HYR1 AS A TARGET FOR ACTIVE AND PASSIVE IMMUNIZATION AGAINST *CANDIDA*

Statement as to Federally Funded Research

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Field of Invention

This invention relates to *Candida* hyphal cell wall proteins, to antibodies resulting from an immune response to vaccination with *Candida* hyphal cell wall proteins and to methods for the prevention and/or treatment of candidiasis and other bacterial infections with *Candida* hyphal cell wall proteins.

Background of the Invention

All documents referred to herein, or the indicated portions, are hereby incorporated by reference herein including U.S. Provisional Application Serial No. 61/223,005, filed July 3, 2009. No document, however, is admitted to be prior art to the claimed subject matter.

Approximately 60,000 cases of disseminated candidiasis occur per year in the United States [1], resulting in billions of dollars of health care expenditures. Given the 40% mortality rate of such infections, there is a need to identify new prophylactic or therapeutic targets for intervention.

The primary host defense mechanism against disseminated candidiasis is phagocytic killing of the organism [2, 3]. Only phagocytic cells are capable of directly killing *Candida in vitro* [4]. Additionally, within thirty-minutes of intravenous inoculation of *Candida* in mice, rabbits, dogs, or humans, yeasts are retained within the reticuloendothelial system, especially in the liver. The liver, rich in Kupffer macrophages, is capable of clearing 99.9% of yeast in the portal system during a single pass [5], underscoring the effectiveness of phagocytic defense mechanisms against the fungus. Hence, resistance of *C. albicans* to phagocyte killing is an important virulence function of the organism.

Cell surface glycosyl phosphatidylinositol (GPI)-anchored proteins are at the critical interface between pathogen and host, making these proteins likely participants in host-pathogen interactions [6].

The identification of effectors in the regulatory pathways of the organism that contribute to virulence offers the opportunity for therapeutic intervention with methods or compositions that are superior to existing antifungal agents. The identification of cell surface proteins or hyphal proteins that affect a regulatory pathway involved in virulence is particularly promising because characterization of the protein enables immunotherapeutic techniques that are likely superior to or synergistic with existing antifungal agents when fighting a candidal infection.

The virulence of *C. albicans* is regulated by several putative virulence factors of which adherence to host constituents and the ability to transform from yeast-to-hyphae are among the most critical in determining pathogenicity. While potent antifungal agents exist that are microbicidal for *Candida*, the attributable mortality of candidemia is approximately 38%, even with treatment with potent anti-fungal agents such as amphotericin B. Also, existing agents such as amphotericin B tend to exhibit undesirable toxicity. Although additional antifungals may be developed that are less toxic than amphotericin B, it is unlikely that agents will be developed that are more potent. Therefore, either passive or active immunotherapy to treat or prevent disseminated candidiasis is a promising alternative to standard antifungal therapy.

Thus, there exists a need for effective immunogens that will provide host immune protection and passive immunoprotection against *Candida* and other immunogenically related pathogens. The present invention satisfies this need and provides related advantages as well.

Summary of the Invention

The present invention features *Candida* HYR1 polypeptide antigens, and the therapeutic uses of such antigens. The HYR1 polypeptide antigens of the present invention may be used to treat or prevent *Candida* infection in a subject.

By screening a novel conditional overexpression/suppression system focusing on GPI-anchored proteins in *C. albicans*, we identified *HYR1* as a virulence factor. *HYR1* is a hyphae co-expressed gene, the null mutant strain of which does not display any morphologic abnormality *in vitro* [7]. Below we provide results demonstrating

that *HYR1* mediates resistance to phagocytic killing *in vitro*, modulates tissue fungal burden *in vivo*, and is therefore a vaccine target to ameliorate the severity of disseminated candidiasis.

Definitions

By a "HYR1" polypeptide is meant a polypeptide that is substantially identical to the amino acid sequence of SEQ ID NO:1. Desirably, a HYR1 polypeptide has at least 70, 75%, 80%, 85%, 90%, 95%, 99%, or even 100% identity to the amino acid sequence of SEQ ID NO: 1.

By "fragment of a HYR1 polypeptide" or a "HYR1 fragment" is meant a fragment of a HYR1 polypeptide containing fewer than 937, 936, or 935 amino acids. Preferred HYR1 fragments are between 300 and 350 or 250 to 500 amino acids in length. Desirably, the fragment is fewer than 937, 936, 935, 934, 933, 932, 931, or 930, 920, 910, 900, 890, 880, 870, 860, 850, 840, 830, 820, 810, 800, 790, 780, 770, 760, 750, 740, 730, 720, 710, 700, 690, 680, 670, 660, 650, 640, 630, 620, 610, 600, 590, 580, 570, 560, 550, 540, 530, 520, 510, 500, 490, 480, 470, 460, 450, 440, 430, 420, 410, 400, 390, 380, 370, 360, 350, 340, 330, 320, 310, 300, 290, 280, 270, 260, 250, 240, 230, 220, 210, 200, 190, 180, 170, 160, 150, 140, 130, 120, 110, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15, or 10 amino acids, and desirably, is immunogenic. A HYR1 fragment, for example, may contain one or more conservative amino acid substitutions in the sequence of SEQ ID NO: 2. Additional desirable HYR1 fragments contain one or more conservative amino acid substitutions in the sequence of SEQ ID NO: 2 and/or at least one flanking amino acid (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 flanking amino acids) at the N- and/or C-terminus of the sequence of SEQ ID NO: 2. Other preferred HYR1 fragments contain seven or more continuous amino acids of the sequence of SEQ ID NO: 2.

Non-limiting examples of a HYR1 fragment include amino acids 1-40, 10-50, 20-60, 30-70, 40-80, 50-90, 60-100, 70-110, 80-120, 90-130, 100-140, 110-150, 120-160, 130-170, 140-180, 150-190, 160-200, 170-210, 180-220, 190-230, 200-240, 210-250, 220-260, 230-270, 240-280, 250-290, and 260-300, 270-310, 280-320, and 290-331 amino acids of the sequence of SEQ ID NO: 2; and these fragments having one or more of the following features: one or more conservative amino acid substitutions (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 conservative amino acid substitutions) in the sequence of SEQ ID NO: 2; one or more amino acids (e.g., 1, 2,

3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 amino acids) truncated from the N and/or C-terminus of the sequence of SEQ ID NO: 2; and at least one flanking amino acid (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 flanking amino acids) at the N- and/or C-terminus of the sequence of SEQ ID NO: 2.

By "substantially identical" is meant a polypeptide exhibiting at least 50%, desirably 60%, 70%, 75%, or 80%, more desirably 85%, 90%, or 95%, and most desirably 99% amino acid sequence identity to a reference amino acid sequence. The length of comparison sequences will generally be at least 10 amino acids, desirably at least 15 contiguous amino acids, more desirably at least 20, 25, 50, 75, 90, 100, 150, 200, 250, 275, 300, 310, 315, 320, 325, 330, 335, 340, 345, or 350 contiguous amino acids, and most desirably the full-length amino acid sequence.

Sequence identity may be measured using sequence analysis software on the default setting (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software may match similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Multiple sequences may also be aligned using the Clustal W(1.4) program (produced by Julie D. Thompson and Toby Gibson of the European Molecular Biology Laboratory, Germany and Desmond Higgins of European Bioinformatics Institute, Cambridge, UK) by setting the pairwise alignment mode to "slow," the pairwise alignment parameters to include an open gap penalty of 10.0 and an extend gap penalty of 0.1, as well as setting the similarity matrix to "blosum." In addition, the multiple alignment parameters may include an open gap penalty of 10.0, an extend gap penalty of 0.1, as well as setting the similarity matrix to "blosum," the delay divergent to 40%, and the gap distance to 8.

By "conservative amino acid substitution," as used herein, is meant replacement, in an amino acid sequence, of an amino acid for another within a family of amino acids that are related in the chemical nature of their side chains.

