VACCINE COMPOSITIONS AND METHODS FOR TREATMENT OF MUCORMYCOSIS AND OTHER FUNGAL DISEASES

BACKGROUND OF THE INVENTION

This invention was made in part with U.S. Government support under NIH grant 011671 awarded by NIAID. The U.S. Government can have certain rights in the invention.

This invention generally relates to compositions and methods to vaccinate subjects against infectious diseases and, more particularly, relates to compositions and methods to vaccinate subjects against opportunistic fungal diseases.

About 180 of the 250,000 known fungal species are recognized to cause disease (*mycosis*) in man and animal. Some of fungi can establish an infection in all exposed subjects, e.g., the systemic pathogens *Histoplasma capsulatum* and *Coccidioides immitis*. Others, such as *Candida*, *Asergillus* species and *Zygomycetes* are opportunist pathogens which ordinarily cause disease only in a compromised host. Fungi of the class *Zygomycetes*, order Mucorales, can cause Mucormycosis, a potentially deadly fungal infection in human. Fungi belonging to the order Mucorales are distributed into at least six families, all of which can cause mucormycosis (Ibrahim et al. *Zygomycosis*, p. 241-251, In W. E. Dismukes, P. G. Pappas, and J. D. Sobel (ed.), *Clinical Mycology*, Oxford University Press, New York (2003); Kwon-Chung, K. J., and J. E. Bennett, *Mucormycosis*, p. 524-559, *Medical Mycology*, Lea & Febiger, Philadelphia (1992), and Ribes et al. *Zygomycetes in Human Disease*, *Clin Microbiol Rev* 13:236-301 (2000)). However, fungi belonging to the family *Mucoraceae*, and specifically the species *Rhizopus oryzae* (*Rhizopus arrhizus*), are by far the most common cause of infection (Ribes et al., *supra*). Increasing cases of mucormycosis have been also reported due to infection with *Cunninghamella spp.* in the *Cunninghamellaceae* family (Cohen-Abbo et al., *Clinical Infectious Diseases* 17:173-77 (1993); Kontoyianis et al., *Clinical Infectious Diseases* 18:925-28 (1994); Kwon-Chung et al., *American Journal of Clinical Pathology* 64:544-48 (1975), and Ventura et al., *Cancer* 58:1534-36 (1986)). The remaining four families of the *Mucorales* order are less frequent causes of disease (Bearer et al., *Journal of Clinical Microbiology* 32:1823-24 (1994); Kamalam and Thambiah, *Sabouraudia* 18:19-20 (1980); Kemna et al., *Journal of Clinical Microbiology* 32:843-45 (1994); Lye et al., *Pathology* 28:364-65 (1996), and Ribes et al., (*supra*)).

The agents of mucormycosis almost uniformly affect immunocompromised hosts (Spellberg et al., *Clin. Microbiol. Rev.* 18:556-69 (2005)). The major risk factors for mucormycosis include uncontrolled diabetes mellitus in ketoacidosis known as diabetes ketoacidosis (DKA), other forms of metabolic acidosis, treatment with corticosteroids, organ or bone marrow transplantation, neutropenia, trauma and burns, malignant hematological disorders, and deferoxamine chelation-therapy in subjects receiving hemodialysis.

Recent reports have demonstrated a striking increase in the number of reported cases of mucormycosis over the last two decades (Gleissner et al., *Leuk. Lymphoma* 45(7):1351-60 (2004)). There has also been an alarming rise in the incidence of mucormycosis at major transplant centers. For example, at the Fred Hutchinson Cancer Center, Marr et al. have described a greater than doubling in the number of cases from 1985-1989 to 1995-1999 (Marr et al., *Clin. Infect. Dis.* 34(7):909-17 (2002)). Similarly, Kontoyiannis et al. have described a greater than doubling in the incidence of mucormycosis in transplant subjects over a similar time-span (Kontoyiannis et al, *Clin. Infect. Dis.* 30(6):851-6 (2000)). Given the increasing prevalence of diabetes, cancer, and organ transplantation in the aging United States population, the rise in incidence of mucormycosis is anticipated to continue unabated for the foreseeable future.

Available therapies for invasive mucormycosis include attempts to reverse the underlying predisposing factors, emergent, wide-spread surgical debridement of the infected area, and adjunctive antifungal therapy (Edwards, J., Jr., Zygomycosis, p. 1192-1199. In P. Hoeprich and M. Jordan (ed.), *Infectious Disease*, 4th ed. J.B. Lippincott Co., Philadelphia (1989); Ibrahim et al., (2003), *supra*; Kwon-Chung and Bennett, *supra*; Sugar, A. M., Agent of Mucormycosis and Related Species, p. 2311-2321. In G. Mandell, J. Bennett, and R. Dolin (ed.), *Principles and Practices of Infectious Diseases*, 4th ed. Churchill Livingstone, New York (1995)).

Currently, Amphotericin B (AmB) remains the only antifungal agent approved for the treatment of invasive mucormycosis (*Id.*). Because the fungus is relatively resistant to AmB, high doses are required, which frequently cause nephrotoxicity and other adverse effects (Sugar, *supra*). Also, in the absence of surgical removal of the infected focus (such as excision of the eye in subjects with rhinocerebral mucormycosis), antifungal therapy alone is rarely curative (Edwards, J. (1989), *supra*; Ibrahim et al., (2003), *supra*). Even when surgical debridement is combined with high-dose AmB, the mortality associated with mucormycosis exceeds 50% (Sugar, *supra*). In subjects with disseminated disease mortality approaches

100% (Husain et al., *Clin Infect Dis* 37:221-29 (2003)). Because of this unacceptably high mortality rate, and the extreme morbidity of highly disfiguring surgical therapy, it has been imperative to develop new strategies to treat and prevent invasive mucormycosis.

One of the underlying factors in predisposition to fungal infection is elevated serum iron levels. Subjects who have elevated available serum iron are hypersusceptible to mucormycosis. Iron is required by virtually all microbial pathogens for growth and virulence. In mammalian hosts, very little serum iron is available to microorganisms because it is highly bound to carrier proteins such as transferrin. Although sequestration of serum iron is a major host defense mechanism against pathogenic fungi, subjects treated with exogenous iron chelators e.g., deferoxamine have a markedly increased incidence of invasive mucormycosis, which is associated with a mortality of >80%. While deferoxamine is a chelator from the perspective of the human host, it predisposes subjects to mucormycosis by acting as a siderophore, supplying previously unavailable iron to the pathogenic fungi.

Therefore, there exists a need for compounds and methods that can reduce the risk of mucormycosis pathogenesis and provide effective therapies without adverse effects. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

In a first aspect, the present invention refers to a vaccine composition, comprising an *FTR* polypeptide, or an antigenic fragment of the polypeptide, and a pharmaceutically acceptable carrier. In a second aspect, the present invention refers to a vaccine composition, comprising a vector comprising a nucleotide sequence that is substantially complimentary to at least 18 contiguous nucleotides of *FTR* sequence, a transcription promoter, and a transcription terminator; wherein the promoter is operably linked to the *FTR* nucleotide sequence, and wherein the *FTR* nucleotide sequence is operably linked to the transcription terminator, and a pharmaceutically acceptable carrier. The vaccine compositions of the present invention can further include an adjuvant.

In addition, the invention describes a pharmaceutical composition for treating or preventing a fungal condition in a subject in need thereof, including an antisense, a small interfering RNA or an antibody inhibitor of *FTR* selected from the group consisting of a nucleotide sequence that is substantially complimentary to a portion of an *FTR* sequence; a nucleotide sequence that is substantially complimentary to at least 12 contiguous nucleotide bases of *FTR* sequence; a nucleotide RNAi sequence that is substantially complimentary to at least 18

contiguous nucleotide bases of *FTR* sequence; an antibody or antibody fragment thereof that specifically binds to an *FTR* polypeptide or a fragment thereof; and a pharmaceutically acceptable excipient or carrier.

In a third aspect, the present invention refers to the use of an antisense, a small interfering RNA or an antibody inhibitor of *FTR* selected from the group consisting of a nucleotide sequence that is substantially complimentary to a portion of an *FTR* sequence encoding for *FTR* polypeptide as defined above; a nucleotide sequence that is substantially complimentary to at least 12 contiguous nucleotide bases of *FTR* sequence encoding for *FTR* polypeptide as defined above and a pharmaceutically acceptable excipient or carrier in the manufacture of a vaccine for treating or preventing a fungal condition in a subject in need thereof.

In a fourth aspect, the present invention refers to the use of an immunogenic amount of an *FTR* polypeptide, or an immunogenic fragment thereof in the manufacture of a vaccine for treating or preventing a fungal condition, wherein said vaccine is to be administered to a subject having, or susceptible to having, a fungal condition, wherein said fungal condition comprises zygomycosis.

In a fifth aspect, the present invention refers to the use of an antisense, a small interfering RNA or an antibody (polyclonal or monoclonal) inhibitor of *FTR* nucleotide sequence encoding for *FTR* polypeptide as defined above in the manufacture of a vaccine for treating or preventing a fungal condition in a subject in need thereof, wherein the use comprises exposing said fungi to said vaccine, wherein said fungal condition comprises zygomycosis.

In addition, the invention describes a method of treating or preventing a fungal condition, including administering to a subject having, or susceptible to having, a fungal condition an immunogenic amount of an *FTR* polypeptide, or an immunogenic fragment thereof. In addition, the invention provides a method for treating or preventing a fungal condition in a subject in need thereof, including exposing said fungi to an antisense, a small interfering RNA or an antibody inhibitor of *FTR*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows Rhizopus *oryzae* high affinity iron permease nucleotide sequence (SEQ ID NO: 1), with Genbank cDNA accession NO. AY344587.

Figure 2 shows Rhizopus *oryzae* high affinity iron permease polypeptide sequence (SEQ ID NO:2), with Genbank protein ID. No. AAQ24109.1.

Figure 3 shows amino acid sequence alignment (a) and dendrogram (b) for *FTR* of *R. oryzae* having 46% and 44% identity with *FTR* of *C. albicans* and *S. cerevisiae*, respectively. Box on amino acid sequence alignment indicates the conserved REGLE motif involved in a direct interaction with iron.

Figure 4 shows mechanisms of iron uptake by *Zygomycetes* in conditions of elevated available serum iron.

Figure 5 shows the *FTR* expression in *R. oryzae* grown in media with varying concentrations of iron.

Figure 6 shows the growth of *S. cerevisiae ftr1* mutant transformed with vector expressing *FTR*.

Figure 7 shows high affinity iron uptake by *S. cerevisiae ftr1* mutant transformed with vector expressing *FTR* as compared with iron uptake by wild-type *S. cerevisiae* and *S. cerevisiae ftr1* mutant transformed with empty vector. $^{*}P < 0.05$.

Figure 8 shows the percent survival of diabetic mice (n = 10) infected with *R. oryzae* as compared with non-diabetic infected and diabetic uninfected mice.

Figure 9 shows a temporal link or an inverse correlation between percent survival and the kidney burden of *R. oryzae* (5×10^4 spores) as determined by TaqMan assay.

Figure 10 shows the percent survival of DKA mice (n=20) (A) and tissue fungal burden (n= 11) (B) infected with 5×10^3 *R. oryzae* spores and treated with: 1) deferasirox (given orally); 2) deferasirox + saturating $FeCl_3$ (given i.p,); 3) intravenous LAmB for 4 days; and 4) placebo. Uninfected DKA mice and uninfected treated with $FeCl_3$ were included as negative controls. *$p < 0.05$ vs. placebo or deferasirox +iron.

Figure 11 shows the expression of *FTR* in the hematogenously disseminated mucormycosis model using DKA mice. Mice were infected with 10^5 spores of *R. oryzae* 99-880 through the tail vein. At indicated time points infected brains were removed and total RNA was then used for real-time-RT-PCR analysis (n=4 mice per time point). Brains from uninfected mice served as a negative control. Values are expressed as average $\pm$ SD.

Figure 12 shows the expression of *FTR* in the brains of DKA mice infected with *R. oryzae* expressing GFP under the control of *FTR* promoter. (A) H & E stain of brain infected with *R. oryzae*; (B) brain section stained with rabbit polyclonal antibody to GFP then counter stained with FITC conjugated anti-rabbit antibody; and (C) DIC confocal image showing non-fluorescent *R. oryzae* at the time of infection. Arrows denote fungal elements in infected brains. Magnification, X 400.

Figure 13 shows the agarose gel electrophoresis result of an RT-PCR assay showing lack of expression of *FTR* in *R. oryzae* transformed with RNA-interference plasmid (T_1 and T_3-T_5) as compared to *R. oryzae* transformed with empty plasmid (C). Primers amplifying the 18s rDNA served as a control to demonstrate the specificity of RNA interference in targeting *FTR*.

Figure 14 shows the percent survival of DKA mice (n=8) infected *i.v.* with *R. oryzae* transformed with empty plasmid (control strain, 2.9×10^3 spores) or with RNAi plasmid targeting expression of *FTR* (*FTR*-i, 4.1×10^3 spores). *, $P<0.001$ by Log Rank test.

Figure 15 shows *Aspergillus fumigatus* high affinity iron permease nucleotide sequence.

Figure 16 shows *Candida guilliermondii* high affinity iron permease nucleotide sequence.

Figure 17 shows *Aspergillus flavus* high affinity iron permease nucleotide sequences.

Figure 18 shows *Candida tropicalis* high affinity iron permease nucleotide sequence.

Figure 19 shows a conceptual model of the *Rhizopus* rFtr1p helical bundle protein and translocation of iron from the extracellular setting into the cytoplasm of *Rhizopus* species.

Figure 20 shows results of an SDS-PAGE demonstrating purified synthetic/recombinant rFtr1p. *E. coli* was transformed with a plasmid expressing 6X-His tagged synthetic *rFTR1* or with empty plasmid. rFtr1p was purified by Ni-agrose column and detected at the expected size of 28 kD in the rFtr1p clone but not when *E. coli* was transformed with empty plasmid.

Figure 21 shows survival of DKA mice (n=8) infected with *R. oryzae* (2.5 x 107 spores) and treated with serum collected from mice immunized with either rFtr1p or empty plasmid.*, P<0.007 by Log Rank test.

Figure 22 shows that *FTR1* is expressed in DKA mice infected intravenously with *R. oryzae*. Panel (A) shows FACS analysis of *R. oryzae* transformed with plasmid containing the reporter gene GFP driven by either the *FTR1* promoter or the constitutively expressed *ACT1* promoter and grown in iron-rich or iron-depleted conditions. *R. oryzae* M16 transformed with an empty plasmid was used as a negative control. Panel (B) shows *FTR1* is expressed in the brains of DKA mice infected with *R. oryzae* expressing GFP under the control of FTR1p. For anti-GFP Ab stain, tissue section was stained with rabbit polyclonal antibody to GFP then counter stained with FITC conjugated anti-rabbit antibody. For DIC, confocal image showing non-fluorescent *R. oryzae* at the time of infection. Arrows denote fungal elements in infected brains. Magnification, X 400.

Figure 23 shows that the disruption cassette integrates in FTR1 locus but complete elimination of FTR1 could not be achieved. Panel (A) A diagram summarizing the strategy we used to achieve FTR1 disruption. PyrF (998 bp) was used as a selectable marker flanked by 606 and 710 bp fragments of FTR1-5' UTR and FTR1-3' UTR, respectively. Panel (B) Gel electrophoresis showing integration of the disruption cassette in a representative putative ftr1 null mutant (KO) but not in the wild-type (WT) (see 5'UTR and 3'UTR). Primers FTR1 P11 and FTR1 P12 were used to amplify 503 bp from the FTR1 ORF only from the wild-type but not from the putative ftr1 null mutant (see FTR1). Primers PyrF P9 and PyrF P18 to test for possible reciculization of the transformed plasmid with expected band of 2094 bp were also used (see self ligation). Panel (C) Comparison of growth rate of *R. oryzae* wild-type, *R. oryzae* PyrF-complemented, or putative ftr1 null mutants grown on different sources of iron on iron-limited or iron-rich media. Growth was measured after 48 h for media containing 10 or 1000 μM (iron-rich) of FeCl3 or FeSO4 or 100 μM of ferrioxamine, while growth was

measured after 72 h for medium supplemented with 100 μM heme. Values are expressed as increase in mycelial diameter growth on solid growth medium in cm/h. * P<0.05 compared to wild-type or *R. oryzae* PyrF-complemented strains. Panel (D) Gel electrophoresis showing lack of amplification of FTR1 after one round of purification of the putative null mutants on iron-rich medium (1000 μM FeCl3) and amplification of the FTR1 from the same isolate following growth on iron-depleted medium (i.e. 100 μM ferrioxamine) for 96 h. Amplification of actin (600 bp) was used to control for DNA loading.

