application the whole document ---	
A	US 5 585 277 A (BOWIE JAMES U ET AL) 17 December 1996 (1996-12-17) the whole document -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

national Application No

PCT/US 00/16560

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9737230 A	09-10-1997	US 5863762 A CA 2250121 A EP 0894269 A	26-01-1999 09-10-1997 03-02-1999
US 5585277 A	17-12-1996	US 5679582 A	21-10-1997