Genetically encoded amino acids can be divided into four families: acidic (aspartate, glutamate); basic (lysine, arginine, histidine); nonpolar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan); and uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine). Phenylalanine, tryptophan, and tyrosine are sometimes grouped as aromatic amino

acids. In similar fashion, the amino acids can also be separated into the following groups: acidic (aspartate, glutamate); basic (lysine, arginine, histidine); alipathic (glycine, alanine, valine, leucine, isoleucine, serine, threonine), with serine and threonine optionally grouped separately as alipathic-hydroxyl; aromatic (phenylalanine, tyrosine, tryptophan); amide (asparagine, glutamine); and sulfur-containing (cysteine, methionine).

Whether a change in the amino acid sequence results in a functional homolog can be determined by assessing the ability of the variant peptide to function in a fashion similar to the wild-type protein using standard methods such as the assays described herein.

Desirable embodiments of the invention, include at least one conservative amino acid substitution in the amino acid sequence of SEQ ID NO: 1 or 2; and more desirably 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitutions in the sequence of SEQ ID NO: 1 or 2.

By "flanking amino acid" is meant an amino acid in a polypeptide sequence that is immediately adjacent to the N- or C-terminus of a particular defined sequence. Desirably, a flanking amino acid is present on the N- and/or C-terminus of the amino acid sequence of SEQ ID NO: 1 or 2 or a fragment thereof; and more desirably, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 flanking amino acids are present at the N- and/or C-terminus of the amino acid sequence of SEQ ID NO: 1 or 2, or fragment thereof.

As used herein "fusion protein" refers to a polypeptide consisting of (1) a HYR1 polypeptide, HYR1 fragment; and (2) a fusion partner.

As used herein "fusion partner" refers to a heterologous sequence that can be fused to a HYR1 polypeptide or HYR1 fragment. Examples of fusion partners are described herein and include detection markers, stabilizing domains, or sequences which aid in production or purification of the protein.

As used herein "immune response" refers to the activation of an organism's immune system in response to an antigen or infectious agent. In vertebrates, this may include, but is not limited to, one or more of the following: naïve B cell maturation into memory B cells; antibody production by plasma cells (effector B cells); induction of cell-mediated immunity; activation and cytokine release by $CD4^+$ T cells; activation and cytokine release of $CD8^+$ T cells; cytokine recruitment and activation of phagocytic cells (e.g., macrophages, neutrophils, eosinophils); and/or complement

activation.

By "immunogenic" is meant any substance that is capable of inducing an immune response in a subject.

By "pharmaceutically acceptable salt" is meant any non-toxic acid addition salt or metal complex used in the pharmaceutical industry. Examples of acid addition salts include organic acids such as acetic, lactic, pamoic, maleic, citric, malic, ascorbic, succinic, benzoic, palmitic, suberic, salicylic, tartaric, methanesulfonic, toluenesulfonic, or trifluoroacetic acids or the like; polymeric acids such as tannic acid, carboxymethyl cellulose, or the like; and inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, or the like. Metal complexes include zinc, iron, and the like.

By "pharmaceutically acceptable carrier" is meant any solution used to solubilize and deliver an agent to a subject. A desirable pharmaceutically acceptable carrier is saline. In desirable embodiments, a pharmaceutically acceptable carrier includes an adjuvant. Exemplary adjuvants are described herein. Other physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in Remington's Pharmaceutical Sciences, (20th edition), ed. A. Gennaro, 2003, Lippincott Williams & Wilkins.

By "isolated" is meant a protein (or a fragment thereof) that has been separated from components that naturally accompany it. Typically, the polypeptide is substantially isolated when it is at least 60%, by weight, free from the proteins and naturally occurring organic molecules with which it is naturally associated. The definition also extends to a polypeptide separated from its flanking amino acids (e.g., for an amino acid sequence, isolated refers to a sequence that is free from the flanking amino acids with which the sequence is naturally associated in a polypeptide). Preferably, the polypeptide is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, isolated. An isolated polypeptide may be obtained by standard techniques, for example, by extraction from a natural source (e.g., purification from a cell infected with *Candida*), by expression of a recombinant nucleic acid encoding a HYR1 fragment; or fusion protein thereof, by chemically synthesizing the polypeptide. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

By a "therapeutically effective amount" is meant the amount of a immunogenic compound (e.g., polypeptide, fragment, fusion protein, or vaccine) required to generate in a subject one or more of the following effects: an immune response; a decrease in the level of *Candida* infection (e.g., a reduction of at least 5%, 10%, 20%, or 30%; more desirably 40%, 50%, 60%, or 70%; and most desirably 80% or 90%); a decrease (e.g., at least a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or even 100% reduction) in one or more symptoms of *Candida* infection in a patient; or increased resistance to a new *Candida* infection (e.g., an increase of at least 5%, 10%, 20%, 30%, 40%, or 50%; more desirably 60%, 70%, 80%, or 90%; or most desirably 100%, 200%, or 300%).

Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

Brief Description of the Drawings

Figure 1. Conditional expression of *IIYR1* enhanced neutrophil and macrophage mediated killing of *C. albicans*. (A) Confirmation of *HYR1* conditional overexpression/suppression strain CAAH-31. RT-PCR results of *HYR1* demonstrating overexpression of the gene in –DOX medium and lack of expression in +DOX medium. *EFB1* fragment was co-amplified and served as a control. Lack of genomic DNA contamination in cDNA preparations was demonstrated by the absence of 919 bp band containing the intron of *EFB1*. THE31 was the wild-type control strain. (B) *C. albicans* strains were grown in YPD with DOX (suppression of *HYR1*) and without DOX (overexpression of *HYR1*) at 30°C overnight and then cocultured with human neutrophil; (C) *C. albicans* cocultured with HL-60 derived neutrophil; (D) with HL-60 derived macrophage.

Figure 2. Expression of *Candida albicans HYR1* increased human neutrophil killing resistance in *bcr1* null mutant or *C. glabrata* strain. (A and B) Autonomously expression of *HYR1* in a *bcr1* deficient strain of *C. albicans* completely complemented the hypersusceptibility of the parent strain to neutrophil killing resistance. *C. albicans* strains DAY185 (wild-type), CJN702 (*bcr1* null mutant) and CJN698 (*BCR1* complemented) CJN114, CJN1153, CJN1222, CJN1259, CJN1276, CJN1281, and CJN1288 (autonomously expressing *ALS1, ALS3, HWP1, HYR1, RBT5, CHT2* and *ECE1* in a *bcr1* null mutant background, respectively) were grown

in YPD overnight at 30°C. Data are displayed as median ± interquartile. * P <0.04 vs. the wild-type and *BCR1*-complemented. (C) Heterologous expression of *C. albicans HYR1* gene increased *C. glabrata* resistance to HL-60 derived neutrophil mediated killing. * P<0.0001.

Figure 3. Detection of *HYR1* expression during disseminated candidiasis although its expression was initially inhibited by neutrophil *in vitro.* (A) Kidneys livers, lungs, spleens and brains were harvested 6 or 24 h after intravenous infection with *C. albicans*. Nested RT-PCR was used to detect expression of *HYR1*. *C. albicans EFB1* and mouse house-keeping gene G3PDH were used as a control. + denotes infected mice, while – denotes uninfected mice. (B) HL-60 derived neutrophil inhibited *C. albicans HYR1* expression. Wild-type *C. albicans* was cultured in RPMI 1640 plus 10% pooled human serum. Without neutrophil, *HYR1* expression was detected after half hour induction. With neutrophil, *HYR1* expression was inhibited two hours.