Figure 24 shows confirmation of the lack of complete disruption of FTR1 in the multinucleated *R. oryzae*. Panel (A) DAPI stain of swollen *R. oryzae* spores showing the presence of multiple nuclei with a single spore. Arrows denote nuclei. Original magnification, x1000. Panel (B) Gel electrophoresis showing lack of amplification of FTR1 after 14 passages of the putative null mutants on iron-rich medium (1000 μM FeCl3) and amplification of the FTR1 from the same isolate following growth on iron-depleted medium (i.e. 100 μM ferrioxamine) for 96 h. Amplification of actin (600 bp) was used to confirm the integrity of DNA used as template and the absence of PCR inhibitors. Panel (C) Southern blot confirming the integration of the disruption cassette in the putative ftr1 (7380 bp band is present only in DNA sample extracted from putative ftr1 grown in iron-rich medium) and almost complete elimination of the FTR1 copy (lack of 1960 bp in DNA sample extracted from putative ftr1 grown in iron-rich medium).

Figure 25 shows that reduced copy number results in compromised ability of *R. oryzae* to take up iron. Panel (A) Quantitative PCR demonstrating reduced copy number in the putative ftr1 null mutant compared to *R. oryzae* PyrF-complemented strain or to the same mutant grown in iron-depleted medium. Panel (B) Gel electrophoresis of samples taken from the qPCR tube showing the amplification specificity for the FTR1 product. Panel (C) The putative ftr1 mutant demonstrated reduced ability to acquire 59Fe compared to *R. oryzae* wild-type or *R. oryzae* PyrF-complemented strains. 59Fe uptake by wild-type, *R. oryzae* PyrF-complemented, or putative ftr1 mutant. Germinated spores were incubated with 0.1 μM 59FeCl3 (a concentration in which high-affinity iron permeases are induced (Fu et al., *FEMS Microbiol Lett* 235: 169-176 (2004)). *P <0.05 when compared with *R. oryzae* wild-type or *R. oryzae* PyrF-complemented strains. Data (n= 9 from three separate experiments) are expressed as medians + interquartile ranges.

Figure 26 shows how the reduction of FTR1 copy number reduces R. oryzae virulence in the DKA mouse models. Panel (A) a representative of the putative ftr1 null mutant demonstrated

comparable growth to *R. oryzae* PyrF-complemented strain on YPD or CSM-URA media. Panel (B) Survival of mice (n=8) infected i.v. with *R. oryzae* wild-type (4.3 x 103), *R. oryzae* PyrF-complemented strain (4.8 x 103 spores) or with putative ftr1 null mutant (3.0 x 103 spores). *, P<0.0005 compared to wild-type or PyrF-complemented strains. Panel (C) Survival of mice (n= 9) infected intranasally with *R. oryzae* wild-type (4.3 x 103 spores), *R. oryzae* PyrF-complemented strain (5.1 x 103 spores) or putative ftr1 null mutant (5.3 x 103 spores). *, P=0.04 compared to wild-type or PyrF-complemented strains.

Figure 27 shows how inhibition of *FTR1* expression reduces *R. oryzae* ability to take up ^{59}Fe *in vitro*. (A) RT-PCR showing lack of expression of *FTR1* in *R. oryzae* transformed with RNA-interference plasmid (T_1 and T_3-T_5) compared to *R. oryzae* transformed with empty plasmid (C, control). Primers amplifying the 18s rDNA served as a control to demonstrate the integrity of starting sample and lack of PCR inhibitors. (B) a representative of the RNAi transformants demonstrated comparable growth to the *R. oryzae* M16 transformed with empty plasmid on YPD or CSM-URA media. (C) ^{59}Fe uptake by wild-type, *R. oryzae* M16 transformed with the empty plasmid, or one of the RNAi transformants. Germinated spores were incubated with 0.1 μM $^{59}FeCl_3$ (a concentration in which high-affinity iron permeases are induced (Fu et al., *FEMS Microbiol Lett* 235: 169-176 (2004)). *P <0.05 when compared with *R. oryzae* wild-type or *R. oryzae* M16 transformed with empty plasmid. Data (n= 9 from three separate experiments) are expressed as medians ± interquartile ranges.

Figure 28 shows how inhibition of *FTR1* expression reduces virulence of *R. oryzae* in the DKA mouse models and passive immunization with anti-Ftr1p sera protects DKA mice from *R. oryzae* infection. Panel (A) Survival of mice (n=8) infected i.v. with *R. oryzae* transformed with empty plasmid (control strain, 2.9 x 10^3 spores) or with RNA-i plasmid targeting expression of *FTR1* (FTR1-i, 4.1 x 10^3 spores). *, P<0.001. Panel (B) Survival of mice (n=9) infected intranasally with *R. oryzae* transformed with empty plasmid (control strain, 2.8 x 10^3 spores) or with RNAi plasmid targeting expression of *FTR1* (FTR1-i, 7.6 x 10^3 spores). *, P<0.02. Panel (C) Kidney or brain Fungal burden of mice (n=8) infected i.v. with *R. oryzae* transformed with empty plasmid (control strain, 4.2 x 10^3 spores) or with RNAi plasmid targeting expression of *FTR1* (FTR1-i, 5.1 x 10^3 spores). *, P<0.0006 and ¥, P<0.04 compared to control strain. Data are expressed as medians + interquartile ranges. The y-axes reflect lower limits of detection of the assay. (D) Survival of mice (n=8) infected intranasally with *R. oryzae* (intended inoculum of 2.5 x 10^7 spores and actual inhaled inoculum of 9 x 10^3 spores)

and treated with serum collected from mice immunized with either Ftr1p or proteins collected form empty plasmid clone. *, $P<0.007$.

DETAILED DESCRIPTION OF THE INVENTION

This invention is directed to the use of compositions and methods that directly and/or indirectly inhibit the high affinity iron permease (*FTR*) of pathogenic fungi, specifically those involved in the onset of mucormycosis. High affinity iron permease is a molecule responsible for the uptake of iron in fungi; targeting and inhibition of this molecule, therefore, will impede the ability of the fungi to uptake and/or use the iron available in the surrounding environment. Inhibition of high affinity iron permease will result in iron-starvation in fungal pathogens hampering their growth and/or virulence. The *FTR* polypeptide in, for example, *R. oryzae* has little or no homology with any known human proteins. For example, homology search of the human proteome identified five open reading frames with extremely limited homology to *R. oryzae*'s *FTR* protein with an alignment score of 30.4, e= 9.0 for all of the five proteins. Three of these proteins are *coiled-coil domain containing 82* (i.e., EAW66982; AAH33726.1; and NP_079001.2), one is a *CCDC82 protein* (i.e., AAH18663.1) and an unnamed protein (i.e., BAB15683.1) As a benchmark, the standard BLAST search e value for identification of unique sequences from fungi compared to other organisms has been set at 10^{-8}, indicating that rFtr1p has no significant homology to the human proteome. Therefore, the compositions and methods of the current invention in targeting and inhibiting *FTR* will only affect the iron levels in the fungal pathogen not the host, which constitutes an effective and targeted therapy against mucormycosis.

In one embodiment, the invention is directed to an immunogenic composition such as a vaccine. The immunogenic composition includes an effective dose of fungal *FTR* polypeptide or an antigenic fragment thereof that confer protection against mucormycosis in a subject. The vaccine composition of the invention induces host humoral and/or cell mediated immune response against fungal *FTR*. In another embodiment, a composition of the invention further includes an adjuvant that can boost the immunogenecity of the vaccine composition.

In yet another embodiment, the invention includes an inhibitor of *FTR* molecule such as siRNA, for example. The *FTR* inhibitor includes a vector expressing one or more siRNAs that include sequences sufficiently complementary to a portion of the *FTR* molecule for inhibiting *FTR* transcription or translation levels. For example as described in Example 9, interfering RNAs against *FTR* of *R. oryzae* were prepared, which were shown to inhibit *FTR* expression

in these fungi. In DKA mice, it was demonstrated that *R. oryzae* transformants harboring anti-*FTR* siRNAs were less virulent than the wild type *R. oryzae.*

As used herein, the term "*FTR*" refers to high affinity iron permease, a membrane protein responsible for iron transport in pathogenic fungi, such as, but not limited to *FTR* in *R. oryzae, A. fumigatus, C. guilliermondii, A flavus,* and *C. tropicalis;* and the nucleic acids encoding the same. As shown in Figure 3 and described in Example 1, for example, *FTR*s from *R. oryzae*, *C. albicans* and *S. cerevisiae* share percent identities of 39% or more with multiple regions of protein sequence homology. The nucleotide sequence of *FTR* in, for example, *R. oryzae* is shown in Figure 1 (SEQ ID NO:1), and the corresponding amino acid sequence is shown in Figure 2 (SEQ ID NO:2). The nucleotide sequence of *FTR*, in *A. fumigatus* is shown in Figure 15; in *C. guilliermondii* is shown in Figure 16; in *A flavus* is shown in Figure 17; and in *C. tropicalis* is shown in Figure 18. Throughout the present specification, the terms "*FTR* expression" or "expressing *FTR*" can be employed to designate indifferently expression of an *FTR* nucleic acid or an *FTR* polypeptide.

Generally, nucleic acid is an RNA, for example, mRNA or pre-mRNA, or DNA, such as cDNA and genomic DNA. An *FTR* nucleic acid, for example, refers to a nucleic acid molecule (RNA, mRNA, cDNA, or genomic DNA, either single-or double-stranded) corresponding to *FTR* polypeptide or an immunogenic fragment thereof. DNA molecules can be doubled-stranded or singled-stranded; single stranded RNA or DNA can be either the coding or sense strand, or the non-coding or antisense strand. The nucleic acid molecule or nucleotide sequence can include all or a portion of the coding sequence of the gene and can further include additional non-coding sequences such as introns and non-coding 3' and 5' sequences (including promoter, regulatory, poly-A stretches or enhancer sequences, for example). In addition, the nucleic acid molecule or nucleotide sequence can be fused to another sequence, for example, a label, a marker or a sequence that encodes a polypeptide that assists in isolation or purification of the polypeptide. Such sequences include, but are not limited to, those that encode a selection marker (e.g. an antibiotic resistance gene, or a reporter sequence), those that encode a repetition of histidine (HIS tag) and those that encode a glutathione-S-transferase (GST) fusion protein. The nucleic acid molecule or nucleotide sequence can include a nucleic acid molecule or nucleotide sequence which is synthesized chemically or by recombinant means, such nucleic acid molecule or nucleotide sequence is suitable for use in recombinant DNA processes and within genetically engineered protein synthesis systems.

The term "polypeptide" refers to a chain of two or more amino acids covalently linked by a peptide bond. Particular polypeptides of interest in the context of this invention are amino acid subsequences having antigenic epitopes. Antigenic epitopes are well known in the art and include sequence and/or structural determinants substantially responsible for the immunogenic properties of a polypeptide and being capable of evoking an immune response. Functional domains of the *FTR* polypeptide are also considered to fall within the scope of the invention. For example, the *REGLE* motif which interacts with iron is one exemplary functional domain of the invention. Another exemplary functional domain is the cell surface EXXE motif of *FTR* which is required for full function of *FTR* in *Saccharomyces cerevisiae* (Stearman et al., *Science* 271: 1552-1557 (1996)). Polypeptides also undergo maturation or post-translational modification processes that can include, for example, glycosylation, proteolytic cleavage, lipidization, signal peptide cleavage, propeptide cleavage, phosphorylation, and such like.

The term "immunogenic" or "antigenic" as it is used herein refers to a portion of a protein that is recognized by a T-cell and/or B-cell antigen receptor. The immunogenic portion generally includes at least 5 amino acid residues, preferably at least 10, more preferably at least 20, and still more preferably at least 30 amino acid residues of an *FTR* polypeptide or a variant thereof. Preferred immunogenic portions can contain a small N-and/or C-terminal fragment (e.g., 5-30 amino acids, preferably 10-25 amino acids).

A variant polypeptide contains at least one amino acid change compared to the target polypeptide. Polypeptide variants of *FTR* can exhibit at least about 39%, more preferably at least about 50%, and even more preferably at least about 70% identity to the *FTR* polypeptide. A polynucleotide variant includes a substantially homologous polynucleotide that deviates in some bases from the identified polynucleotide, usually caused by mutations such as substitution, insertion, deletion or transposition. Polynucleotide variants preferably exhibit at least about 60% (for fragments with 10 or more nucleotides), more preferably at least about 70%, 80% or 90%, and even more preferably at least about 95%, 98% or 99% identity to the identified polynucleotide.

The term "fragment" as used herein with reference to an *FTR* polypeptide is intended to refer to a polypeptide having a portion of *FTR* amino acid sequence. Useful fragments include those that retain one or more of the biological activities of the polypeptide. Such biologically active fragments can have a wide range of lengths including, for example, 4, 6, 10, 15, 20, 25, 30, 40, 50, 100, or more amino acid in length. In addition to activity, biologically active fragments also can be characterized by, for example, a motif, domain, or segment that has been

identified by analysis of the polypeptide sequence using methods well known in the art. Such regions can include, for example, a signal peptide, extracellular domain, transmembrane segment, ligand binding region, zinc finger domain and/or glycosylation site.

The term "vaccine", as used herein, refers to a composition that can be administered to an animal to protect the animal against an infectious disease. Vaccines protect against diseases by inducing or increasing an immune response in an animal against the infectious disease. An exemplary infectious disease amenable to treatment with the vaccines of the invention is mucormycosis. The vaccine-mediated protection can be humoral and/or cell mediated immunity induced in host when a subject is challenged with, for example, *FTR* or an immunogenic portion or fragment thereof.

The term "adjuvant" is intended to mean a composition with the ability to enhance an immune response to an antigen generally by being delivered with the antigen at or near the site of the antigen. Ability to increase an immune response is manifested by an increase in immune mediated protection. Enhancement of humoral immunity can be determined by, for example, an increase in the titer of antibody raised to the antigen. Enhancement of cellular immunity can be measured by, for example, a positive skin test, cytotoxic T-cell assay, ELISPOT assay for IFN-gamma or IL-2. Adjuvants are well known in the art. Exemplary adjuvants include, for example, Freud's complete adjuvant, Freud's incomplete adjuvant, aluminum adjuvants, MF59 and QS21.

The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portion of immunoglobulin molecules. Antibodies can be prepared by any of a variety of techniques known to those skilled in the art (see, for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1988). The present invention provides polyclonal and monoclonal antibodies that bind specifically to a polypeptide of the invention or fragment or variant thereof. Monoclonal antibodies of the invention, for example, include a population of antibody molecules that contain only one species of antigen binding site capable of immunoreacting with a particular epitope of a polypeptide of the invention or a fragment or variant thereof. Monoclonal antibodies can be coupled to one or more therapeutic agents. Suitable agents in this regard include differentiation inducers, drugs, toxins, and derivatives thereof. A therapeutic agent can be coupled (e.g., covalently bonded) to a suitable monoclonal antibody either directly or indirectly (e.g., via a linker group).

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a nucleic acid can be introduced into a host cell. The vector can be used for propagation or harboring a nucleic acid or for polypeptide expression of an encoded sequence. A wide variety of vectors are known in the art and include, for example, plasmids, phages and viruses Exemplary vectors can be found described in, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Third Ed., Cold Spring Harbor Laboratory, New York (2001); Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, MD (1999).