Figure 4. *HYR1 in vivo* expression and its effect on fungal burden. To detect *HYR1* expression during disseminated candidiasis, kidneys livers, lungs, spleens and brains were harvested 6 or 24 h after intravenous infection with *C. albicans*. Nested RT-PCR was used to detect expression of *HYR1*. *C. albicans EFB1* and mouse house-keeping gene G3PDH were used as a control. + denotes infected mice, while – denotes uninfected mice (A). Conditional expression *HYR1* increased fungal burden in organs with extensive tissue phagocytes. Burden of *C. albicans* in livers and spleens of immunocompetent mice (n=8 per group) infected with *C. albicans IIYR1* or control strain grown in conditional expressing (-DOX) or suppressing (+DOX) conditions (A) and the *in vivo HYR1* expression (B). Livers and spleens were harvested one day post infection. The y-axes reflect lower limits of detection of the assay. Data are displayed as median ± interquartile. * P <0.0001 vs. no expression of *HYR1* strain (HYR1-DOX) or control strain.

Figure 5. Indirect immunofluorescence with anti-Hyr1p serum demonstrates surface expression of Hyr1p on *C. albicans* hyphae. Hyphal formation was induced by incubating *C. albicans* in RPMI 1640 for 90 min. Cells were stained with either the *hyr1* null mutant pre-absorbed anti-Hyr1p serum (1:100) or the anti-protein preparation from the empty plasmid clone serum (negative control), followed by staining with Alexa labeled anti-mouse Ab.

Figure 6. Recombinant N-terminus of Hyr1p (rHyr1p-N) remarkably protected against murine hematogenously disseminated candidiasis. (A) Survival of mice (8 mice per group) vaccinated with rHyr1p-N mixed with complete or incomplete Freund's adjuvant and infected by means of the tail vein with 2.2×10^5 blastospores of *Candida albicans* SC5314. (B) Survival of mice (8 mice per group, except the control group, which had 17 mice) vaccinated with rHyr1p-N or detoxified rHyr1p-N mixed with 0.1% alhydrogel and infected with 7×10^5 blastopsores of *Candida albicans* 15563. *P = .001 by log-rank test. (C) Effect of vaccinated or control $F(ab)'_2$ on blocking mouse neutrophil killing of *C. albicans* conditionally expressed or suppressed Hyr1. Control denotes assay performed in the absence of either $F(ab)'_2$. Data are displayed as median ± interquartile range. *P = .001 by Mann-Whitney test.

Detailed Description

Candida albicans is a common pathogen in humans. For example, *C. albicans*, while normally a harmless commensal, can cause a variety of conditions ranging from superficial mucocutaneous infection such as vaginal and/or oropharyngeal candidiasis, to deep organ involvement in disseminated candidiasis. Prior to causing disease, the fungus colonizes the gastrointestinal tract, and in some cases skin and mucous membranes. Adherence to host mucosal surfaces is a key prerequisite for this initial step. After colonization, C. *albicans* enters the bloodstream via infected intravascular devices or by transmigration through gastrointestinal mucosa compromised by chemotherapy or stress ulcerations. Organisms then disseminate via the bloodstream, bind to and penetrate the vascular endothelium to egress from the vascular tree, and invade deep organs such as liver, spleen, and kidney.

The identification and functional characterizations of a HYR1 fragment described herein allows this polypeptide to be effectively utilized in the treatment of candidiasis.

The nature of the pathogenesis of *C. albicans* by adherence to endothelial cells is discussed in U.S. Pat. No. 5,578,309 which is specifically incorporated herein by reference in its entirety. For a description of an HYR1 gene and characteristics thereof, including the characterization of the gene product see, Bailey et al. (*Journal*

of Bacteriology 178:5353-5360, 1996).

The invention provides a vaccine having an isolated HYR1 fragment, and optionally an adjuvant in a pharmaceutically acceptable medium. The vaccine can be an HYR1 fragment derived from a *Candida* species such as *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Candida glabrata*, or *Candida parapsilosis*.

The invention utilizes the gene product of *C. albicans* HYR1 sequence as a vaccine to treat, prevent, or alleviate disseminated candidiasis. The vaccine is effective against different strains of *C. albicans* as well as against different *Candida* species.

Thus, according to one aspect, the invention provides an HYR1 fragment useful when formulated in a pharmaceutical composition and administered as a vaccine with or without an adjuvant. An HYR1 fragment can be of candidal origin and can be obtainable, for example, from species belonging to the genera *Candida*, for example *Candida parapsilosis*, *Candida krusei*, *Candida glabrata*, and *Candida tropicalis*. An HYR1 fragment can be obtained in isolated or purified form, and thus, according to one embodiment of the invention an HYR1 fragment is formulated as a vaccine to cause an immune response in a patient to elicit an immune response against *Candida*.

The invention also provides a method of treating or preventing disseminated candidiasis. The method includes administering an immunogenic amount of a vaccine of an HYR1 fragment. The vaccine can be administered with or without an adjuvant. The HYR1 fragment can be derived from different *Candida* strains as well as from different *Candida* species such as *Candida* albicans, *Candida krusei*, *Candida tropicalis*, *Candida glabrata*, and *Candida parapsilosis*.

The effectiveness of the vaccines of the invention against different *Candida* strains, different *Candida* species, other bacteria and infectious agents and their wide range of immune activity are assessed according to standard methods such as those described further below.

Given the teachings and guidance provided herein, those skilled in the art will understand that immunotherapeutic methods well know in the art can be employed with one or more HYR1 fragments in a pharmaceutically acceptable composition administered as a vaccine with or without an adjuvant. For the purposes of this invention, the terms "pharmaceutical" or "pharmaceutically acceptable" refer to

compositions formulated by known techniques to be non-toxic and, when desired, used with carriers or additives that can be safely administered to humans. Administration can be performed using well known routes including, for example, intravenous, intramuscular, intraperitoneal or sub-cutaneous injection. Such vaccines of the inventions also can include buffers, salts or other solvents known to these skilled in the art to preserve the activity of the vaccine in solution. Similarly, any of a wide range of adjuvants well known in the art can be employed with the vaccine of the invention to elicit, promote or enhance a therapeutically effective immune response capable of reducing or blocking binding, invasion and/or infection of *Candida* to a susceptible host cell.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

Example I

Candida albicans Hyr1p confers resistance to neutrophil killing and is a vaccine target

As is discussed above, *Candida albicans* is the most common cause of invasive fungal infections in humans. It is unclear how *C. albicans* escapes from phagocytic attack and survives in the hostile blood environment during life-threatening systemic infections. Using a conditional overexpression/suppression genetic strategy, we discovered that the *HYR1* gene reduced phagocytic killing of *C. albicans in vitro* and increased tissue fungal burden *in vivo*. Concordant with its positive regulation by the transcription factor Bcr1p, *HYR1* complemented the hyper-susceptibility to phagocyte-mediated killing of a *bcr1* null mutant of *C. albicans in vitro*. Furthermore, heterologous expression of *HYR1* in *Candida glabrata* rendered the organism more resistant to neutrophil killing. Finally, vaccination with recombinant Hyr1p significantly protected mice against hematogenously disseminated candidiasis. Thus, Hyr1 is an important virulence factor for *C. albicans*, mediating resistance to phagocyte killing. Hyr1p is accordingly a target for vaccine or other immunological or small molecule intervention to improve the outcomes of

disseminated candidiasis.

Results

Conditional expression of HYR1 in blastospores significantly enhanced C. albicans resistance to phagocyte-mediated killing in vitro

To study the function of *HYR1*, we constructed a conditional overexpression/suppression strain of *C. albicans*, CAAH-31. In CAAH-31, one allele of *HYR1* was controlled by the tetracycline regulated (TR)-promoter and the other allele was disrupted. By semi-quantitative RT-PCR, we confirmed that *HYR1* was abundantly expressed when blastospores of CAAH-31 were grown in media without DOX, and was not detected in the presence of DOX (Fig. 1A). As expected, *HYR1* was not detected in wild-type (THE31) blastospores because *HYR1* is a hyphal co-expressed gene (Fig. 1A).

The *HYR1* conditional overexpression strain, CAAH-31, and THE31 wild-type control had identical growth rates, irrespective of the presence or absence of DOX (doubling time for wild-type control strain without DOX= 1.51 ± 0.29 hr and with DOX= 1.51 ± 0.38 hr; doubling time for CAAH-31 strain without DOX= 1.39 ± 0.30 hr and with DOX= 1.35 ± 0.19 hr). We also evaluated the impact of *HYR1* overexpression on the normal accumulation of other GPI-anchored proteins on the cell surface. By direct immunofluorescence, we confirmed that *HYR1* overexpression had no impact on the accumulation of the GPI-anchored protein Als1p (data not shown) [8].

During routine screening for virulence-associated phenotypes, we determined the impact of conditional overexpression of *HYR1* on candidal killing by human phagocytes. *HYR1*-expressing *C. albicans* (CAAH-31 –DOX) was significantly more resistant to human neutrophil-mediated killing than wild-type *C. albicans* (which does not express *HYR1* in the blastospore phase) and *HYR1*-suppressed *C. albicans* (CAAII-31 +DOX) (Fig. 1B). This phenotype was not due to DOX, as killing was not significantly different between the wild-type control and *HYR1*-suppressed *C. albicans* (+DOX).

We also performed candidal killing assays using the HL-60 cell line, which can be differentiated into either neutrophil-like or macrophage-like cells [9, 10]. Like freshly harvested human neutrophils, conditional overexpression of *HYR1* reduced killing of *C. albicans* blastospores by both HL-60 neutrophil-like (Fig.1C) and

macrophage-like (Fig. 1D) cells *in vitro*.

Hyper-susceptibility to neutrophil killing of bcr1 null mutant C. albicans was complemented by HYR1 expression in vitro

Since *HYR1* is a downstream gene of the positive transcription regulator Bcr1p [17], we hypothesized that disruption of *bcr1* would exacerbate susceptibility to neutrophil killing under conditions promoting wild-type *C. albicans* to express *HYR1* (i.e. during hyphal formation). We therefore induced *C. albicans* to form germ tubes by incubating the cells in RPMI plus 10% FBS at 37°C for 40 min. This condition is known to induce expression of *HYR1* [18], and resulted in germ tubes short enough such that extensive hyphae were not formed, thereby enabling quantification of colony forming units (CFUs) in our kill assay. We compared neutrophil killing of the *bcr1* null mutant strain (CJN702), a *BCR1* complemented strain in the *bcr1* null mutant background (CJN698), and a wild-type *C. albicans* strain (DAY185). The *bcr1* null mutant was hyper-susceptible to neutrophil-mediated killing compared to the *BCR1*-complemented and wild-type control strains (Fig. 2A). Furthermore, the hyper-susceptibility to killing of *bcr1*-deficient *C. albicans* was fully complemented by autonomous expression of *HYR1* in the *bcr1* mutant background, but not by other cell surface encoding genes regulated by Bcr1p [17] (Fig. 2A and 2B).

Resistance of C. albicans overexpressing HYR1 to phagocyte killing can be recapitulated by heterologous expression of HYR1 in C. glabrata

To further define the virulence phenotype mediated by *HYR1*, we expressed the gene heterologously in *C. glabrata* BG14 [19] using a plasmid pGRB2.2 carrying a constitutive *PGK1* promoter [11]. We also generated a *C. glabrata* BG14 transformed with the empty plasmid, as a negative control. Expression of *HYR1* in *C. glabrata* resulted in a 75% reduction in killing by HL-60-derived neutrophils *in vitro*, compared to the *C. glabrata* transformed with an empty plasmid (Fig. 2C).

Neutrophils inhibit candidal HYR1 expression

Because conditional overexpression of *HYR1* conferred *Candida* resistance to neutrophil-mediated killing, we used RT-PCR to study the expression of wild-type *C. albicans* (SC5314) *HYR1* in response to HL-60-derived neutrophils *in vitro*. *HYR1* was expressed as early as 30 min after exposure to medium containing serum, and maintained high expression for 2.5 hr during culture (Fig. 3A). However, when *C. albicans* was exposed to HL-60-derived neutrophils in culture, even in the presence of

serum, *HYR1* expression was inhibited for up to 2 hr into the co-culture (Fig. 3A).

Wild-type C. albicans expresses HYR1 during hematogenously disseminated candidiasis, resulting in increased tissue fungal burden in organs rich in phagocytes

To determine whether *HYR1* was expressed during hematogenously disseminated candidiasis, five major organs - brain, liver, lung, spleen and kidney - were harvested from mice infected with *C. albicans* wild-type strain after 6 and 24 hr of infection. An improved nested-RT-PCR assay [20] was used to assess *HYR1* expression *in vivo*. *HYR1* expression was detected in all five organs (Fig. 3B).

Overexpression of *HYR1* increased and suppression of *HYR1* decreased fungal burden in the liver and spleen (Fig. 4A). Furthermore, we confirmed that the overexpressing strain had significantly higher levels of *HYR1* than the wild-type strain in livers; the suppressed strain demonstrated a trend towards reduced levels (Fig. 4B). In contrast, *HYR1* expression did not significantly alter fungal burden in the kidney (data not shown), an organ lacking resident phagocytes.

rHyr1p-N as a vaccine candidate

Based on sequence analysis, Hyr1p is predicted to be a cell surface protein [7]. To confirm this, we generated a recombinant N-terminal Hyr1p in *E. coli* transformed with an expression clone that includes amino acids 25-350 of the coding sequence (rHyr1p-N). Serum from mice immunized with rHyr1p-N was pre-absorbed against the *hyr1* null mutant of *C. albicans* [7], followed by indirect immuno-staining on wild-type hyphae. We found that the cell wall of wild-type *C. albicans* hyphae was heavily stained (Fig. 5), confirming that it is cell-surface expressed, and hence exposed to the immune system.

Because Hyr1p is as a cell surface protein, which confers resistant to candidal killing by phagocytes, we sought to determine its potential as a vaccine candidate. Mice were vaccinated with rHyr1p-N plus adjuvant or adjuvant alone. Two weeks after the boost, mice were infected via the tail vein with highly virulent *C. albicans* SC5314. Vaccination with rHyr1p-N markedly improved survival of mice compared to those vaccinated with adjuvant alone (62.5 and 0% survival at 35 days, respectively) (Fig. 6A).

Vaccination with rHyr1p-N mixed with complete or incomplete Freund's adjuvant or alum markedly improved survival of mice compared with those

vaccinated with either adjuvant alone (Figure 6A and 6B).

Anti-rHyr1p serum enhanced neutrophil killing in mice by directly inhibiting Hyr1p neutrophil resistance function.

The protective effect of the rHyr1p-N vaccine suggested that the anti-rHyr1p serum might be able to neutralize the protective function of Hyr1p in *C. albicans*. To determine whether anti-rHyr1p antibodies could directly inhibit Hyr1p function, we isolated and prepared F(ab)'$_2$ fragments from total IgG of mice immunized with either rHyr1p-N or the control (preparation produced from *E. coli* cells transformed with the empty plasmid). We found that F(ab)'$_2$ from immune but not control serum was able to restore neutrophil killing of the *HYR1* conditional expressing strain to levels equivalent to that of the suppressing strain (Figure 6C).

Summary

In this study, we demonstrated that *HYR1*, a hyphal co-expressed gene [7, 21], encodes a candidal phagocyte resistance factor. Conditional expression of *HYR1* in *Candida* blastospores caused the fungus to be more resistant to killing by phagocytes compared to wild-type *C. albicans* blastospores. Additionally, the function of *HYR1* in *C. albicans* was recapitulated by heterologously expressing the gene in *C. glabrata in vitro*. We also found that a strain deficient in Bcr1p, a transcription factor that positively regulates *HYR1* expression [17], exhibited enhanced susceptibility to phagocyte-mediated killing. The hyper-susceptibility to phagocyte killing of the *bcr1* null mutant was fully complemented by autonomously expressed *HYR1*, but not other genes which encode GPI-proteins positively regulated by Bcr1p. Hence, *HYR1* is a downstream gene of *BCR1* in a phagocyte killing resistance pathway.