The term "antibody inhibitor" as used herein refers to an antibody that reduces the biological activity or function of the target antigen (i.e., *FTR*). Such reduction in activity or function can be, for example, in connection with a cellular component (e.g., membrane localization), or in connection with a cellular process (e.g., iron transport), or in connection with an overall process of a cell (e.g., cell growth or survival). In reference to cell growth, the inhibitory effects can be fungicidal (killing of fungi) or fungistatic (i.e., stopping or at least slowing fungal growth). The latter slows or prevents fungal growth such that fewer fungi are produced relative to uninhibited fungi over a given time period. From a molecular standpoint, such inhibition can equate with a reduction in the level of, or elimination of, the transcription and/or translation of *FTR* molecule, or reduction or elimination of activity of *FTR* molecule.

The term "treating" or "treatment," as it is used herein is intended to mean an amelioration of a clinical symptom indicative of a fungal condition. Amelioration of a clinical symptom includes, for example, a decrease or reduction in at least one symptom of a fungal condition in a treated individual compared to pretreatment levels or compared to an individual with a fungal condition. The term "treating" also is intended to include the reduction in severity of a pathological condition, a chronic complication or an opportunistic fungal infection which is associated with a fungal condition. Such pathological conditions, chronic complications or opportunistic infections are exemplified below with reference to mucormycosis. Mucormycosis and other such pathological conditions, chronic complications and opportunistic infections also can be found described in, for example, Merck Manual, Sixteenth Edition, 1992, and Spellberg et al., *Clin. Microbio. Rev.* 18:556-69 (2005).

The term "preventing" or "prevention," as it is used herein is intended to mean a forestalling of a clinical symptom indicative of a fungal condition. Such forestalling includes, for example, the maintenance of normal physiological indicators in an individual at risk of infection by a fungus or fungi prior to the development of overt symptoms of the condition or prior to

diagnosis of the condition. Therefore, the term "preventing" includes the prophylactic treatment of individuals to guard them from the occurrence of a fungal condition. Preventing a fungal condition in an individual also is intended to include inhibiting or arresting the development of the fungal condition. Inhibiting or arresting the development of the condition includes, for example, inhibiting or arresting the occurrence of abnormal physiological indicators or clinical symptoms such as those described above and/or well known in the art. Therefore, effective prevention of a fungal condition would include maintenance of normal body temperature, weight, psychological state as well as lack of lesions or other pathological manifestations in an individual predisposed to a fungal condition. Individuals predisposed to a fungal condition include, for example, an individual with AIDS, azotemia, diabetes mellitus, bronchiectasis, emphysema, TB, lymphoma, leukemia, or burns, or an individual with a history of susceptibility to a fungal condition. Inhibiting or arresting the development of the condition also includes, for example, inhibiting or arresting the progression of one or more pathological conditions, chronic complications or susceptibility to an opportunistic infection associated with a fungal condition.

The term "fungal condition" as used herein refers to fungal diseases, infection, or colonization including superficial mycoses (i.e., fungal diseases of skin, hair, nail and mucous membranes; for example, ringworm or yeast infection), subcutaneous mycoses (i.e., fungal diseases of subcutaneous tissues, fascia and bone; for example, mycetoma, chromomycosis, or sporotichosis), and systemic mycoses (i.e., deep-seated fungal infections generally resulting from the inhalation of air-borne spores produced by causal moulds; for example, zygomycosis, mucormycosis, coccidioidomycosis, blastomycosis, histoplasmosis, or paracoccidioidomycosis)

As used herein, the term "zygomycosis" is intended to mean a fungal condition caused by fungi of the class *Zygomycetes*, comprised of the orders Mucorales and Entomophthorales. The Entomophthorales are causes of subcutaneous and mucocutaneous infections known as entomophthoromycosis, which largely afflict immunocompetent hosts in developing countries. Zygomycosis is also referred to as mucormycosis and the two terms are used interchangeably to refer to similar types of fungal infections.

As used herein, the term "mucormycosis" is intended to mean a fungal condition caused by fungi of the order Mucorales. Mucormycosis is a life-threatening fungal infection almost uniformly affecting immunocompromised hosts in either developing or industrialized countries. Fungi belonging to the order Mucorales are distributed into at least six families, all

of which can cause cutaneous and deep infections. Species belonging to the family Mucoraceae are isolated more frequently from patients with mucormycosis than any other family. Among the Mucoraceae, *Rhizopus oryzae* (*Rhizopus arrhizus*) is a common cause of infection. Other exemplary species of the Mucoraceae family that cause a similar spectrum of infections include, for example, *Rhizopus microsporus* var. *rhizopodiformis*, *Absidia corymbifera*, *Apophysomyces elegans*, *Mucor* species, *Rhizomucor pusillus* and *Cunninghamella* spp (*Cunninghamellaceae* family). Mucormycosis is well known in the art and includes, for example, rinocerebral mucormycosis, pulmonary mucormycosis, gastrointestinal mucormycosis, disseminated mucormycosis, bone mucormycosis, mediastinum mucormycosis, trachea mucormycosis, kidney mucormycosis, peritoneum mucormycosis, superior vena cava mucormycosis or external otitis mucormycosis.

Fungi belonging to the order Mucorales are currently distributed into the families of *Choanephoraceae; Cunninghamellaceae; Mucoraceae; Mycotyphaceae; Phycomycetaceae; Pilobolaceae; Saksenaeaceae; Syncephalastraceae;* and *Umbelopsidaceae*. Each of these fungi families consists of one or more genera. For example, fungi belonging to the order Mucorales, family *Mucoraceae*, are further classified into the genera of *Absidia* (e.g., *A. corymbifera*); *Actinomucor* (e.g., *A. elegans*); *Amylomyces* (e.g., *A. rouxii*); *Apophysomyces; Backusella* (e.g., *B. circina*); *Benjaminiella* (e.g., *B. multispora*); *Chaetocladium* (e.g., *C. brefeldii*); *Circinella* (e.g., *C. angarensis*); *Cokeromyces* (e.g., *C. recurvatus*); *Dicranophora* (e.g., *D. fulva*); *Ellisomyces* (e.g., *E. anomalus; Helicostylum* (e.g., *H. elegans*); *Hyphomucor* (e.g., *H. assamensis*); *Kirkomyces* (e.g., *K. cordensis*); *Mucor* (e.g., *M amphibiorum*); *Parasitella* (e.g., *P. parasitica*); *Philophora* (e.g., *P. agaricina*); *Pilaira* (e.g., *P. anomala*); *Pirella* (e.g., *P. circinans*); *Rhizomucor* (e.g., *R. endophyticus*); *Rhizopodopsis* (e.g., *R. javensis*); *Rhizopus; Sporodiniella* (e.g., *S. umbellata*); *Syzygites* (e.g., *S. megalocarpus*); *Thamnidium* (e.g., *T. elegans*); *Thermomucor* (e.g., *T. indicae-seudaticae*); and *Zygorhynchus* (e.g., *Z. californiensis*). The genus *Rhizopus*, for example, consists of *R. azygosporus; R. caespitosus; R. homothallicus; R. oryzae;* and *R. schipperae* species.

The *Choanephoraceae* family consists of fungi genera *Blakeslea* (e.g., *B. monospora*), *Choanephora* (e.g., *C. cucurbitarum*), *Gilbertella* (e.g., *G. hainanensis*), and *Poitrasia* (e.g., *P. circinans*). The *Cunninghamellaceae* family consists of genera *Chlamydoabsidia* (e.g., *C. padenii*); *Cunninghamella* (e.g., *C. antarctica*); *Gongronella* (e.g., *G. butleri*); *Halteromyces* (e.g., *H. radiatus*); and *Hesseltinella* (e.g., *H. vesiculosa*). The *Mycotyphaceae* family consists of fungi genus *Mycotypha* (e.g., *M. africana*). The *Phycomycetaceae* family consists of fungi

genus *Phycomyces* (e.g., *P. blakesleeanus*) and *Spinellus* (e.g., *S. chalybeus*). The *Pilobolaceae* family consists of fungi genera *Pilobolus* (e.g., *P. longipes*) and *Utharomyces* (e.g., *U. epallocaulus*). The *Saksenaeaceae* family consists of fungi genera *Apophysomyces* (e.g., *A. elegans*) and *Saksenaea* (e.g., *S. vasiformis*). The *Syncephalastraceae* family consists of fungi genera *Dichotomocladium* (e.g., D. elegans); *Fennellomyces* (e.g., *F. gigacellularis*); *Mycocladus* (e.g., *M. blakesleeanus*); *Phascolomyces*(e.g., *P. articulosus*); *Protomycocladus* (e.g., *P. faisalabadensis*); *Syncephalastrum* (e.g., *S. monosporum*); *Thamnostylum*(e.g., *T. lucknowense*); *Zychaea* (e.g., *Z. mexicana*). Finally, the *Umbelopsidaceae* family consists of fungi genus *Umbelopsis* (e.g., *U. angularis*).

As used herein, the term "pharmaceutically acceptable carrier" includes any and all pharmaceutical grade solvents, buffers, oils, lipids, dispersion media, coatings, isotonic and absorption facilitating agents and the like that are compatible with the active ingredient. These pharmaceutically acceptable carriers can be prepared from a wide range of pharmaceutical grade materials appropriate for the chosen mode of administration, e.g., injection, intranasal administration, oral administration, etc. For the purposes of this invention, the terms "pharmaceutical" or "pharmaceutically acceptable" further 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. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like.

The term "immunogenic amount" as used herein refers an effective amount of a particular epitope of a polypeptide of the invention or a fragment or variant thereof that can induce the host immune response against the polypeptide or the infectious agent expressing the

polypeptide. This amount is generally in the range of 20 μg to 10 mg of antigen per dose of vaccine and depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. The precise amount of immunogen required can be calculated by various methods such as, for example, antibody titration. The term effective amount refers to an amount of a compound or compositions that is sufficient to provide a desired result. Thus, as used to describe a vaccine, an effective amount refers to an amount of a compound or composition (e.g., an antigen) that is sufficient to produce or elicit a protective immune response. An effective amount with respect to an immunological composition is an amount that is sufficient to elicit an immune response, whether or not the response is protective.

The present invention, in part, relates to the discovery that *FTR* gene product is required for full virulence of a fungal pathogen such as *R. oryzae* in hematogenous dissemination or mucormycosis. Moreover, inhibition of *FTR* polypeptide formation in a host having mucormycosis conferred prolonged survival. As described herein, abrogation of FTR1 function resulted in diminished iron uptake and diminished virulence *in vivo*, and passive immunization with anti-Ftr1p antibody significantly improved survival in infected mice. As disclosed herein, [assive immunotherapy against FTR1 is a viable strategy to improve outcomes of these deadly infections.

Accordingly, different compositions are disclosed herein for effective inhibition of *FTR* molecule and/or its function in treating mucormycosis or other fungal diseases. These inhibitory compositions include vaccines, antisense, siRNA, antibodoy or any other compositions capable of effectively targeting and inhibiting the function of *FTR*. Such compositions will reduce and/or prevent the growth of the fungus in the infected tissues and will cause organism death. The compositions of the invention also are useful in prophylactic settings to decrease onset and/or prevent infection from occurring. In addition, any of the *FTR* inhibitory compositions disclosed herein can further be supplemented and/or combined with other known antifungal therapies including, for example, Amphotericin B or iron chelators. Exemplary iron chelators include Deferiprone and Deferasirox.

In one aspect, the invention provides a vaccine composition having an *FTR* polypeptide or an antigenic fragment or variant of the polypeptide. The vaccine composition also can include an adjuvant. In certain embodiments, the vaccine composition of the invention has an *FTR* polypeptide (SEQ ID NO: 2) shown in Figure 2 or an antigenic fragment of the *FTR* polypeptide (e.g., REGLE motif), a pharmaceutically acceptable carrier and/or an adjuvant.

Similarly, the vaccine composition has an *FTR* polypeptide corresponding to the nucleotides shown in Figure 15-18. The formulation of the vaccine composition of the invention is effective in inducing protective immunity in a subject by stimulating both specific humoral (neutralizing antibodies) and effector cell mediated immune responses against fungal pathogens' *FTRs*. The vaccine composition of the invention is also used in the treatment or prophylaxis of fungal infections such as, for example, mucormycosis.

The vaccine of the present invention will contain an immunoprotective quantity of *FTR* antigens and is prepared by methods well known in the art. The preparation of vaccines is generally described in, for example, M. F. Powell and M. J. Newman, eds., "Vaccine Design (the subunit and adjuvant approach)," Plenum Press (1995); A. Robinson, M. Cranage, and M. Hudson, eds., "Vaccine Protocols (Methods in Molecular Medicine)," Humana Press (2003); and D. Ohagan, ed., "Vaccine Ajuvants: Preparation Methods and Research Protocols (Methods in Molecular Medicine)," Humana Press (2000).

FTR polypeptide, and peptide fragments or variants thereof can include immunogenic epitopes, which can be identified using methods known in the art and described in, for example, Geysen et al. *Proc. Natl. Acad. Sci.* USA 81: 3998 (1984)). Briefly, hundreds of overlapping short peptides, e.g., hexapeptides, can be synthesized covering the entire amino acid sequence of the target polypeptide (i.e., *FTR*). The peptides while still attached to the solid support used for their synthesis are then tested for antigenicity by an ELISA method using a variety of antisera. Antiserum against *FTR* protein can be obtained by known techniques, Kohler and Milstein, Nature 256: 495-499 (1975), and can be humanized to reduce antigenicity, see, for example, U.S. Patent No. 5,693,762, or produced in transgenic mice leaving an unrearranged human immunoglobulin gene, see, for example, U.S. Patent No. 5,877,397. Once an epitope bearing hexapeptide reactive with antibody raised against the intact protein is identified, the peptide can be further tested for specificity by amino acid substitution at every position and/or extension at both C and/or N terminal ends. Such epitope bearing polypeptides typically contain at least six to fourteen amino acid residues of SEQ ID NO: 2, and can be produced, for example, by polypeptide synthesis using methods well known in the art or by fragmenting an *FTR* polypeptide. With respect to the molecule used as immunogens pursuant to the present invention, those skilled in the art will recognize that the *FTR* polypeptide can be truncated or fragmented without losing the essential qualities as an immunogenic vaccine. For example, *FTR* polypeptide can be truncated to yield an N-terminal fragment by truncation from the C-terminal end with preservation of the functional properties

of the molecule as an immunogen. Similarly, C-terminal fragments can be generated by truncation from the N-terminal end with preservation of the functional properties of the molecule as an immunogen. Other modifications in accord with the teachings and guidance provided herein can be made pursuant to this invention to create other *FTR* polypeptide functional fragments, immunogenic fragments, variants, analogs or derivatives thereof, to achieve the therapeutically useful properties described herein with the native protein.

The vaccine compositions of the invention further contain conventional pharmaceutical carriers. Suitable carriers are well known to those of skill in the art. These vaccine compositions can be prepared in liquid unit dose forms. Other optional components, e.g., pharmaceutical grade stabilizers, buffers, preservatives, excipients and the like can be readily selected by one of skill in the art. However, the compositions can be lyophilized and reconstituted prior to use. Alternatively, the vaccine compositions can be prepared in any manner appropriate for the chosen mode of administration, e.g., intranasal administration, oral administration, etc. The preparation of a pharmaceutically acceptable vaccine, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

The immunogenicity of the vaccine compositions of the invention can further be enhanced if the vaccine further comprises an adjuvant substance. Various methods of achieving adjuvant effect for the vaccine are known. General principles and methods are detailed in "The Theory and Practical Application of Adjuvants", 1995, Duncan E.S. Stewart-Tull (ed.), John Wiley & Sons Ltd, ISBN 0-471-95170-6, and also in "Vaccines: New Generationn Immunological Adjuvants", 1995, Gregoriadis G et al. (eds.), Plenum Press, New York, ISBN 0-306-45283-9, both of which are hereby incorporated by reference herein.