It is interesting that HL-60 derived neutrophils were able to inhibit the expression of *HYR1* in wild-type *C. albicans* during the initial contact between the phagocytes and *Candida*. Thus, a dynamic interaction occurred between host phagocytes and *C. albicans*, in which the phagocytes mediated a delay in the expression of a phagocyte-resistance gene in the wild-type fungus. This is consistent with a previous finding that human neutrophils delayed the formation of *C. albicans* hyphae and the expression of hyphae co-expressed genes [18].

Resistance to phagocyte killing is a complex phenotype, likely attributable to multiple factors. Phagocytes can kill *Candida* extracellularly or intracellularly.

Recently, *C. albicans* cell surface superoxide dismutases were characterized as virulence factors that help the fungi escape from killing by degrading host-derived highly reactive oxygen species [22]. Neutrophils typically attach to and spread over the surfaces of hyphal forms of fungi, as extended hyphae are too large for phagocytes to ingest completely. Since *C. albicans* expresses *HYR1* in the hyphal form, it is possible that Hyr1p contributes to resistance to phagocyte killing by preventing surface contact to the phagocyte. Alternatively, Hyr1p might interfere with oxidative or non-oxidative killing mechanisms of phagocytes.

The *in vitro* phenotype of *HYR1* overexpression was recapitulated *in vivo*. During murine disseminated infection, overexpression of *HYR1* led to a significant increase, and suppression of *HYR1* led to a significant decrease, in tissue fungal burden compared to the wild-type strain in organs with resident phagocytes. The lack of phenotype in the kidney likely reflects the fact that kidneys do not have resident phagocytes, and that neutrophil influx into the kidneys during lethal disseminated candidiasis does not begin until > 24 hr of infection [15]. Nevertheless, vaccination with rHyr1p-N resulted in considerable protection against hematogenously disseminated candidiasis. The efficacy seen for the rHyr1p-N vaccine was greater than that previously seen in mice vaccinated with the rAls1p-N and rAls3p-N vaccines. Hence, rHyr1p-N is a promising vaccine candidate for disseminated candidiasis.

Our data also demonstrate the advantage of using a conditional overexpression/suppression approach to explore potential virulence functions of a given gene. Large-scale forward genetic approaches to explore virulence genes *in vitro* require functional screening assays, and are limited to screening for genes that are expressed while conducting the assay. When a gene is not expressed significantly in the wild-type strain under conditions used for the *in vitro* screening assay, forced overexpression of the gene still allows for detection of a gain-of-function phenomenon in the assay, as in the case of *HYR1* during *Candida* blastospore growth. When a gene is strongly expressed, conditional suppression of the gene yields a loss-of-function phenotype in the same assay. Furthermore, when overexpression and suppression are used simultaneously, the phenotype can be amplified, as in the case of the effect of *HYR1* on liver and spleen fungal burdens *in vivo*. The ability to detect a phenotype by comparing gene overexpression and suppression enables conditional

gene expression to overcome the limitations of functional redundancy, which particularly plague forward genetic approaches when members of a gene family are being studied.

In summary, we used a conditional gene expression approach to identify Hyr1p as a surface expressed, virulence factor for *C. albicans*. *HYR1* expression mediated resistance to neutrophil killing *in vitro* and increased tissue fungal burden *in vivo*. Finally, we demonstrated that *HYR1* is a promising vaccine target which merits further development as a prophylactic strategy for disseminated candidiasis.

Materials and Methods

The above-described Results were obtained using the following materials and methods.

Strains and culture conditions

All strains used are listed in Table 1 and grown as previously described [8].

Conditional HYR1 overexpression/suppression mutant construction

To generate a conditional *HYR1* expression strain, a *HIS1-TR* promoter cassette [8] was inserted in front of one allele of the *HYR1* gene of strain THE4, yielding strain CAAH. The *URA3* at the *HIS1* locus in strain CAAH was looped out, generating CAAH-1. The second allele of *HYR1* in CAAH-1 was disrupted by a recyclable *URA3* cassette, generating strain CAAH-2, followed by looping out of *URA3*, yielding strain CAAH-3. A 3.9-kb *Nhe I-Pst I* fragment containing the *URA3-IRO1* gene was inserted into its original locus on the CAAH-3 genome, yielding CAAH-31. Primers used are listed in Table 1.

Semi-quantitative RT-PCR

The semi-quantitative RT-PCR to detect gene expression *in vitro* was described previously. Primers used to detect *EFB1* expression were EFB1a and EFB1b; primers used to amplify *HYR1* were HYR1 specific1 and HYR1 specific2 (Table 1). To study the impact of neutrophils on *C. albicans HYR1* expression, 1 x 10^6 overnight cells of SC5314 grown in YPD were either co-cultured with 1 x 10^7 HL-60 derived neutrophils or cultured alone in RPMI 1640 plus 10% pooled human serum. Samples were taken at 30 min intervals for 3 hr until RNA was extracted, and semi-quantitative RT-PCR was performed.

Phagocyte killing assay

Human neutrophils were isolated, HL-60 cells were differentiated into neutrophils or macrophages, and the phagocyte killing assay was performed as previously described [8-10]. Briefly, phagocytes were incubated with fungi for 1 hr, and then sonicated and quantitatively cultured. Percent killing was calculated by dividing the number of fungal colonies after co-incubation with phagocytes by the number of fungal colonies incubated with media without phagocytes. Human neutrophils and HL-60 derived neutrophils or macrophages were tested at a 2:1 and 20:1 phagocyte:fungus ratio, respectively. For *bcr1* and related mutants, the blastospores were pre-germinated for 40 min in RPMI plus 10% FBS at 37°C before performing the assay.

Heterologous expression of HYR1 in C. glabrata BG14

C. glabrata BG14 was transformed with either an *HYR1* expression vector pGRB2.2-HYR1 or an empty control plasmid pGRB2.2 [11]. *HYR1* coding sequence was amplified by CG-Hyr1-a and CG-Hyr1-b (Table 1) and was cloned into *Xba I*, *Xho I* sites of pGRB2.2 using In-Fusion™ 2.0 Dry-Down PCR Cloning Kit per manufacture's instruction (Clontech Laboratories, Mountain View, CA).

C. albicans HYR1 expression during hematogenous infection

HYR1 expression by wild-type *C. albicans* SC5314 was examined during hematogenously disseminated candidiasis as described. Brains, livers, lungs, kidneys, and spleens of BALB/C mice were collected 6 and 24 hr post infection. Primers used are listed in Table 1. Reverse transcription was performed with RETROscript (Ambion, Texas). For amplification of mouse *G3PDH* housekeeping gene, primers *G3PDHF* and *G3PDHR* were used. For detection of *HYR1* and *EFB1* of *C. albicans*, two rounds of PCR were performed. Round one used outer primer set (EFB1F and EFB1R for *EFB1*, or P2 and P5 for *HYR1*); round two used an aliquot (1 μl) of round one PCR product as a template. The inner primer sets were as follows: EFB1nF and EFB1nR (for *EFB1*), or P2 and P4 (for *HYR1*). All PCR conditions were as follows: denaturing at 95°C, 2 min and amplification for 35 cycles at 94°C, 30 s (denaturing), 55°C, 30 s (annealing), and 72°C, 90 s (extension). For qRT-PCR, cDNA was prepared as above. Optimization of amplification efficiency and real-time RT-PCR SYBR green assays were carried out as described [12]. Constitutively expressed *ACT1* was used as a control for all reactions. Calculations and statistical analyses

were carried out as described in ABI PRISM 7000 Sequence Detection System User Bulletin 2 (Applied Biosystems, USA).

Tissue fungal burden

Mice were given water with or without DOX (2 mg/ml) dissolved in 5% sucrose solution throughout the period of the experiment starting from day −3 relative to infection [13], and were given food and water ad libitum. Tissue fungal burden was carried out as previously described [8] except that organs were removed 1 day post infection. All procedures involving mice were approved by the institutional animal use and care committee, following NIH guidelines.

rHyr1p-N production

rHyr1p-N (from amino acids 25–350 of Hyr1p) was produced in an Escherichia coli pQE-32 expression system (Qiagen), and the 6XHis tagged protein was purified as described elsewhere [14], with the exception of using a HisPur Cobalt resin (Thermo Scientific) affinity column. Endotoxin was removed from rHyr1p-N by using Detoxi-Gel Endotoxin Removing Columns (Thermo Scientific), and the endotoxin level was determined with Limulus Amebocyte Lysate endochrome (Charles River) per manufacturer's instruction. Using this procedure, endotoxin was reduced to <0.28 EU per dose used for vaccination.

Immunofluorescence detection of Hyr1p cellular localization

Indirect immunofluorescence was performed using polyclonal anti-Hyr1p antisera generated by immunization of mice with rHyr1p-N (from amino acids 25-350). An inoculum of 1 x 10^7 blastospores of *hyr1* null strain were incubated in RPMI 1640 for 90 min at 37 °C and pelleted twice to absorb the antiserum.

C. albicans blastospores (1x 10^5) were pre-germinated in RPMI 1640 for 90 min at 37 °C and transferred into a 4-well chamber slide (Nalge Nunc International Corp, IL, USA). After incubation at 4 °C for 30 min, the cells were blocked with 300 µl of 1.5% goat serum, stained with polyclonal antiserum at a 1:100 dilution or PBS as a negative control, and then by fluorescein isothiocyanate-labeled goat anti-mouse IgG at 1:200. The cells were imaged by confocal scanning laser microscopy [15].

Immunization Protocol

All vaccinations were subcutaneous, at the base of the neck. Eight juvenile (10–12-week) C57BL/6 mice were vaccinated with 20 µg of affinity-purified rHyr1p-N in complete Freund's adjuvant and boosted in incomplete Freund's adjuvant (IFA)

at 3 weeks. Eight additional juvenile mice received adjuvant alone mixed with the preparation produced from *E. coli* cells transformed with the empty plasmid. Fourteen days after the boost, mice were infected via the tail vein with 5 x 10^5 cells of wild-type *C. albicans* SC5314 [16].

The efficacy of rHyr1p-N in protecting against hematogenously disseminated candidiasis was also evaluated using alum (2% Alhydrogel; Brenntag Biosector), an adjuvant approved by the Food and Drug Administration (FDA) for use in humans. Additionally, to determine that rHyr1p-N was protective against other strains of *C. albicans*, we used another clinical isolate, strain 15563. For these experiments, 33 μg of affinity-purified rHyr1p-N was mixed with 0.1% alhydrogel and administered to BALB/c mice as above on day 0, boosted on day 21, and then infected on day 35 with *C. albicans* through tail vein injection. For all vaccination experiments, survival of mice for 35 days after infection was used as an end point.

F(ab)'$_2$ blocking assay. Pooled anti-Hyr1p or control serum was collected from 5 mice that were vaccinated either with rHyr1p-N or with the preparation produced from *E. coli* cells transformed with the empty plasmid. The total IgG from both sera was isolated using Nab Spin Kit (Thermo Scientific). The F(ab)'$_2$ fragments were purified with Pierce F(ab)'$_2$ Preparation Kit according to the manufacturer's instruction. *Candida* cells were opsonized on ice for 45 min with 5% normal mouse serum (Santa Cruz Biotechnology) or 5% normal mouse serum plus 5% F(ab)'$_2$ prepared from either rHyr1p-N vaccinated or control mice IgG before mixing with mouse neutrophils. Mouse neutrophil killing assay was described as above.

Statistical analysis

Phagocyte mediated killing and tissue fungal burdens among different groups were compared by the Mann-Whitney U test for unpaired comparisons, as appropriate. The non-parametric Log Rank test was utilized to determine differences in survival times. *P* values of <0.05 were considered significant.

Table 1 Strains and oligonucleotides used in this study

Strains

Candida albicans Strains	Genotype	Source
THE31	*ade2::hisG/ade2::hisG* *HIS1/his1::dpl200* *ura3-iro1::imm434/ura3-iro1::imm434::URA3-IRO1* *ENO1/ENO1-tetR-ScHAP4AD-3XHA-ADE2*	[8]
CAAH	*ade2::hisG/ade2::hisG* *his1::URA3-dpl200/his1::dpl200* *ura3-iro1::imm434/ura3-iro1::imm434* *ENO1/ENO1-tetR-ScHAP4AD-3XHA-ADE2* *HYR1/HIS1-pTR-HYR1*	[8]
CAAH-1	*ade2::hisG/ade2::hisG* *his1::dpl200/his1::dpl200* *ura3-iro1::imm434/ura3-iro1::imm434* *ENO1/ENO1-tetR-ScHAP4AD-3XHA-ADE2* *HYR1/HIS1-pTR-HYR1*	This study
CAAH-2	*ade2::hisG/ade2::hisG* *his1::dpl200/his1::dpl200* *ura3-iro1::imm434/ura3-iro1::imm434* *ENO1/ENO1-tetR-ScHAP4AD-3XHA-ADE2* *hyr1::URA3-dpl200/HIS1-pTR-HYR1*	This study
CAAH-3	*ade2::hisG/ade2::hisG* *his1::dpl200/his1::dpl200* *ura3-iro1::imm434/ura3-iro1::imm434* *ENO1/ENO1-tetR-ScHAP4AD-3XHA-ADE2* *hyr1::dpl200/HIS1-pTR-HYR1*	This study
CAAH-31	*ade2::hisG/ade2::hisG*	This study

	his1::dpl200/his1::dpl200 *ura3-iro1::imm434/ura3-iro1::imm434::URA3-IRO1* *ENO1/ENO1-tetR-ScHAP4AD-3XHA-ADE2* *hyr1::dpl200/HIS1-pTR-HYR1*	
DAY185	*ura3-iro1::imm434/ura3-iro1::imm434* *his1::hisG::pHIS1/his1::hisG* *arg4::hisG::ARG4-URA3/arg4::hisG*	[17]
CJN702	*ura3-iro1::imm434/ura3-iro1::imm434* *his1::hisG::pHIS1/his1::hisG* *arg4::hisG/arg4::hisG* *bcr1::ARG4/bcr1::URA3*	[17]
CJN698	*ura3-iro1::imm434/ura3-iro1::imm434* *his1::hisG::pHIS1-BCR1/his1::hisG* *arg4::hisG/arg4::hisG* *bcr1::ARG4/bcr1::URA3*	[17]
CJN1144	*ura3-iro1::imm434/ura3-iro1::imm434* *his1::hisG::pHIS1-P_{TEF1}-ALS1-t_{TEF1}/his1::hisG* *arg4::hisG/arg4::hisG* *bcr1::ARG4/bcr1::URA3*	[17]
CJN1153	*ura3-iro1::imm434/ura3-iro1::imm434* *his1::hisG::pHIS1-P_{TEF1}-ALS3-t_{TEF1}/his1::hisG* *arg4::hisG/arg4::hisG* *bcr1::ARG4/bcr1::URA3*	[17]
CJN1222	*ura3-iro1::imm434/ura3-iro1::imm434* *his1::hisG::pHIS1-P_{TEF1}-HWP1-t_{TEF1}/his1::hisG* *arg4::hisG/arg4::hisG* *bcr1::ARG4/bcr1::URA3*	[17]
CJN1259	*ura3-iro1::imm434/ura3-iro1::imm434* *his1::hisG::pHIS1-P_{TEF1}-HYR1-t_{TEF1}/his1::hisG* *arg4::hisG/arg4::hisG* *bcr1::ARG4/bcr1::URA3*	[17]