Preferred adjuvants facilitate uptake of the vaccine molecules by antigen presenting cells (APCs), such as dendritic cells, and activate these cells. Non-limiting examples are selected from the group consisting of an immune targeting adjuvant; an immune modulating adjuvant such as a toxin, a cytokine, and a mycobacterial derivative; an oil formulation; a polymer; a micelle forming adjuvant; a saponin; an immunostimulating complex matrix (ISCOM® matrix); a particle; DDA (dimethyldioctadecylammonium bromide); aluminium adjuvants; DNA adjuvants; and an encapsulating adjuvant. Liposome formulations are also known to confer adjuvant effects, and therefore liposome adjuvants are included according to the invention.

Another aspect of the invention relates to a vaccine composition having a vector containing a nucleotide sequence that is substantially complimentary to at least 12 contiguous nucleotides of *FTR* sequence (e.g., SEQ ID NO: 1) shown in Figures 1, 15-18, a transcription promoter, and a transcription terminator; wherein the promoter is operably linked to the *FTR* nucleotide sequence, and wherein the *FTR* nucleotide sequence is operably linked to the transcription terminator. The preparation of DNA vaccines is generally described in, for example, M. Saltzman, H. Shen, and J. Brandsma, eds., "DNA Vaccines (Methods in Molecular Medicine)," Humana Press (2006); H. Ertl, ed., "DNA Vaccines," Kluwer Academic/ Plenum Publishers (2003). In one embodiment, the vaccine composition further contains pharmaceutically acceptable carrier and/or adjuvant. Combination of DNA vaccines with adjuvants have been shown to induce a stronger and more specific immune response in human (Hokey et al. *Springer Semin Immun* 28:267-279 (2006)). In general, the potency of DNA vaccines increases when combined with adjuvants that can provide additional immune stimuli. For example, chemokines such as, for example, MIP-1α when used as adjuvants for DNA vaccines have the ability to recruit a variety of cells including professional antigen presenting cells (APCs) to the immunization site. The requirement of APCs to the sites such as muscle where there are relatively low levels of APCs will greatly increase the potency of DNA vaccines for intramuscular injections. Cytokines such as, for example, GM-CSF when used as adjuvant for DNA vaccines can recruit dendritic cells and promote their survival at the immunization site. Molecular adjuvants such as, for example, *Fas* that induce cell death can also increase the potency and efficacy of DNA vaccines. Adjuvant-mediated apoptosis and necrosis have been shown to provide more antigens to APCs. Other molecules such as for example, poly(lactide-co-glycolide) (PLG) and heat shock proteins have also been shown to act as adjuvants for DNA vaccines. It is well known to those skilled in the art that adjuvants can be combined with DNA vaccines as intact molecules such as, for example, intact molecules, or as vectors expressing such molecules; for example, plasmids expressing GM-CSF.

In addition to vaccination of subjects susceptible to fungal infections such as mucormycosis, the vaccine compositions of the present invention can be used to treat, immunotherapeutically, subjects suffering from a variety of fungal infections. Accordingly, vaccines that contain one or more of *FTR* polynucleotides, polypeptides and/or antibody compositions described herein in combination with adjuvants, and that act for the purposes of prophylactic or therapeutic use, are also within the scope of the invention. In an embodiment, vaccines of the present

invention will induce the body's own immune system to seek out and inhibit fungal *FTR* molecules.

Another aspect of the invention relates to a pharmaceutical composition for treating or preventing a fungal condition having an antisense, a small interfering RNA or antibody inhibitor of *FTR* selected from the group consisting of a nucleotide sequence that is substantially complimentary to a portion of an *FTR* sequence; a nucleotide sequence that is substantially complimentary to at least 12 contiguous nucleotide bases of *FTR* sequence; a nucleotide RNAi sequence that is substantially complimentary to at least 18 contiguous nucleotide bases of *FTR* sequence; an antibody or antibody fragment thereof that specifically binds to an *FTR* nucleotide sequence, polypeptide or a fragment thereof; and a pharmaceutically acceptable excipient or carrier. In one embodiment, the pharmaceutical composition further includes an adjuvant.

Antisense nucleic acid molecules of the invention can be designed using the nucleotide sequences of SEQ ID NO: 1, Figures 15-18, their complementary strands, and/or a portion or variant thereof, constructed using enzymatic ligation reactions by procedures known in the art of the genetic engineering. For example, an antisense nucleic acid molecule (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to hybridize with a control region of a gene (e.g., promoter, enhancer, or transcription initiation region) to inhibit the expression of the *FTR* gene through triple-helix formation. Alternatively, the antisense nucleic acid molecule can be designed to hybridize with the transcript of a gene (i.e., mRNA), and thus inhibit the translation of *FTR* by inhibiting the binding of the transcript to ribosomes. The antisense methods and protocols are generally described in, for example, C. Stein, A. Krieg, eds., "Applied Antisense Oligonucleotide Technology" Wiley-Liss, Inc. (1998); or U.S. Patent Nos. 5,965,722; 6,339,066; 6,358,931; and 6,359,124.

The present invention also provides, as antisense molecules, nucleic acids or nucleotide sequences that contain a fragment, portion or variant that hybridizes under high stringency conditions to a nucleotide sequence including a nucleotide sequence selected from SEQ ID NO: 1, Figures 15-18, or their complementary strands. The nucleic acid fragments of the invention are at least about 12, generally at least about 15, 18, 21, or 25 nucleotides, and can be 40, 50, 70, 100, 200, or more nucleotides in length. Longer fragments, for example, 30 or more nucleotides in length, which encode antigenic polypeptides described hereinafter, are particularly useful, such as for the generation of antibodies.

Particular small nucleic acid molecules that are of use in the invention are short stretches of double stranded RNA that are known as short interfering RNAs (siRNAs). These interfering RNA (RNAi) allow for the selective inhibition of *FTR* gene function *in vivo*. In the present invention, RNAi has been used to knock-down *FTR* expression in a DKA mouse model of mucormycosis infection, and in doing so it demonstrates a dramatic effect on survival and protection against the infection. The RNAi approach relies on an innate cellular response to combat viral infection. In this process, double stranded mRNAs are recognized and cleaved by the dicer RNase resulting in 21-23 nucleotide long stretches of RNAi. These RNAis are incorporated into and unwound by the RNA-inducing silencing complex (RISC). The single antisense strand then guides the RISC to mRNA containing the complementary sequence resulting in endonucleolytic cleavage of the mRNA, see Elbashir et al. (Nature 411; 494-498 (2001)). Hence, this technique provides a means for the targeting and degradation of *FTR* mRNA *in vivo* in fungal pathogen infecting a subject.

The present invention further provides inhibitory antibodies (monoclonal or polyclonal) and antigen-binding fragments thereof, that are capable of binding to and inhibition of *FTR* function. The antibody inhibitors of the present invention can bind to *FTR*, or a portion, fragment, variant thereof, and interfere with or inhibit the protein function, i.e., iron transportation. Furthermore, such antibodies can bind to *FTR* and interfere with or inhibit the proper localization or conformation of the protein within the fungal membrane. An antibody, or antigen-binding fragment thereof, is said to "specifically bind," "immunologically bind," and/or is "immunologically reactive" to an *FTR* polypeptide of the invention if it reacts at a detectable level with the *FTR* polypeptide, and does not react detectably with unrelated polypeptides under similar conditions.

In addition, recombinant antibodies, such as chimeric and humanized antibodies, including both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Also included within the term "antibody" are fragments, such as the Fab, F(ab'). The *FTR* specific monoclonal antibodies of the invention have specific binding activity to *FTR*, or a functional fragment thereof, in pathogenic fungi responsible for mucormycosis.

Monoclonal antibodies can be prepared using methods such as, for example, hybridoma, recombinant, phage display, and combinatorial antibody technologies or a combination thereof. The techniques and protocols for production of monoclonal antibodies are generally described in, for example, Harlow and lane, eds., "Antibodies: A laboratory Manual," Cold

Spring harbor Laboratory Press (1999); Harlow et al., *Using Antibodies: A Laboratory Manual*, Cold Spring harbor Laboratory Press (1999); C. Borrebaeck, ed., *Antibody Engineering: A Practical Guide*, W.H. Freeman and Co., Publishers, pp. 130-120 (1991).

Moreover, portions or fragments or variants of the *FTR* nucleotide sequence identified herein (and the corresponding complete gene sequence) can be used in various ways as polynucleotide reagents. For example, these sequences can be used to identify and express recombinant polypeptides for analysis, characterization, or therapeutic use. The sequences can additionally be used as reagents in the screening and/or diagnostic assays described hereinafter, and can also be included as components of kits (e.g., diagnostic kits) for use in the screening and/or diagnostic assays.

The compositions of the present invention in inhibiting *FTR* can be applied to subjects who are suffering from a wide variety of fungal infections including zygomycosis and mucormycosis. The compositions of the invention can further be supplemented with other antifungal agents (e.g., Amphotericin, Deferiprone, Deferasirox). Alternatively, the compositions of the invention can be applied prophylactically to all subjects who are at high risk of developing mucormycosis or other fungal infections (e.g., via active immunization). This would not be considered an over treatment giving the high mortality and morbidity of mucormycosis in view of the current antifungal and surgical debridement treatment.

Further, the invention is also directed to host cells in which immunogenic *FTR* polypeptides or *FTR* inhibitory nucleotides (e.g., RNAi, antisense molecules) can be produced. The term "host cell" is understood to refer not only to the particular subject cell but also to the progeny or potential progeny of the foregoing cell. A host cell can be any prokaryotic (e.g., *E. coli*) or eukaryotic cell (e.g., yeast, insect cells, or mammalian cells, such as CHO or COS cells). Other suitable host cells are known to those skilled in the art. Vectors expressing such immunogenic inhibitory molecules can be introduced into prokaryotic or eukaryotic cells via conventional transfection or transformation techniques (see, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1989).

According to another aspect of the present invention, any of the above-described compositions can be used for treating or prevention of a fungal condition. A fungal condition is an aberrant condition or infection causes by a pathogenic fungus. Symptoms of a fungal condition that can be ameliorated by a method of the invention include, for example, fever, chills, night

sweats, anorexia, weight loss, malaise, depression and lung, skin or other lesions. Other symptoms or characteristic manifestations include, for example, dissemination from a primary focus, acute or subacute presentations, progressive pneumonia, fungemia, manifestations of extrapulmonary dissemination, chronic meningitis, progressive disseminated histoplasmosis as a generalized involvement of the reticuloendothelial system (liver, spleen, bone marrow) and blastomycosis as single or multiple skin lesions. Effective treatment of an individual with a fungal condition, for example, will result in a reduction one or more of such symptoms in the treated individual. Numerous other clinical symptoms of fungal conditions are well known in the art and also can be used as a measure of amelioration or reduction in the severity of a fungal condition using the methods of the invention described herein.

Diagnosis of a fungal condition can be confirmed by isolating causative fungi from, for example, sputum, urine, blood, bone marrow, or specimens from infected tissues. For example, fungal infections can be diagnosed histopathologically with a high degree of reliability based on distinctive morphologic characteristics of invading fungi and/or by immunohistochemistry and the like selective for identifying antigens. Assessment of the activity of the infection also can be based on cultures taken from many different sites, fever, leukocyte counts, clinical and laboratory parameters related to specific involved organs (eg, liver function tests), and immunoserologic tests. The clinical significance of positive sputum cultures also can be corroborated by confirmation of tissue invasion.

Fungal infection, or mycoses, of humans and animals include, for example, superficial fungal infections that affect the outer layers of skin; fungal infections of the mucous membranes including the mouth (thrush), vaginal and anal regions, such as those caused by *Candida albicans*, and fungal infections that affect the deeper layers of skin and internal organs are capable of causing serious, often fatal illness, such as those caused by, for example, *Rhizopus oryzae*. Fungal infections are well known in the art and include, for example, zygomycosis, mucormycosis, aspergillosis, cryptococcosis, candidiasis, histoplasmosis, coccidiomycosis, paracoccidiomycosis, fusariosis (hyalohyphomycoses), blastomycosis, penicilliosis or sporotrichosis. These and other fungal infections can be found described in, for example, Merck Manual, Sixteenth Edition, 1992, and in Spellberg et al., *Clin. Microbio. Rev.* 18:556-69 (2005).

The fungal conditions caused by fungi of the genus *Candida*, candidiasis, can occur, for example, in the skin and mucous membranes of the mouth, respiratory tract and/or vagina as well as invade the bloodstream, especially in immunocompromised individuals. Candidiasis

also is known in the art as candidosis or moniliasis. Exemplary species of the genus *Candida* include, for example, *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Candida glabrata* and *Candida parapsilosis*.

The fungal diseases caused by the genus *Aspergillus* include, for example, allergic aspergillosis, which affects asthma, cystic fibrosis and sinusitis patients; acute invasive aspergillosis, which shows increased incidence in patients with weakened immunity such as in cancer patients, patients undergoing chemotherapy and AIDS patients; disseminated invasive aspergillosis, which is widespread throughout the body, and opportunistic *Aspergillus* infection, which is characterized by inflammation and lesions of the ear and other organs. *Aspergillus* is a genus of around 200 fungi. *Aspergillus* species causing invasive disease include, for example, *Aspergillus fumigatus* and *Aspergillus flavus*. *Aspergillus* species causing allergic disease include, for example, *Aspergillus fumigatus* and *Aspergillus clavatus*. Other exemplary *Aspergillus* infectious species include, for example, *Aspergillus terreus* and *Aspergillus nidulans*.

The fungal conditions such as, for example, zygomycosis and mucormycosis which are caused by saprophytic mould fungi include rinocerebral mucormycosis, pulmonary mucormycosis, gastrointestinal mucormycosis, disseminated mucormycosis, bone mucormycosis, mediastinum mucormycosis, trachea mucormycosis, kidney mucormycosis, peritoneum mucormycosis, superior vena cava mucormycosis or external otitis mucormycosis. Infectious agents causing mucormycosis are of the order Mucorales which include species from *Rhizopus* genus such as, for example, *Rhizopus oryzae* (*Rhizopus arrhizus*), *Rhizopus microsporus* var. *rhizopodiformis*; or species from *Absidia* genus such as, for example, *Absidia corymbifera*; or species from *Apophysomyces* genus such as, for example, *Apophysomyces elegans;* or species from *Mucor* genus such as, for example, *Mucor amphibiorum*; or species from *Rhizomucor* genus such as, for example, *Rhizomucor pusillus;* or species from *Cunninghamell* genus (in the *Cunninghamellaceae* family) such as, for example, *Cunninghamella bertholletiae*.

Various methods are described herein for effective inhibition of *FTR* molecule and/or its function in treatment of mucormycosis and other fungal diseases. These inhibiting methods involve vaccines, antisense, siRNA, antibodoy, or any other compositions capable of effectively targeting and inhibiting the function of *FTR*. Such methods will reduce or prevent the growth of the fungus in the infected tissues by inhibiting the main iron transporter that functions in supplying the pathogenic organism with iron. An immunotherapeutic inhibition of iron transportation using a soluble *FTR* polypeptide or functional fragment or a variant

thereof is useful in this context because: (i) the morbidity and mortality associated with mucormycosis, for example, continues to increase, even with currently available antifungal therapy; (ii) a rising incidence of antifungal resistance is associated with the increasing use of antifungal agents; iii) the population of patients at risk for serious zygomycosis, mucormycosis, candidosis, or aspergillosis, for example, is well-defined and very large, and includes, e.g., post-operative patients, transplant patients, cancer patients, low birth weight infants, subjects with diabetes ketoacidosis (DKA) and other forms of metabolic acidosis, subjects receiving treatment with corticosteroids, subjects with neutropenia, trauma, burns, and malignant hematological disorders, and subjects receiving deferoxamine chelation-therapy or hemodialysis; and iv) a high percentage of the patients who develop serious fungal infections are not neutropenic, and thus can respond to a vaccine or a competitive polypeptide or compound inhibitor. For these reasons, *Zygomycetes* or *Candida*, for example, are fungal targets for passive immunotherapy, active immunotherapy or a combination of passive or active immunotherapy.

Mechanistically, *FTR* polypeptide physically complexes with copper oxidase in yeast, transports ferric iron nearly simultaneously to the oxidation step. In subjects with DKA, low pH conditions cause proton-mediated displacement of ferric iron (Fe^{3+}) from serum carrier molecules, including transferrin (T). See Figure 4. Fe^{3+} is then reduced at the cell surface to ferrous iron (Fe^{2+}). In contrast, deferoxamine (D) directly chelates iron from transferrin, resulting in ferrioxamine (iron-deferoxamine complex). Ferrioxamine then binds to unidentified receptor(s) on the surface of fungi, e.g., *Zygomycetes*. The fungus then liberates ferrous iron from ferrioxamine by reduction at the cell surface. In both cases, ferrous iron is reoxidized back to ferric iron by copper oxidase (Cu-oxidase).

Therefore, the methods of the present invention in inhibiting *FTR* can be applied to subjects who are suffering from a wide variety of fungal infections including zygomycosis and mucormycosis. The methods of the invention can further be supplemented with other antifungal agents (e.g., Amphotericin, Deferiprone, Deferasirox). Alternatively, the methods of the invention can be applied prophylactically to all subjects who are at high risk of developing mucormycosis or other fungal infections (e.g., via active immunization). This would not be considered an over treatment giving the high mortality and morbidity of mucormycosis in view of the current antifungal and surgical debridement treatment.

Accordingly, in one aspect, the invention provides a method of treating or preventing disseminated mucormycosis or other fungal diseases. The method includes administering an

immunogenic amount of a vaccine having an *FTR* polypeptide (SEQ ID NO: 2) shown in Figure 2, or an antigenic or immunogenic fragment of the polypeptide or a variant thereof in a pharmaceutically acceptable medium. The preparation of vaccines is generally described in, for example, M. F. Powell and M. J. Newman, eds., "Vaccine Design (the subunit and adjuvant approach)," Plenum Press (1995); A. Robinson, M. Cranage, and M. Hudson, eds., "Vaccine Protocols (Methods in Molecular Medicine)," Humana Press (2003); and D. Ohagan, ed., "Vaccine Ajuvants: Preparation Methods and Research Protocols (Methods in Molecular Medicine)," Humana Press (2000).

The *FTR* polypeptide, or an antigenic or immunogenic fragment of the polypeptide or a variant thereof can be derived from different pathogenic fungal species of *Zygomycetes* such as *Rhizopus oryzae* (*Rhizopus arrhizus*), *Rhizopus microsporus* var. *rhizopodiformis*, *Absidia corymbifera*, *Apophysomyces elegans*, *Mucor* species, *Rhizomucor pusillus* and *Cunninghamella* spp (*Cunninghamellaceae* family); or from different *Candida* species such as *Candida albicans*, *Candida krusei, Candida tropicalis, Candida glabrata,* and *Candida parapsilosis;* or from different *Aspargillus* species such as *Aspargillus fumigatus, Aspargillus niger, Aspargillus flavus, Aspargillusterreus,* and *Aspargillus nidulans*. Administration of a vaccine of the invention will result in inhibition of the growth and/or virulence of fungal pathogen in a subject.

The sequence homology of, for example, *FTR* of *R. oryzae* with that of *S. cerevisiae* and *C. albicans* are described further below in Example I. Given the teachings and guidance provided herein, those skilled in the art will understand that the vaccines and methods of the invention can be applied to the treatment of mucormycosis or other fungal infections alike. Similarly, given the teachings and methods described herein, those skilled in the art also will understand that the vaccines and methods of the invention also can be applied to other pathogens having iron permease polypeptides with similar immunogenicity, sequence and/or structural homology to the *FTR* protein described herein, including fungus, bacteria and the like.

The vaccine compositions are administrated in a manner compatible with the dosage formulation and in such amount as will be prophylactically effective with or without an adjuvant. The quantity to be administered, which is generally in the range of 1 to 10 mg, preferably 1 to 1000 μg of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered can depend on the judgement of the practitioner and can be peculiar to each subject. Moreover, the amount of polypeptide

in each vaccine dose is selected as an immunogenic amount which induces an immunoprotective response. Particularly useful immunogenic amounts include an amount of *FTR* polypeptide that also is devoid of significant, adverse side effects. Such amount will vary depending upon the immunogenic strength of an *FTR* polypeptide selected for vaccination. Useful immunogenic amounts of an *FTR* polypeptide or immunogenic fragment thereof include, for example, doses ranging from about 1-1000 μg. In certain embodiments, useful immunogenic amounts of an *FTR* polypeptide or immunogenic fragment thereof include about 2-100 μg, and particularly useful dose ranges can range from about 4-40 μg, including for example, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 35 and 40 μg as well as all values in between the above exemplified amounts. An optimal immunogenic amount for a selected *FTR* polypeptide vaccine of the invention can be ascertained using methods well known in the art such as determination of antibody titres and other immune responses in subjects as exemplified previously. Following an initial vaccination, subjects receive a boost in about 3-4 weeks. Vaccine delivery methods is further described, for example, in S. Cohen and H. Bernstein, eds., "Microparticulate Systems for the Delivery of Proteins and Vaccines (Drugs and The Pharmaceutical Sciences)," Vol. 77, Marcel Dekker, Inc. (1996). Encapsulation within liposomes is described, for example, by Fullerton, U.S. Pat. No. 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Pat. No. 4,372,945 and by Armor et al., U.S. Pat. No. 4,474,757.

Furthermore, the vaccine compositions of the present invention include DNA vaccines encoding antigenic *FTR* molecules. As mentioned earlier, the preparation of DNA vaccines is generally described in, for example, M. Saltzman, H. Shen, and J. Brandsma, eds., "DNA Vaccines (Methods in Molecular Medicine)," Humana Press (2006); H. Ertl, ed., "DNA Vaccines," Kluwer Academic/ Plenum Publishers (2003). DNA vaccines can be introduced into the host cells of the subject by a variety of expression systems. These expression systems include prokaryotic, mammalian, and yeast expression systems. For example, one approach is to utilize a viral vector, such as vaccinia virus incorporating the new genetic material, to innoculate the host cells. Alternatively, the genetic material can be incorporated in a vector or can be delivered directly to the host cells as a "naked" polynucleotide, i.e. simply as purified DNA. In addition, the DNA can be stably transfected into attenuated bacteria such as *Salmonella typhimurium*. When a subject is orally vaccinated with the transformed Salmonella, the bacteria are transported to Peyer's patches in the gut (i.e., secondary lymphoid tissues), which then stimulate an immune response. In addition, DNA vaccines can be delivered by variety of well-known delivery vehicles such as, for example, lipid monolayers,

bilayers, or vesicles such as liposomes. Agents such as saponins and block-copolymers, which are commonly used to permeablilize cells, can also be used with DNA vaccines. As described earlier, DNA vaccine compositions of the invention can include pharmaceutically acceptable carriers and/or adjuvants.

The DNA vaccine compositions as described herein can be administered by a variety of routes contemplated by the present invention. Such routes include intranasal, oral, rectal, vaginal, intramuscular, intradermal and subcutaneous administration.

The DNA vaccine compositions for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions or emulsions, the protein vaccine, and an adjuvant as described herein. The composition can be in the form of a liquid, a slurry, or a sterile solid which can be dissolved in a sterile injectable medium before use. The parenteral administration is preferably intramuscular. Intramuscular inoculation involves injection via a syringe into the muscle. This injection can be via a syringe or comparable means. The vaccine composition can contain a pharmaceutically acceptable carrier and/or an adjuvant. Alternatively, the present vaccine compositions can be administered via a mucosal route, in a suitable dose, and in a liquid form. For oral administration, the vaccine composition can be administered in liquid, or solid form with a suitable carrier.

The invention also provides a method of treating or preventing a fungal condition in a subject in need thereof, including exposing said fungi to an antisense against *FTR*. In one embodiment, the antisense includes a nucleotide sequence that is substantially complimentary to a portion of an *FTR* nucleotide sequence. In another embodiment the nucleotide sequence of the antisense is substantially complimentary to at least 12 contiguous nucleotide bases of *FTR* sequence.

The antisense oligonucleotides used in accordance with this invention can be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis can also be employed, however the actual synthesis of the oligonucleotides are well within the talents of those skilled in the art. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives. As described earlier, an antisense nucleic acid molecule (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to hybridize with a control region of a gene (e.g.,

promoter, enhancer, or transcription initiation region) to inhibit the expression of the *FTR* gene through triple-helix formation. Alternatively, the antisense nucleic acid molecule can be designed to hybridize with the transcript of *FTR* (i.e., mRNA), and thus inhibit the translation of *FTR* by inhibiting the binding of the transcript to ribosomes. The antisense methods and protocols are generally described in, for example, C. Stein, A. Krieg, eds., "Applied Antisense Oligonucleotide Technology" Wiley-Liss, Inc. (1998); or U.S. Patent Nos. 5,965,722; 6,339,066; 6,358,931; and 6,359,124.

The antisense compositions of the invention can be delivered to a subject in need thereof with variety of means known in the art. For example, microparticles such as polystyrene microparticles, biodegradable particles, liposomes or microbubbles containing the antisense compositions in releasable form can be used for direct delivery of the compositions into tissues via injection. In some embodiments of the invention, the antisense oligonucleotides can be prepared and delivered in a viral vector such as hepatitis B virus (see, for example, Ji et al., J. Viral Hepat. 4:167 173 (1997)); in adeno-associated virus (see, for example, Xiao et al. Brain Res. 756:76 83 (1997)); or in other systems including but not limited to an HVJ(Sendai virus)-liposome gene delivery system (see, for example, Kaneda et al. Ann. N.Y. Acad. Sci. 811:299 308 (1997)); a "peptide vector" (see, for example, Vidal et al. CR Acad. Sci III 32):279 287 (1997)); as a gene in an episomal or plasmid vector (see, for example, Cooper et al. Proc. Natl. Acad. Sci. U.S.A. 94:6450 6455 (1997), Yew et al. Hum Gene Ther. 8:575 584 (1997)); as a gene in a peptide-DNA aggregate (see, for example, Niidome et al. J. Biol. Chem. 272:15307 15312 (1997)); as "naked DNA" (see, for example, U.S. Pat. No. 5,580,859 and U.S. Pat. No. 5,589,466); in lipidic vector systems (see, for example, Lee et al. Crit Rev Ther Drug Carrier Syst. 14:173 206 (1997)); polymer coated liposomes (Marin et al., U.S. Pat. No. 5,213,804 issued Can 25, 1993; Woodle et al., U.S. Pat. No. 5,013,556 issued Can 7, 1991); cationic liposomes (Epand et al., U.S. Pat. No. 5,283,185 issued Feb. 1, 1994; Jessee, J. A. U.S. Pat. No. 5,578,475 issued Nov. 26, 1996; Rose et al, U.S. Pat. No. 5,279,833 issued Jan. 18, 1994; Gebeyehu et al., U.S. Pat. No. 5,334,761 issued Aug. 2, 1994); gas filled microspheres (Unger et al., U.S. Pat. No. 5,542,935 issued Aug. 6, 1996), ligand-targeted encapsulated macromolecules (Low et al. U.S. Pat. No. 5,108,921 issued Apr. 28, 1992; Curiel et al., U.S. Pat. No. 5,521,291 issued Can 28, 1996; Groman et al., U.S. Pat. No. 5,554,386 issued Sep. 10, 1996; Wu et al., U.S. Pat. No. 5,166,320 issued Nov. 24, 1992).

The invention also provides a method of treating or preventing a fungal condition in a subject in need thereof, including exposing said fungi to a small interfering RNA against *FTR*. In one

embodiment, a nucleotide RNAi sequence that is substantially complimentary to at least 18 contiguous nucleotide bases of *FTR* sequence is used that is capable of binding to an *FTR* nucleotide sequence or a fragment thereof.

Double-stranded RNA (dsRNA) also known as small-interfering RNA (siRNA) induces sequence-specific post-transcriptional gene silencing in many organisms by a process known as RNA interference (RNAi). In the present invention, as described in Example 9, RNAi has been prepared and used to knock-down *FTR* expression in a DKA mouse model of mucormycosis infection, and in doing so it demonstrates a dramatic effect on survival and protection against the infection.

The siRNA is usually administered as a pharmaceutical composition. The administration can be carried out by known methods, wherein a nucleic acid is introduced into a desired target cell *in vitro* or *in vivo*. Commonly used gene transfer techniques include calcium phosphate, DEAE-dextran, electroporation and microinjection and viral methods (Graham et al. Virol. 52, 456 (1973); McCutchan et al. J. Natl. Cancer Inst. 41, 351(1968); Chu et al. Nucl. Acids Res. 15, 1311 (1987); Fraley et al. J. Biol. Chem. 255, 10431 (1980); Capecchi, Cell 22, 479 (1980); and cationic liposomes (Feigner et al. Proc. Natl. Acad. Sci USA 84, 7413 (1987)). Commercially available cationic lipid formulations are e.g. Tfx 50™ (Promega) or Lipofectamin2000™ (Invitrogen).

The invention also provides a method of treating or preventing a fungal condition in a subject in need thereof, including an antibody inhibitor of *FTR*. In one embodiment, the antibody inhibitor of *FTR* is an antibody or antibody fragment that specifically binds to an *FTR* nucleotide polypeptide or a fragment thereof.

As described earlier the antibody inhibitors of *FTR* are are capable of binding to and inhibition of *FTR* function. The antibody inhibitors of the present invention can bind to *FTR*, a portion, fragment, or variant thereof, and interfere with or inhibit the protein function, i.e., iron transportation. These antibodies can inhibit *FTR* by negatively affecting, for example, the protein's proper membrane localization, folding or conformation, its substrate binding ability.

The antibodies of the present invention can be generated by any suitable method known in the art. Polyclonal antibodies against *FTR* can be produced by various procedures well known in the art. For example, an *FTR* peptide antigenic can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants can be used to

increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, alum (alhydrogel), surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *corynebacterium parvum*. Such adjuvants are also well known in the art.

FTR peptide antigens suitable for producing antibodies of the invention can be designed, constructed and employed in accordance with well-known techniques. *See, e.g.,* ANTIBODIES: A LABORATORY MANUAL, Chapter 5, p. 75-76, Harlow & Lane Eds., Cold Spring Harbor Laboratory (1988); Czernik, *Methods In Enzymology, 201:* 264-283 (1991); Merrifield, *J. Am. Chem. Soc. 85:* 21-49 (1962)). Monoclonal antibodies of the present invention can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties).

The antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in U.S. Pat. Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

The antibodies of the invention can be assayed for immunospecific binding by any method known in the art. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art. See, e.g., Sambrook, Fitsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Specific binding can be determined by any of a variety of measurements known to those skilled in the art including, for example, affinity (K_a or K_d), association rate (k_{on}), dissociation rate (k_{off}), avidity or a combination thereof. Antibodies of the present invention can also be described or specified in terms of their binding affinity to *FTR*. Preferred binding affinities include those with a dissociation constant or K_d less than 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6}M, 5×10^{-7} M, 10^{7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M.

An exemplary approach in which the antibodies of the present invention can be used as *FTR* inhibitors includes binding to and inhibiting *FTR* polypeptides locally or systemically in the body or by direct cytotoxicity of the antibody, e.g. as mediated by complement (CDC) or by effector cells (ADCC). The antibodies of this invention can be advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hematopoietic growth factors (such as, e.g., IL-2, IL-3 and IL-7), for example, which serve to increase the number or activity of effector cells which interact with the antibodies.

The antibodies of the invention can be administered alone or in combination with other types of treatments such as, for example, anti-fungal therapies. In one embodiment, *FTR* inhibitor antibodies are administered to a human patient for therapy or prophylaxis.