CJN1276	*ura3-iro1::imm434/ura3-iro1::imm434* *his1::hisG::pHIS1-P_{TEF1}-RBT51-t_{TEF1}/his1::hisG* *arg4::hisG/arg4::hisG* *bcr1::ARG4/bcr1::URA3*	[17]
CJN1281	*ura3-iro1::imm434/ura3-iro1::imm434* *his1::hisG::pHIS1-P_{TEF1}-CHT2-t_{TEF1}/his1::hisG* *arg4::hisG/arg4::hisG* *bcr1::ARG4/bcr1::URA3*	[17]
CJN1288	*ura3-iro1::imm434/ura3-iro1::imm434* *his1::hisG::pHIS1-P_{TEF1}-ECE1-t_{TEF1}/his1::hisG* *arg4::hisG/arg4::hisG* *bcr1::ARG4/bcr1::URA3*	[17]
Candida glabrata strain BG14	*ura3Δ (-85 + 932)::Tn903NeoR*	[17]

Oligonucleotides

Oligonucleotides used for making and confirming *HYR1* conditional overexpression/suppression strain	
P1	5'-ACTTGGCACCAGGAACAAC
P2	5'-ACAGCTTTATCTCAGAAAAACTAGTAATAACAACATGAAAGTGGTATCA
P3	5'-CGACAAACACAACGGCACATTCTGGTTTCAACAAACTGGAATACTTTG
P4	5'-AGCAGTAACACAACCAGTACCT
PH1	5'-GTCGTCGCTGTGTTTGTC
PH2	5'-CGTTGGAGAAGGTAATTGTGA
P5	5'-CAGCATGAACAATCAAAGACGA
P6	5'-CAAAGTATTCCAGTTTGTTGAAACC
Oligonucleotides used for detecting *in vitro* expression	
HYR1 specific1	5'-CGTCAACCTGACTGTTACATC
HYR1 specific2	5'-TCTACGGTGGTATGTGGAAC
EFB1a	5'-ATTGAACGAATTCTTGGCTGAC
EFB1b	5'-CATCTTCTTCAACAGCAGCTTG
Oligonucleotides used for for *in vivo* expression	
EFB1F	5'-CACAAACCAATACATAATG
EFB1R	5'-GTAGACAGTGACATCAGC
EFB1nF	5'-TCAGATTTCTCTAAAGTCG
EFB1nR	5'-TGACATCAGCTTGAGTGG
G3PDHF	5'-GTCTTCACCACCATGGAGAAGG
G3PDHR	5'-TCGCTGTTGAAGTCAGAGGAGA

Oligonucleotides used for confirming *URA3-IRO1* in its original locus	
URA3 Conf1	5'-TGCTGGTTGGAATGCTTATTTG
URA3 Conf2	5'-TGCAAATTCTGCTACTGGAGTT
Oligonucleotides used for *HYR1* expression construct in *C. glabrata*	
CG-Hyr1-a	5'-ATATAAAACATCTAGATGAAAGTGGTATCAAACTTTATATTC
CG-Hyr1-b	5'-GGGTTGTGTTCTCGATCACATGAATAAAACAACCATG

EXAMPLE II

rHyr1p-N is a Vaccine Against Disseminated Candidiasis

Background: We have found that overexpression of *HYR1* by *Candida albicans* mediates resistance to neutrophil killing in vitro. We sought to determine the impact of *HYR1* overexpression on tissue fungal burden in vivo during infection, and to define the potential for vaccination with rHyr1p-N to protect against disseminated candidiasis in mice.

Methods: Mice were infected via the tail-vein with *HYR1* overexpression/suppression or wild-type *C. albicans*. Livers and spleens were harvested 1 day post infection and the expression levels of *HYR1* and tissue fungal burden were determined by qRT-PCR and quantitative culturing, respectively. For vaccination, rHyr1p-N was produced in *E. coli* pQE-32 expression system and purified per the manufactures' instruction (Qiagen). Mice were vaccinated with 20 μg of rHyr1p-N in complete Freund's adjuvant (CFA), boosted in incomplete Freund's adjuvant (IFA) at 3 weeks, and infected with *C. albcians* strain SC5314 two weeks post the boost. Control mice received adjuvant plus cell extract from *E. coli* transformed with empty plasmid.

Results: Overexpression of *HYR1* significantly increased fungal burden in both livers and spleens compared to control strain. Suppression of *HYR1* significantly reduced fungal burden in both livers and spleens compared to control strain. The relative level of expression of *HYR1* was 2.5 and 0.8 in livers infected with overexpression or suppression strain versus the control strain, respectively. The rHyr1p-N vaccine resulted in 62.5% long-term survival of infected mice, versos 0% survival in control mice.

Conclusions: *HYR1* expression affects the ability of *C. albicans* to infect tissues in vivo. Furthermore, vaccination with rHyr1p-N markedly protected mice from disseminated candidiasis. The rHyr1p-N vaccine is useful to prevent disseminated candidiasis.

References

1. Spellberg BJ, Filler SG, and Edwards JE, Jr. Current treatment strategies for disseminated candidiasis. Clin Infect Dis. 2006;42:244-251

2. Del Poeta M. Role of Phagocytosis in the Virulence of *Cryptococcus neoformans.* Eukaryot Cell 2004;3:1067-1075

3. Koh AY, Kohler JR, Coggshall KT, Van Rooijen N and Pier GB. Mucosal damage and neutropenia are required for *Candida albicans* dissemination. PLoS Pathog. 2008;4:DOI:10.1371/journal.ppat.0040035.

4. Gulay Z, Imir T. Anti-candidial activity of natural killer (NK) and lymphokine activated killer (LAK) lymphocytes *in vitro*. Immunobiology 1996;195:220-230

5. Stone HH. Studies in the pathogenesis, diagnosis, and treatment of *Candida* sepsis in children. J Pediatr Surg. 1974;9:127-133

6. Richard M, Ibata-Ombetta S, Dromer F, Bordon-Pallier F, Jouault T and Gaillardin C. Complete glycosylphosphatidylinositol anchors are required in *Candida albicans* for full morphogenesis, virulence and resistance to macrophages. Mol. Microbiol. 2002;44

7. Bailey DA, Feldmann PJ, Bovey M, Gow NA and Brown AJ. The *Candida albicans* HYR1 gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. J Bacteriol. 1996;178:5353-5360

8. Fu Y, Luo G., Spellberg BJ, Edwards JE, Jr, and Ibrahim AS. Gene overexpression/suppression analysis of candidate virulence factors of *Candida albicans*. Eukaryot Cell. 2008;7:483-492

9. Nusing R, Goerig M, Habenicht AJ and Ullrich V. Selective eicosanoid formation during HL-60 macrophage differentiation. Regulation of thromboxane synthase. Eur J Biochem 1993;212:371-376

10. Spellberg BJ, Collins M, French SW, Edwards JE, Jr., Fu Y and Ibrahim AS. A phagocytic cell line markedly improves survival of infected neutropenic mice. J Leukoc Biol 2005;78:338-344

11. Eiden-Plach A, Zagorc T, Heintel T, Carius Y, Breinig F. Viral preprotoxin signal sequence allows efficient secretion of green fluorescent protein by *Candida glabrata, Pichia pastoris, Saccharomyces cerevsiae,* and *Schizosaccharomyces pombe.* Appl.Environ.Microbiol 2004;70:961-966

12. Avrova AO, Venter E, Birch PR and Whisson SC. Profiling and quantifying differential gene transcription in *Phytophthora infestans* prior to and during the early stages of potato infection. Fungal Genetics & Biology 2003;40:4-14

13. Saville SP, Lazzell AL, Monteagudo C and Lopez-Ribot JL. Engineered control of cell morphology *in vivo* reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. Eukaryot Cell 2003;2:1053-1060.