Various delivery systems are known and can be used to administer the antibody inhibitors of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes.

The compounds or compositions can be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and can be administered together with other biologically active agents. Administration can be systemic or local. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

For antibodies, the dosage administered to a subject is typically 0.1 mg/kg to 100 mg/kg of the subject's body weight. Preferably, the dosage administered to a subject is between 0.1 mg/kg and 20 mg/kg of the subject's body weight, more preferably 1 mg/kg to 10 mg/kg of the subject's body weight. Generally, humanized or human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of humanized antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention can be reduced by enhancing uptake and tissue penetration (e.g., into the brain) of the antibodies by modifications such as, for example, lipidation.

In pharmaceutical dosage forms, the compositions of the invention including vaccine, antisense, siRNA and antibodies can be used alone or in appropriate association, as well as in combination, with each other or with other pharmaceutically active compounds. Administration of the agents can be achieved in various ways, including oral, buccal, nasal, rectal, parenteral, intraperitoneal, intradermal, transdermal, subcutaneous, intravenous, intra-arterial, intracardiac, intraventricular, intracranial, intratracheal, and intrathecal administration, etc., or otherwise by implantation or inhalation. Thus, the subject compositions can be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, enemas, injections, inhalants and aerosols. The following methods and excipients are merely exemplary and are in no way limiting.

Any of treatment modalities disclosed herein can be combined and administered to a subject suffering from a fungal infection or being at risk for developing a fungal infection (prophylactic vaccination or treatment). In a combination therapy, for example, a subject can first receive a vaccine of the invention to generate an immune response towards the fungi, then an antisense, siRNA and/or antibody that can target *FTR* of the fungi and further augment the fungal treatment. In one embodiment of the treatment, the vaccine of the invention is used in combination with an antisese, siRNA and/or antibody against *FTR* for treating or preventing a

fungal condition such as, for example, mucormycosis. In another embodiment, the antibodies of the invention are used in combination with antisense and/or siRNA for treating the fungal condition.

The compositions of the inventions, either alone or in combination, can further be combined one or more methods or compositions available for fungal therapy. In one embodiment, the compositions of the invention can be used in concert with a surgical method to treat a fungal infection. In yet another embodiment, the compositions of the invention can be used in combination with a drug or radiation therapy for treating a fungal condition. Antifungal drugs that are useful for combination therapy with the compositions of the invention include, but are not limited to, amphotericin B, iron chelators such as, for example, deferasirox, deferiprone, POSACONAZOLE®, FLUCONAZOLE®, ITRACONAZOLE® and/or KETOCONAZOLE®. Radiations useful in combination therapies for treating fungal infections include electromagnetic radiations such as, for example, near infrared radiation with specific wavelength and energy useful for treating fungal infections. In combination therapy, chemotherapy or irradiation is typically followed by administration of the vaccine in such a way that the formation of an effective anti-fungal immune response is not compromised by potential residual effects of the prior treatment.

In a further embodiment of combination therapy, the compositions of the invention can be combined with immunocytokine treatments. Without wishing to be bound by theory, it is believed that, for example, a vaccine generates a more effective immune response against, for example, an infection when a cytokine promoting the immune response is present at the site of the infection. For example, useful immunocytokines are those that elicit Th1 response, such as IL-2 or IL-12. During a combination therapy, for example, a subject can first receive a vaccine of the invention to generate an immune response towards a fungal infection, then an immunocytokine that can target the fungi and support the immune response in fighting the infection. Preferred immunocytokines typically have, for example, an antibody moiety that recognizes a surface antigen characteristic of the fungi such as, for example, *FTR*. Immunocytokines typically also have a cytokine moiety such as IL-2, IL-12, or others that preferentially direct a Thl response. Immunocytokines suitable for the invention are described in U.S. Pat. No. 5,650,150, the contents of which are hereby incorporated by reference.

In another embodiment of combination therapy, combinations of the compositions of the invention can be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the

combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second. In another specific embodiment, compositions of the invention are used in any combination with amphotericin B, deferasirox, deferiprone, POSACONAZOLE®, FLUCONAZOLE®, ITRACONAZOLE®, and/or KETOCONAZOLE® to prophylactically treat, prevent, and/or diagnose an opportunistic fungal infection.

The invention, therefore, provides methods of treatment, inhibition and prophylaxis by administration to a subject of an effective amount of one or more compounds or pharmaceutical compositions of the invention. In a preferred aspect, the compositions of the invention are substantially purified (e.g., substantially free from substances that limit their effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

As discussed above, various delivery systems are known and can be used to administer the compositions of the invention. The compositions can be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and can be administered together with other biologically active agents. Administration can be systemic or local.

In a specific embodiment, it can be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this can be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a protein, including a vaccine or antibody, of the invention, care must be taken to use materials to which the protein does not absorb. In another embodiment, the compound or composition can be delivered in liposomes. In yet another embodiment, the compounds or compositions can be delivered in a controlled release system.

In an embodiment, the compositions are formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition can also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule indicating the quantity of active agent. Where the compositions are to be administered by infusion, they can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the compositions are administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients can be mixed prior to administration.

The compounds of the invention can also be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compounds or compositions of the invention which will be effective in the treatment, inhibition and prevention of a fungal disease or condition can be determined by standard clinical techniques. In addition, *in vitro* assays can optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or condition, and should be decided according to the judgment of the practitioner and each subject's circumstances. Effective doses can be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

The following Examples illustrate the therapeutic utility of the *FTR* as the basis for preventive measures or treatment of disseminated mucormycosis. Example 1 describes cloning and identification of *FTR*. Example 2 describes *FTR* expression in *R. oryzae* under iron-depleted condition. Example 3 describes *FTR* expression in *S. cerevisiae ftr1* null mutant. Example 4 describes *FTR* function in *S. cerevisiae ftr1* null mutant. Example 5, describes development of animal model of mucormycosis. Example describes the effect of serum iron availability on susceptibility of DKA mice to *R. oryzae*. Example 7 describes the expression of *FTR in vivo* in DKA mice infected with *R. oryzae*. Example 8 describes *FTR* polypeptide and its

homology to other proteins. Example 9 describes the role of *FTR* gene product in virulence of *R. oryzae* in the DKA mouse model of hematogenous dissemination of mucormycosis.

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 1

Cloning and Identification of *FTR*

This Example describes the cloning and identification of *FTR* of *R. oryzae* which showed considerable sequence homology to high affinity iron permeases of *S. cerevisiae* and *C. albicans* (Figure 3).

The following describes materials and methods used in the procedures described in this example. In accordance with the present invention, there can be employed conventional molecular biology, microbiology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fitsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Rhizopus oryzae 99-880 was obtained from the Fungus Testing Laboratory (University of Texas Health Science Center at San Antonio). This strain was isolated from a brain abscess in a diabetic subject with rhinocerebral mucormycosis.

To clone the *FTR* of *R. oryzae*, we used degenerate primers designed from the conserved regions of the *S. cerevisiae FTR* to amplify a 0.6 kb fragment from *R. oryzae* genomic DNA. This fragment showed 43% homology to *S. cerevisiae FTR* and hybridized to a 2.0 kb fragment of *R. oryzae* genomic DNA cut with *EcoRI.* We used this PCR-generated fragment to screen an *R. oryzae* genomic library made in λ-phage. Five different plaques were isolated and each contained a 2 kb fragment upon treatment with different restriction enzymes. Sequence analysis of this 2.0 kb genomic clone revealed a single open reading frame of 1101 bp that lacked introns. Comparison of the putative *FTR* polypeptide with those of other proteins in GenBank data-base revealed 46% and 44% identity to known fungal high affinity iron permeases from *C. albicans* and *S. cerevisiae,* respectively (Fu et al. *FEMS Micorbiol. Lett.* 235:169-176 (2004)). Multiple regions of the predicted amino acid sequence of *FTR*

polypeptide showed significant homology with putative transmembrane domains from *S. cerevisiae* and *C. albicans FTR*. Importantly, the putative REGLE motif in which the glutamic acid residue is believed to interact directly with iron was conserved in the predicted amino acid sequences of *FTR* polypeptide from the three organisms and was embedded in a hydrophobic region of the protein. Additionally, Southern blot analysis of *R. oryzae* genomic DNA cut with *EcoRI, DraI,* or *EcoRI* + *DraI* and probed with the ORF of *FTR* confirmed the gene map of the *FTR*. Southern blot analysis of *R. oryzae* gDNA using the ORF of *FTR* under low stringency did not reveal any other bands, thus indicating that the *FTR* is not a member of a gene family (data not shown).

EXAMPLE 2

Expression Of *FTR* In *R. oryzae* Under Iron-Depleted Conditions

This Example shows that *FTR* is expression at higher levels under iron-depleted conditions.

Expression of high affinity iron permeases is usually induced in iron-limited environments and suppressed in iron-rich environments. To verify that *FTR* polypeptide functions as a high-affinity iron permease, we examined *FTR* expression in response to different concentrations of $FeCl_3$. *R. oryzae* mycelia were collected by filtration and used to inoculate potato dextrose broth (PDB) supplemented with the iron chelators, 1 mM ferrozine and 100 μM of 2,2'bipyridyl, to induce iron starvation. The mycelia were transferred to PDB previously chelated for iron, and supplemented with varying concentrations of $FeCl_3$ and incubated at 37°C for selected intervals. As expected, *FTR* expression was induced at all time points when the organism was exposed to media deficient in $FeCl_3$. The addition of $FeCl_3$ resulted in rapid suppression of *FTR* expression as early as 5 minutes after exposure. Further, this suppression of *FTR* expression appeared to be dose dependent, with a more marked, and rapid decrease in *FTR* mRNA at 350μM $FeCl_3$ as compared with 50μM $FeCl_3$. Consistent with these results, *FTR* expression was undetectable when mycelia were grown in the iron-rich medium, PDB. These results demonstrate that *FTR* is induced in iron-depleted environments, suppressed in iron-rich environments, and that its transcription is tightly regulated by the amount of iron in the medium (Figure 5). This tight transcriptional regulation has been reported in yeast and is likely due to the sensitivity of transcriptional activation to changes in intracellular iron concentration. Such tight regulation likely allows the organism to avoid toxicity caused by excess iron. Of note, these results also demonstrate that *FTR* is likely to be expressed *in vivo* (see below) even in a host that has elevated available serum iron because free iron concentration in these hosts is still expected to be several orders of magnitude less than the

highest concentration shown to induce expression of *FTR* (i.e. 50 μM). For example, we found that DKA mice have 7.29 μM available iron in their serum (see below). Additionally, Artis *et al.* demonstrated that sera collected from subjects in DKA contain 12.4 μM available iron (Artis et al., *Diabetes* 31(12):1109-14 (1982)).

EXAMPLE 3

Expression of *FTR* in *S. cerevisiae ftr1* Null Mutant

This Example shows that expression of *R. oryzae FTR* in *S. cerevisiae ftr1* null mutant restores *S. cerevisiae*'s ability to grow in iron-depleted environment.

To determine whether *FTR* is functionally equivalent to *S. cerevisiae FTR*, we tested whether *FTR* could rescue the iron-dependent growth defect of a *S. cerevisiae ftr1* null mutant. *S. cerevisiae* was transformed with a plasmid containing *FTR* under the control of the inducible *GAL1* promoter (i.e. expression only in the presence of galactose). *S. cerevisiae* transformed with *FTR* grew when cultured on iron-limited medium (50 μM iron) containing galactose. In contrast, no growth was noted when the *FTR*-transformed cells were cultured on plates containing glucose, which failed to induce activation of the *GAL1* promoter, and hence transcription of the *FTR* (Figure 6). As expected, *S. cerevisiae* transformants carrying vector alone (negative control) did not grow on iron-depleted medium even in the presence of galactose. All *S. cerevisiae* transformants grew equally well on iron-rich plates (350 μM iron) containing either glucose or galactose, likely due to the presence of the low-affinity iron permease of *S. cerevisiae,* which is believed to function in iron-rich environments (Figure 8).

EXAMPLE 4

FTR Complements *S. cerevisiae ftr1* Null Mutant Uptake of Iron

This example shows that *R. oryzae FTR* encodes a functional polypeptide in *S. cerevisiae*.

To confirm that *FTR*-mediated growth rescue of the *S. cerevisiae ftr1* mutant was due to increased iron uptake, we compared the kinetics of ^{59}Fe uptake of *S. cerevisiae* transformed with *R. oryzae FTR* under the *GAL1* promoter to transformants containing the empty vector. The *ftr1* null mutant cells transformed with the empty vector showed no intracellular iron accumulation when $^{59}FeCl_3$ was supplied at 0.1 μM (a concentration in which only high affinity iron permeases are active). In contrast, introduction of *FTR* into *S. cerevisiae ftr1* null mutant restored the iron uptake to between 48-60% of the amount exhibited by the wild-type strain (Figure 7).

In summary, in Examples 3 and 4 we showed that we have cloned a gene (*FTR)* that is expressed in *R. oryzae* in iron-depleted media, suppressed in iron-rich media, and complements the growth defect of high-affinity iron permease null mutant of *S. cerevisiae* by rescuing the mutant's ability to take up iron in iron-poor media. In aggregate, these data strongly indicate that *FTR* polypeptide encodes a high-affinity *R. oryzae* iron permease and also justifies the production of *FTR* polypeptide in *S. cerevisiae* because *R. oryzae* genes can be functionally expressed in *S. cerevisiae*.

EXAMPLE 5

Development of Animal Model of Mucormycosis

To study the pathogenesis of any disease, it is essential to develop an animal model that recapitulates relevant clinical factors. This Example shows that we have developed an animal model relevant to mucormycosis, a DKA mouse model of hematogenously disseminated *R. oryzae* infection.

We successfully developed a DKA mouse model of hematogenously disseminated mucormycosis by using a single injection of streptozotocin given intraperitoneally. We chose this model because subjects with DKA are at high risk of developing mucormycosis. As expected, we found that mice with DKA are more susceptible to *R. oryzae* infection than normal mice. Seven days after intravenous challenge of 10^4 spores, all mice with DKA died, whereas 40% of infected non-diabetic mice survived (Figure 8).

To assess the severity of infection, we compared tissue colony counts to a quantitative PCR-based (qPCR) (TaqMan) assay that was developed originally to determine disease progression in animal models of *A. fumigatus*. The TaqMan technique was developed because the colony count method is unreliable for determining tissue fungal burden of molds since hyphal structures are disrupted by tissue homogenization, resulting in death of the fungus and inaccurately low estimate of the organ fungal burden. Indeed, as anticipated, colony counts did not increase during infection with *R. oryzae* and did not correlate with mortality. In contrast, a temporal correlation between increase in tissue fungal burden and onset of mortality was found when a qPCR-based (TaqMan) technique, using primers designed to amplify *R. oryzae* 18s rDNA, was used to quantify tissue *R. oryzae* burden (Figure 9). These results were consistent with our preliminary results, in which mouse tissues spiked with varying inocula of *R. oryzae* showed a linear range of detection. Therefore, this qPCR- assay is a sensitive and reliable method for assessing the progression of mucormycosis in the DKA mouse model.

This assay will be utilized to elucidate the role of iron metabolism in the pathogenesis of the disease.

EXAMPLE 6

The Effects of Serum Iron Availability on Susceptibility of DKA Mice to *R. oryzae*

This Example shows that susceptibility of DKA mice to R. *oryzae* is due in part to elevated available serum iron.

To confirm that available iron renders diabetic mice more susceptible to *R. oryzae* infection, we compared levels of serum iron in DKA mice to those of normal mice by using the method of Artis et al. (1982, *supra*). In concordance with the results found in humans DKA mice (n = 11) had approximately 5 fold higher levels of available serum iron than normal mice [median (75^{th} quartile, 25^{th} quartile)] = 7.29 (11.8, 4.3) μM vs. 1.69 (2.3, 1.3) μM, p = 0.03 by Wilcoxon Rank Sum). These data underscore the clinical relevance of our DKA mouse model.

To confirm the role of elevated available serum iron in the pathogenicity of *R. oryzae* we investigated the effect of iron chelation on the susceptibility of DKA mice to *R. oryzae* infection. Mice were infected via the tail-vein with spores of *R. oryzae*. The mice were treated by oral gavage with 1, 3, or 10 mg/kg deferasirox (a newly FDA approved iron chelator to treat subjects with iron overload) in 0.5% hydroxypropylcellulose twice daily (bid) for seven days starting the day after infection. Negative control mice were treated with hydroxypropylcellulose carrier (placebo) or deferasirox plus saturating ferric chloride (administered i.p.). An additional negative control consisted of uninfected mice treated with ferric chloride. Deferasirox given at all doses significantly improved survival compared to controls (Figure 10A). This improved survival paralleled the survival we get in this model when a high dose of liposomal amphotericin B (LAmB) is used to treat infection. To determine the impact of deferasirox on tissue fungal burden, DKA mice were infected i.v. as above. Mice were treated with deferasirox (10 mg/kg bid), deferasirox plus saturating ferric chloride, or placebo. Treatment was begun 16 h after infection and administered daily for 3 days. Kidneys and brains were removed on day four, homogenized, and quantitatively cultured. Deferasirox resulted in a greater than 10-fold decrease in both brain and kidney (primary target organs) fungal burden compared to mice treated with placebo or deferasirox plus saturating ferric chloride (Figure 10B). By histopathology, kidneys of deferasirox-treated mice had no visible hyphae, whereas kidneys of mice treated with placebo or deferasirox plus

saturating ferric chloride had extensively filamented fungi. Furthermore, mice treated with saturating iron had a striking absence of neutrophil influx to the sites of infection, while neutrophil influx was prominent in the kidneys of mice treated with deferasirox (data not shown). The reversal of protection when deferasirox was administered to mice with a saturating dose of $FeCl_3$ further proved that the mechanism of protection was due to iron chelation. Of note, these results are in agreement with our previous work showing that deferiprone (another chelating agent that is not used as a siderophore by *Rhizopus*) protected animals from *Rhizopus* infection and confirm the link between iron availability and *R. oryzae*. These results further confirmed the unique importance of iron in the pathogenesis of mucormycosis.

EXAMPLE 7

Expression of *FTR in vivo* in DKA mice

This Example shows that *R. oryzae*'s *FTR* is expressed *in vivo* in DKA mice.

In order for *FTR* polypeptide to play a role in the pathogenesis of mucormycosis, it must be expressed during infection. We used a real time RT-PCR-based approach to investigate the expression of *FTR* polypeptide in the brains of diabetic ketoacidic (DKA) mice infected with 10^5 spores of *R. oryzae* through tail vein injection. The brain was chosen because it is the primary target organ in this model. Mice were sacrificed 24 or 48 h post infection and brains were collected and immediately flash frozen in liquid nitrogen prior to grinding and RNA extraction with phenol. Brains collected from uninfected DKA mice were processed in parallel and served as negative controls. Following DNase treatment to eliminate contaminating genomic DNA, and reverse transcription (Ambion RETROscript® system), cDNA was analyzed by real-time PCR using the SYBR-Green method and an ABI® Prism 7000 cycler. Gene-expression was normalized to *R. oryzae ACT1* or 18S rRNA-expression. *FTR* was found to have been expressed in the brains of 4 infected mice 48 h post infection but not after 24 h (Fig 11). The lack of *FTR* expression after 24 h of infection cannot be attributed to the presence of lower fungal elements in the brains of infected mice since the expression of both 18S rRNA and *ACT1* genes were detected in these tissues. The pattern of delayed *FTR* polypeptide expression (i.e. expression after 48 h but not 24 h of infection) can be explained by the fact that after 24 h fungal elements were not sufficiently iron-starved because spores had been grown on iron-rich medium during preparation of the inoculum. Forty eight hours following infection, as the fungal spores started to proliferate in the brain, *R. oryzae* started to

express *FTR* polypeptide to scavenge iron from the host. As expected, brains from uninfected mice did not show any expression of *FTR* polypeptide.

These results clearly demonstrate that *FTR* polypeptide is expressed during infection and is involved in the pathogenesis of mucormycosis.

To confirm the expression of *FTR in vivo* during active infection, we used GFP as a reporter system for *FTR* expression. *R. oryzae* was transformed with a plasmid containing GFP cloned down stream of a 2 kb fragment containing *FTR* promoter. This strain fluoresced green when grown in iron-depleted but not in iron-rich environments *in vitro* (data not shown). DKA mice were infected with 1 x 10^5 spores of this *R. oryzae* strain grown under iron-rich conditions. Forty eight hours post infection mice were sacrificed and brains were collected, and fixed in 10% zinc formalin. Paraffin sections of the brains were stained with anti-GFP polyclonal rabbit Ab and counter stained with anti-rabbit FITC conjugated Ab. As shown in Figure 14, fungal elements in the brains of mice infected with *R. oryzae* expressing GFP under the control of the *FTR*p fluoresced green, therefore confirming our earlier findings that *FTR* polypeptide is expressed during active infection an is involved in pathogenesis of the mucormycosis.

EXAMPLE 8

FTR Polypeptide and its Homology to Known Proteins

This Example shows that *R. oryzae FTR* polypeptide little or no homology with any known human proteins.

In order to minimize the potential for induction of autoimmune responses, it is desirable that a protein vaccine being utilized as a human vaccine not have significant homology to numerous human proteins. To investigate the potential for homology between the *FTR* polypeptide and human proteins, a PubMed BLAST search was performed comparing the amino acids 16-368 of the *FTR* polypeptide (i.e. the amino acids in the intended *FTR* polypeptide vaccine) to the human proteome. The search identified five open reading frames with extremely limited homology with an alignment score of 30.4, e= 9.0 for all of the five proteins. Three of these proteins are coiled-coil domain containing 82 (i.e., EAW66982; AAH33726.1; and NP_079001.2), one is a CCDC82 protein (i.e., AAH18663.1) and an unnamed protein (i.e., BAB15683.1). As a benchmark, the standard BLAST search e-value for identification of unique sequences from fungi compared to other organisms has been set at 10^{-8}, indicating that *R. oryzae FTR* has no significant homology to the human proteome.

EXAMPLE 9

The Role of *FTR* gene product in Virulence of *R. oryzae* in the DKA Mouse Model of Hematogenous Dissemination or Mucormycosis

This Example shows that *FTR* gene product (e.g., mRNA or polypeptide) is required for full virulence of *R. oryzae* in the DKA mouse model mucormycosis.

We have utilized RNA interference (RNAi) technology to inhibit the expression of *FTR* in *R. oryzae*. A 400 bp fragment of *FTR* ORF containing the REGLE motif (believed to interact with iron during uptake) was cloned in plasmid pRNAi-pdc upstream of an intron segment. The reverse complement sequence of the same fragment was cloned downstream of the intron. The generated plasmid was transformed into *R. oryzae pyrf* mutant using the biolistic® delivery system (BioRad®) and transformants were selected on minimal medium lacking uracil. Southern blot analysis showed that all obtained transformants maintained the transformed plasmid episomally (data not shown). RT-PCR was used to compare expression of *FTR* by five selected transformants to a control strain, which was transformed with the empty plasmid. *FTR* expression was almost completely inhibited in 4 of the 5 transformants tested and reduced in one transformant compared to control strain (Figure 13). The expression of 18s *rDNA* was not altered in any transformant indicating the specificity of RNAi in inhibiting expression of *FTR*.

The virulence of one of the RNAi transformants was compared to the control strain in the DKA mouse model of hematogenously disseminated mucormycosis. Mice were infected with the control strain transformed or with a transformant harboring the RNAi plasmid (*FTR*-i strain). There was delayed and reduced virulence of the RNAi-transformant compared to the control strain. Interestingly, we found that *R. oryzae* recovered from brains and kidneys of moribund mice infected with the *FTR*-i strain lost the RNAi plasmid since *R. oryzae* failed to grow on minimal medium without uracil but did grow on rich medium (potato dextrose agar). In contrast, *R. oryzae* recovered from the two mice that survived the infection for 25 days (with no signs of disease) was able to grow on both minimal medium without uracil and on rich medium, indicating that the RNAi plasmid was still present in these spores and that inhibiting of *FTR* expression during infection inhibits virulence of *R. oryzae* (Figure 14).

These data demonstrate that the *FTR* is a pivotal virulence factor for *R. oryzae* in the DKA mouse model, and provide additional rational in support of development of an *FTR* vaccine to prevent mucormycosis infections.

EXAMPLE 10

The *rFtr1p* is Exposed Extracellularly and has Limited Homology to Known Human Proteins but is Conserved Among other *Mucorales*

Homology modeling predicts rFtr1p to have a poly-helical bundle structure which is the hallmark of ion-binding or transport proteins found in other microorganisms. In the most robust models, the crucial Glu154 and Glu157 residues of the REGLE iron-binding motif are exposed upon the extracellular facet of the protein, making them accessible to potential binding and inhibition by antibodies (Figure 19).

In order to minimize the potential for induction of autoimmune responses, it is desirable that a protein vaccine being utilized as a human vaccine not have significant homology to numerous human proteins. To investigate the potential for homology between the rFtr1p and human proteins, a PubMed BLAST search was performed comparing the amino acids 16-368 of the rFtr1p (i.e. the amino acids in the intended rFtr1p vaccine) to the human proteome. The search identified five open reading frames with extremely limited homology with an alignment score of 30.4, e= 9.0 for all of the five proteins. Three of these proteins are coiled-coil domain containing 82 (i.e. EAW66982; AAH33726.1; and NP_079001.2), one is a CCDC82 protein (i.e. AAH18663.1) and an unnamed protein (i.e.BAB15683.1). As a benchmark, the standard BLAST search e value for identification of unique sequences from fungi compared to other organisms has been set at 10^{-8}, (Jones et al., *Proc Natl Acad Sci U S A* 2004;101:7329-34 (2004)) indicating that rFtr1p has no significant homology to the human proteome. By comparison, a PubMed BLAST search of the Hepatitis B Surface Antigen, which is utilized as an extremely safe vaccine in humans against the Hepatitis B Virus, revealed 18 hits, one of which was significant (score 75.9, e = $3x10^{-14}$), with the remainder ranging from scores of 27 to 29, with e values of 5 to 10. Hence, the proposed rFtr1p vaccine has comparable or less homology to the human proteome as does the widely utilized HBSAg vaccine.

In contrast, a recent publication demonstrated that *rFTR1* is highly conserved among other pathogenic *Mucorales* including *R. microsporus, R. niveus, R. stolonifer, Rhizomucor miehei, Rhizomucor pusillus, Mucor circinelloides, M. racemosus, M. rouxii,* and *M. plumbeus,* with nucleotide homology of >70%. Interestingly, the putative REGLE iron-binding functional motif is 100% conserved among all *Mucorales*. Nyilasi et al., Clin Microbiol Infect. (2008). This indicates that the proposed vaccine will be cross-immunogenic against other agents of mucormycosis. Moreover, it is expected that cross-genera protection will occur because *R.*

oryzae rFtr1p has a high degree of identity with high iron permeases from a very diverse array of fungi, even beyond molds, including *Aspergillus* spp., *C. albicans*, and *Cryptococcus neoformans*. In all of these fungi, the core REGLE iron-binding functional motif is 100% conserved.

EXAMPLE 11

Passive Immunization with Sera Collected from Mice Vaccinated with *rFtr1p* Protects Mice from *R. oryzae* Infection

To maximize protein production a gene was synthesized (Genscript) encoding a more hydrophilic protein by removing the signal peptide and 6 transmembrane domains that direct localization of the protein to the cell membrane. While the synthesized gene had sequence elements removed, none of the remaining sequence was altered, so as to avoid altering potential epitopes in the exposed, hydrophilic regions of the protein. The synthetic gene also included a 6X-His-tag to affinity purify the expressed protein. This gene was cloned into pQE32 expression vector and transformed into *E. coli*. Log phase bacterial cells were induced with IPTG and the cells were harvested and the recombinant protein was purified over a Ni-agrose affinity column according to the manufacturer instructions (Qiagen) with a production of ~1-1.3 mg of purified protein per liter of culture (Fig 20). The generated protein was used to raise murine antibodies as described below.

To generate immune serum for passive immunization, Balb/c mice were immunized by SQ injection of rFtr1p (20 μg) mixed with complete Freund's adjuvant (CFA) at day 0, boosted with another dose of the antigen with incomplete Freund's adjuvant (IFA) at day 21, and bled for serum collection two weeks later. Pooled sera collected from vaccinated mice demonstrated Ab titer against rFtr1p of >1:800,000, whereas pooled sera collected from mice vaccinated with empty plasmid had an Ab titer of 1:200. Immune or control sera (0.25 ml) were administered i.p. to DKA recipient mice 2 h before intranasal infection with *R. oryzae*. Sera doses were repeated 3 days post infection. Infected mice treated with immune serum improved survival compared to mice treated with control serum (Figure 21). These studies clearly demonstrate the feasibility of using passive immunization targeting rFtr1p to improve survival during mucormycosis.

EXAMPLE 12

FTR1 is expressed by *R. oryzae* during infection in DKA mice

For *FTR1* to play a role in the pathogenesis of mucormycosis, it must be expressed during infection. Quantitative real time PCR (qPCR) was used to investigate the expression of *FTR1* in the brains of DKA mice infected intravenously with 10^5 spores of *R. oryzae*, an inoculum that causes a 100% mortality within 2-3 days (Ibrahim et al., *Antimicrob Agents Chemother* 49: 721-727 (2005). The brain was chosen for analysis because it is a primary target organ in this model (Ibrahim et al., *Antimicrob Agents Chemother* 49: 721-727 (2005)). Expression of *FTR1* from mice (n=5) sacrificed 24 h post infection increased by 4 fold [median (25^{th} quartile, 75^{th} quartile) = 4.12 (1.03, 0.27), p = 0.03 by Wilcoxon Rank Sum)] relative to the constitutive *ACT1* gene. As expected, brains from uninfected mice did not show any expression of *FTR1*.

The non-parametric log-rank test was used to determine differences in survival times, whereas differences in kidney fungal burden, iron uptake, growth rate and *in vivo FTR1* expression were compared by the non-parametric Wilcoxon Rank Sum test.

To directly visualize expression of *FTR1 in vivo* during infection, *R. oryzae* was transformed with a plasmid containing GFP under the control of the *FTR1* promoter. *R. oryzae* strains used in this study are listed in Table 1. Briefly, organisms were grown on potato dextrose agar (PDA) or on YPD plates [1% yeast extract (Difco Laboratories), 2% bacto-peptone (Difco), and 2% D-glucose] for 4 days at 37°C. For *R. oryzae* M16 (a pyrF null mutant that is unable to synthesize its own uracil), PDA was supplemented with 100 µg/ml uracil. An 815 bp partial pyrF PCR fragment (pyrF P11/P13) was used to restore *R. oryzae* M16 to prototrophy. This fragment overlaps the pyrF mutation present in M16 (i.e. point mutation at nt +205 of G to A) (Skory and Ibrahim, *Curr Genet* 52: 23-33 (2007)) and is capable of restoring functionality through gene replacement. In some experiments, *R. oryzae* was starved for iron by growth on yeast nitrogen base (YNB) (Difco/Becton Dickinson, Sparks, MD) supplemented with complete supplemental media without uracil (CSM-URA) (Q-Biogene), (YNB+CSM-URA) [formulation/100ml, 1.7g YNB without amino acids, 20g glucose, 0.77g CSM-URA] in the presence of 1 mM of ascorbic acid and ferrozine. The sporangiospores were collected in endotoxin free PBS containing 0.01% Tween 80, washed with PBS, and counted with a hemacytometer to prepare the final inocula.