14. Spellberg B, Ibrahim AS, Yeaman MR, et al. The antifungal vaccine derived from the recombinant N terminus of Als3p protects mice against the bacterium *Staphylococcus aureus*. Infect Immun. 2008;76:4574-4580

15. Fu Y, Ibrahim AS, Sheppard DC, Chen YC, French SW and Cutler JEea. *Candida albicans* Als1p: an adhesin that is a downstream effector of the EFG1 filamentation pathway. Mol Microbiol 2002;44:61-72

16. Ibrahim AS, Spellberg BJ, Avenissian V, Fu Y, Filler SG and Edwards JEJ. Vaccination with rAls1p-N improves survival during murine disseminated candidiasis by enhancing cell-mediated, not humoral, immunity. Infect Immun 2005;73:999-1005

17. Nobile CJ, Andes DR, Nett JE, et al. Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation *in vitro* and *in vivo*. PLoS Pathog. 2006;2:e63

18. Fradin C, De Groot P, MacCallum D, et al. Granulocytes govern the transcriptional response, morphology and proliferation of *Candida abicans* in human blood. Molecular Microbiology 2005;56:397-415

19. Castano I, Pan SJ, Zupancic M, Hennequin C, Dujon B and Cormack BP. Telomere length control and transcriptional regulation of subtelomeric adhesins in *Candida glabrata.* Mol Microbiol 2005;55:1246-1258

20. Schofield DA, Westwater C, Warner T, Nicholas PJ, Paulling EE and Balish E. Hydrolytic gene expression during oroesophageal and gastric candidiasis in immunocompetent and immunodeficient gnotobiotic mice. J Infect Dis 2003;188:591-599

21. Kumamoto CA, Vinces MD. Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. Cell Microbiol. 2005;7:1546-1554

22. Frohner IE, Bourgeois C, Yatsyk K, Majer O and Kuchler K. *Candida albicans* cell surface superoxide dismutases degrade host-derived reactive oxygen

species to escape innate immune surveillance. Mol Microbiol 2009;71:240-252

What is claimed is:

5

CLAIMS

1. A vaccine comprising a polypeptide substantially identical to a fragment of a HYR1 polypeptide.

2. The vaccine of claim 1, wherein said HYR1 polypeptide is

```
1     MKVVSNFIFTILLTLNLSAALEVVTSRIDRGGIQGFHGDVKVHSGATWAILGTTLCSFFG
61    GLEVEKGASLFIKSDNGPVLALNVALSTLVRPVINNGVISLNSKSSTSFSNFDIGGSSFT
121   NNGEIYLDSSGLVKSTAYLYAREWTNNGLIVAYQNQKAAGNIAFGTAYQTITNNGQICLR
181   HQDFVPATKIKGTGCVTADEDTWIKLGNTILSVEPTHNFYLKDSKSSLIVHAVSSNQTFT
241   VHGFGNGNKLGLTLPLTGNRDHFRFEYYPDTGILQLRADALPQYFKIGKGYDSKLFRIVN
301   SRGLKNAVTYDGPVPNNEIPAVCLIPCTNGPSAPESESDLNTPTTSSIETSSYSSAATES
361   SVVSESSSAVDSLTSSSLSSKSESSDVVSSTTNIESSSTAIETTMNSESSTDAGSSSISQ
421   SESSSTAITSSSETSSSESMSASSTTASNTSIETDSGIVSQSESSSNALSSTEQSITSSP
481   GQSTIYVNSTVTSTITSCDENKCTEDVVTIFTTVPCSTDCVPTTGDIPMSTSYTQRTVTS
541   TITNCDEVSCSQDVVTYTTNVPHTTVDATTTTTTSTGGDNSTGGNESGSNHGPGNGSTEG
601   SGNGSGAGSNEGSQSGPNNGSGSGSEGGSNNGSGSDSGSNNGSGSGSNNGSGSGSTEGSE
661   GGSGSNEGSQSGSGSQPGPNEGSEGGSGSNEGSNHGSNEGSGSGSGSGSNNGSGSGSQSG
721   SGSGSQSGSESGSNSGSNEGSNPGAGNGSNEGSGQGSGNGSEAGSGQGSGPNNGSGSGHN
781   DGSGSGSNQGSNPGAGSGSGSESGSKAGSHSGSNEGAKTDSIEGFHTESKPGFNTGAHTD
841   ATVTGNSVANPVTTSTESDTTISVTVSITSYMTGFDGKPKPFTTVDVIPVPHSMPSNTTD
901   SSSSVPTIDTNENGSSIVTGGKSILFGLIVSMVVLFM (SEQ ID NO.:1).
```

3. The vaccine of claim 1, further comprising an adjuvant.

4. The vaccine of claim 1, wherein said fragment of the HYR1 polypeptide is expressed in a *Candida* strain selected from the group consisting of *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Candida glabrata*, and *Candida parapsilosis*.

5. The vaccine of claim 1, wherein said fragment consists of an N-terminal region fragment of the HYR1 polypeptide.

6. The vaccine of claim 4, wherein said fragment is

```
1     TSRIDRGGIQ GFHGDVKVHS
21    GATWAILGTT LCSFFGGLEV
41    EKGASLFIKS DNGPVLALNV
61    ALSTLVRPVI NNGVISLNSK
81    SSTSFSNFDI GGSSFTNNGE
101   IYLASSGLVK STAYLYAREW
121   TNNGLIVAYQ NQKAAGNIAF
141   GTAYQTITNN GQICLRHQDF
161   VPATKIKGTG CVTADEDTWI
181   KLGNTILSVE PTHNFYLKDS
201   KSSLIVHAVS SNQTFTVHGF
221   GNGNKLGLTL PLTGNRDHFR
241   FEYYPDTGIL QLRAAALPQY
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261        FKIGKGYDSK LFRIVNSRGL
281        KNAVTYDGPV PNNEIPAVCL
301        IPCTNGPSAP ESESDLNTPT
321        TSSIET (SEQ ID NO.:2).
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7. The vaccine of claim 6, wherein said fragment is a fusion polypeptide.

8. The vaccine of claim 7, wherein in said fragment is fused to a heterologous leader sequence.

9. The vaccine of claim 7, wherein said fragment is fused to a tag or a linker sequence.

10. The vaccine of claim 6, wherein said tag is a histidine tag.

11. The vaccine of claim 1, wherein said fragment is obtained from a transformed cell.

12. The vaccine of claim 11, wherein said transformed cell is a transformed *Saccharomyces cerevisae* cell.

13. A method of treating or preventing a candidiasis infection, said method comprising administering an immunogenic amount of a vaccine of any one of claims 1 to 12.

14. The method of claim 13, wherein said candidiasis infection is disseminated candidiasis.

15. The method of claim 13, wherein said administering comprises active immunization, passive immunization, or a combination thereof.

16. A method of treating or preventing a candidiasis infection, said method comprising administering an effective amount of an isolated polypeptide substantially identical to a fragment of a HYR1 polypeptide.

17. The method of claim 16, wherein said fragment of the HYR1 polypeptide is expressed in a *Candida* strain selected from the group consisting of *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Candida glabrata*, and *Candida parapsilosis*.

18. The method of claim 16, wherein said fragment consists of an N-terminal region fragment of the HYR1 polypeptide.

19. The method of claim 18, wherein said fragment is SEQ ID NO:2.

20. The method of claim 19, wherein said fragment is a fusion polypeptide.

21. The method of claim 20, wherein in said fragment is fused to a heterologous leader sequence.

22. The method of claim 21, wherein said fragment is fused to a tag or a linker sequence.

23. The method of claim 22, wherein said tag is a histidine tag.

24 The method of claim 16, wherein said fragment is obtained from a transformed cell.

25. The method of claim 24, wherein said transformed cell is a transformed *Saccharomyces cerevisae* cell.