Table 1. Strains used in this study

Strain	Genotype	Description and Source
R. oryzae 99-880	Wild-type	Clinical isolate (Ibrahim et al., *J Clin Invest* 117: 2649-2657 (2007)).
R. oryzae M16	*pyrF205*	Uracil deficient (Skory and Ibrahim, *Curr Genet* 52: 23-33 (2007)).
R. oryzae PyrF complemented	*pyrF205::PyrF*	M16 complemented with a wild-type copy of *PyrF* at its original locus, this work
R. oryzae GFP1	M16 (pP_{Ftr1}-*GFP*)	M16 transformed with a plasmid containing a *FTR1* promoter driven *GFP* (Ibrahim et al., *J Clin Invest* 117: 2649-2657 (2007)).
R. oryzae FTR1Ko	*pyrF205, ftr1::PyrF*	*ftr1* knock out, this work
R. oryzae FTR1Inh	M16 (p*FTRi-pdc* intron)	*FTR1* inhibited by RNAi, this work
R. oryzae Empty	M16 (p*RNAi-pdc* intron)	M16 transformed with empty plasmid, this work

This strain fluoresced green when grown in iron-depleted but not iron-rich media *in vitro*, whereas *R. oryzae* transformed with GFP under the control of the constitutive actin promoter (positive control) fluoresced regardless of the iron concentration in the medium (Figure 22A). DKA mice were infected with the GFP reporter strain or *PyrF*-complemented *R. oryzae* grown under iron-rich conditions to suppress GFP expression prior to infection. Twenty four or 48 h post infection brains were collected and processed for histopathology. Because the paraffin embedding process abrogated the intrinsic fluorescence of the GFP protein, the sections were stained with fluorescent anti-GFP antibody. Samples taken 24 h post infection did not show any fungal elements, which was expected since 48 h post infection is the earliest time point that fungal elements can be detected histopathologically in infected tissues (Ibrahim et al., *Antimicrob Agents Chemother* 49: 721-727 (2005)). At 48 h of infection in the brain, the *FTR1* reporter strain of *R. oryzae* expressed GFP, whereas the negative control, *PyrF*-complemented *R. oryzae* did not (Figure 22B). Additionally, GFP expression was induced by

low iron levels in the host environment since spores used for infecting mice were grown in iron-rich medium (condition that suppresses the expression of *FTR1*) and did not fluoresce green when used to infect mice (Figure 22B, DIC overlaid with fluorescence).

Example 13

Isolation of a homokaryotic ftr1 null could not be achieved in multinucleated *R. oryzae* despite integration of the disruption cassette at the FTR1 locus

The expression of FTR1 during active infection suggested a role for FTR1 in the pathogenicity of *R. oryzae*. The effect of FTR1 gene disruption on the ability of *R. oryzae* to take up iron *in vitro* and cause disease *in vivo* was studied. Isolates obtained from two separate transformations were purified with one round of sporulation and single colony isolation. To achieve single colony isolation, transformants were grown on chemically defined medium (YNB+CSM-URA) supplemented with 1 mM $FeCl_3$ (iron rich) to favor the segregation of the ftr1 null allele, since FTR1 is poorly expressed in concentrations $\geq$ 350 μM of $FeCl_3$ (Fu et al., *FEMS Microbiol Lett* **235**: 169-176 (2004)). Isolates were screened for integration of the disruption cassette with PCR primer pairs FTR1-P3/PyrF-P9 (expected 1054 bp) and PyrF-P18/FTR1-P4 (expected 1140 bp). Disruption of the FTR1 locus was tested by the absence of a PCR amplification product using primers FTR1-P5/FTR1-P6 (expected 503), which amplified a segment from the ORF of FTR1 (Table 2 and Figure 23A). PCR confirmed integration of the disruption cassette in the FTR1 locus, and absence of FTR1 ORF from several putative null mutant strains (Figure 23B). Furthermore, these amplification products were also sequenced to demonstrate that the disruption cassette had integrated into the FTR1 locus by homologous recombination (data not shown). Finally, integration of the disruption cassette in the FTR1 locus was confirmed by Southern blotting (see below).

To study the expression of FTR1, we utilized GFP as a reporter system for FTR1 promoter expression. *R. oryzae* M16 was transformed with a plasmid containing the reporter gene GFP driven by the FTR1 promoter (*R. oryzae* GFP1) as previously described (Ibrahim et al., *J Clin Invest* 117: 2649-2657 (2007)). GFP was also cloned under the constitutively expressed actin promoter (Act1p) then transformed into *R. oryzae* M16 to serve as a positive control. Prior to studying the expression of FTR1 *in vivo* we examined the expression of FTR1 *in vitro* using FACS analysis. Briefly, *R. oryzae* transformed with either GFP driven by Ftr1p or Act1p were grown in YNB+CSM-URA with (iron-depleted conditions) or without (iron-replete conditions) 1 mM of ascorbic acid and ferrozine at 37°C for 12 h. These conditions produced

germlings of *R. oryzae* rather than hyphae. Fluorescence of 1 ml of germlings was determined using a FACSCaliber (Becton Dickinson) instrument equipped with an argon laser emitting at 488 nm. Fluorescence emission was read with 515/40 bandpass filter. Fluorescence data were collected with logarithmic amplifiers. The mean fluorescence intensities of 104 events were calculated using CELLQUEST software.

For *in vivo* infection, BALB/c male mice (>20g) were rendered diabetic with a single i.p. injection of 190 mg/kg streptozotocin in 0.2 ml citrate buffer 10 days prior to fungal challenge (Ibrahim et al. *Antimicrob Agents Chemother* 47: 3343-3344 (2003)). Glycosuria and ketonuria were confirmed in all mice 7 days after streptozotocin treatment. Diabetic ketoacidotic mice were infected with fungal spores by tail vein injection with a target inoculum of 5 x 103 spores. To confirm the inoculum, dilutions were streaked on PDA plates containing 0.1% triton and colonies were counted following a 24 h incubation period at 37°C. For the intranasal infection, 107 spores in 20 µl of 0.01% Tween 80 in PBS were placed on the nostrils of ketamine (100 mg/kg) sedated mice ((Waldorf et al, *Journal of Clinical Investigation* 74: 150-160 (1984)). To confirm the inoculum, mice were sacrificed immediately after inhaling *R. oryzae* spores, and lungs were homogenized, plated on PDA containing 0.1% triton and colonies were counted following incubation at 37°C. For both models, the primary efficacy endpoint was time to death. In some experiments, as a secondary endpoint, brain and kidney fungal burden (primary target organs) (Ibrahim et al., *Antimicrob Agents Chemother* 49: 721-727 (2005)) was determined by homogenization by rolling a pipette on organs placed in Whirl-Pak bags (Nasco, Fort Atkinson, WI) containing 1 ml saline. The homogenate was serially diluted in 0.85% saline and then quantitatively cultured on PDA plates containing 0.1% triton. Values were expressed as log10 cfu g-1 tissue. To detect GFP expression, anti-GFP rabbit polyclonal antibody (Novus) was used to stain the histopathological samples then counter stained with FITC conjugated anti-rabbit antibody.

To quantify the expression of FTR1 in infected tissues, brains of BALB/C mice infected with *R. oryzae* wild type (99-880) through tail vein injection were collected 24 or 48 hr post infection and immediately flash frozen in liquid nitrogen prior to grinding and RNA extraction with phenol. Brains collected from uninfected DKA mice were processed in parallel and served as negative controls. Frozen brains were then ground under liquid nitrogen and total RNA was then isolated using the hot phenol method (Gravelat et al. *Infect Immun* 76: 3632-3639 (2008)). Contaminating genomic DNA was removed from RNA samples by treatment with 1 µl of Turbo-DNase (Ambion) for 30 min at room temperature. DNase was then

removed using an RNA Clean-Up kit (Zymo Research). First-strand cDNA synthesis was performed using the Retroscript first-strand synthesis kit (Ambion). FTR1 specific primers (listed in Table 2) were designed with the assistance of online primer design software (Genscript). The amplification efficiency was determined by serial dilution experiments, and the resulting efficiency coefficient was used for the quantification of the products (Pfaffl et al., *Nucleic Acids Res* 29: e45 (2001)).

Gene expression was analyzed by an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the QuantiTect Sybr Green PCR kit (Qiagen). PCR conditions were were 10 min at 90°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Single PCR products were confirmed with the heat dissociation protocol at the end of the PCR cycles. The amount of FTR1 expression in infected brains was normalized to either 18S rRNA or ACT1 (Table 2) and the quantified using the 2(-ΔΔC(T)) method (Livak and Schmittgen, (2001) Methods 25: 402-408 (2001)). All reactions were performed in duplicate, and the mixture included a negative no-reverse transcription (RT) control in which reverse transcriptase was omitted.

Table 2. Oligonucleotides used in this study.

Primers	Sequence	Description
Primers used for detecting *in vivo* expression of *FTR1*		
FTR1-RT5'	GGTGGTGTCTCCTTGGGTAT	5' primer
FTR1-RT3'	AAGGAAACCGACCAAACAAC	3' primer
18S-RT5'	CCAGACTGGCTTGTCTGTAATC	5' primer annealing to rRNA
18S-RT3'	AAGTCAAATTGTCGTTGGCA	3' primer annealing to rRNA
ACT1-RT5'	TGAACAAGAAATGCAAACTGC	5' primer
ACT1-RT3'	CAGTAATGACTTGACCATCAGGA	3' primer
Primers used for making the *ftr1* disruption cassette and confirming integration in the *FTR1* locus		
FTR1 P1	TTCGAAAAGACCGTCAGGATTAGC	Annealing to *FTR1*-5' UTR
FTR1 P2	GAGGGACACAAGCAAGCAGAAAGT	Annealing to *FTR1*-3' UTR
FTR1 P3	CACTTACGGCCATTTTCCATTGAC	Annealing to *FTR1*-5' UTR upstream of the disruption cassette
FTR1 P4	CGCGCTAAATGAACAAAGAAT	Annealing to *FTR1*-3' UTR

		downstream of the disruption cassette
FTR1 P5	ATGTCTCAAGATCTCTTCAACCGTACC	5' primer testing for the entire *FTR1* ORF (1100 bp)
FTR1 P6	TTAAGCCTTAATAGCATCAGATTCG	3' primer testing for the entire *FTR1* ORF (1100 bp)
FTR1 P11	GATCACTGCCATGGGTCTTGCTAT	5' primer to test for 503 bp of *FTR1* ORF
FTR1 P12	TATCATGTTGGCTTCTGGGTCTC	3' primer to test for 503 bp of *FTR1* ORF
PyrF P9	GCCGTGGCGCAGACAAGAG	3' primer annealing to pyrF
PyrF P18	GTGCCGAAATCGCTCCAGA	5' primer annealing to pyrF
ACT1 P1	GTCTTTCCTTCTATTGTTGGTC	5' primer to test for functional template DNA (600bp)
ACT1-P2	CCATCAGGAAGTTCATAAGAC	3' primer to test for functional template DNA (600bp)
Primers used in making *PyrF*-complemented *R. oryzae*		
PyrF P11	CAAAGCCAATTCAGCCTCAAATG	5' primer to amplify partial PyrF (815 bp)
PyrF P13	CTTGGATCAGGGTGGACTCGTAG	3' primer to amplify partial PyrF (815 bp)
Primers used to determine *FTR1* copy number		
FTR1 P9	CCAACAGTGAAAAGTCATCCTTT	5' primer to amplify *FTR1* (250 bp)
FTR1 P10	GCAATAGGAATTGATTTTCCTTG	3' primer to amplify *FTR1* (250 bp)
ACT1 P3	TATCGTTCTTGACTCTGGTGATG	5' primer to amplify actin (250 bp)
ACT1 P4	GAAAGAGTGACCACGTTCAGC	3' primer to amplify actin (250 bp)
Primers used for making RNAi strain		
PyrF14	CTCGAGGCTTTAGGTCAAATTGTGG	5' primer to amplify 1641bp of *PyrF* to clone in

		pRNAi-pdc
PyrF15	CCCGGGTTATTGCTTGATACCATATTGTG	3' primer to amplify 1641bp of *PyrF* to clone in pRNAi-pdc
FTR1 P7	GCGGCCGCGCTAGCGCATGCATGTCTCA AGATCTCTTCAACGTACCGATC	5' primer to amplify 450bp of *FTR1*to clone in pRNAi-pdc
FTR1 P8	GACGTCCCGCGGGGCGCGCCGGTGATAA AAGGCAAGACAAAGAACGCGTA	3' primer to amplify 450bp of *FTR1* to clone in pRNAi-pdc
18S rRNA P1	CATGGTTGAGATTGTAAGATAG	5' primer to amplify 18S rRNA
18S rRNA P2	AGTCAATGGACGTGGAGTC	3' primer to amplify 18S rRNA
Primers used for making synthetic FTR1p in *E. coli*		
SynFtr1p P5	CATCACCATGGGATCAAAAGAAT GTTTAATACTGAATCTCCA	5' primer to amplify synthetic Ftr1p
SynFtr1p P6	CTAATTAAGCTTGGCTTAAGCTTT AATAGCATCAGATTCAATTTTTTC	3' primer to amplify synthetic Ftr1p

To disrupt the FTR1, we constructed a gene disruption cassette encompassing a functional PyrF copy (998 bp) amplified from *R. oryzae* wild-type flanked by 606 and 710 bp fragments of FTR1-5' UTR and FTR1-3' UTR, respectively (Fig. 23A). The gene disruption construct was PCR amplified using primers FTR1 P1/P2 (Table 2) in order to obtain a 2.3 kb disruption fragment containing only the pyrF flanked by homologous FTR1 UTR sequence (Fig. 23A). This was then used to transform *R. oryzae* M16 (pyrF mutant) with biolistic bombardment (Skory, *Mol Genet Genomics* 268: 397-406 (2002)). The disruption cassette replaces the entire FTR1 coding region from -16 to the stop codon, with the pyrF gene fragment. Isolates obtained from two separate transformations were purified with one round of sporulation and single colony isolation on chemically defined medium (YNB+CSM-URA) supplemented with 1 mM FeCl3 (iron rich) to favor the segregation of the FTR1 null allele, since FTR1 expression in this iron concentration is suppressed (Fu et al., *FEMS Microbiol Lett* 235: 169-176 (2004)). Isolates were tested for integration of the disruption cassette with PCR primer pairs FTR1-P3/PyrF-P9 (expected 1054 bp) and PyrF-P18/FTR1-P4 (expected 1140 bp). Disruption of FTR1 was confirmed by the absence of a PCR amplification product using

primers FTR1-P5/FTR1-P6 (expected 503) to amplify the ORF of FTR1 and by Southern blot analysis. In an effort to obtain a homokaryotic isolate containing the FTR1 null allele, transformants with confirmed integration in the FTR1 locus were further taken through 14 rounds of sporulation and single colony isolation on YNB+CSM-URA supplemented with 1 mM FeCl3 .

We previously found that *FTR1* is expressed *in vitro* in iron-depleted conditions ($FeCl_3$ concentration between 0-50 μM) and suppressed in iron replete media ($FeCl_3$ concentrations of ≥350 μM) (Fu et al., *FEMS Microbiol Lett* 235: 169-176 (2004)). To investigate if *FTR1* disruption had an effect on the ability of *R. oryzae* to grow in media with different sources and concentration of iron, we compared growth of several putative null mutant strains to growth of wild-type or *PyrF*-complemented *R. oryzae*. Growth was compared on media (CSM-URA) which had been previously chelated for iron and then supplemented with defined concentrations of free iron (i.e. $FeCl_3$ or $FeSO_4$) or iron complexed to deferoxamine [ferrioxamine] or heme. Compared to wild-type or *PyrF*-complemented *R. oryzae*, putative *ftr1* null mutant strains had significantly less growth at 48 h in iron-depleted media (i.e. free iron at 10 μM) (Figure 23C). Ferrioxamine or iron complexed with heme at 100 μM (relatively depleted because iron is complexed) supported the growth of the wild-type and *PyrF*-complemented strains better than the putative *ftr1* null mutant. However, free iron at 1000 μM (iron-rich media) supported the growth of all strains equally (Figure 23C) consistent with our previous findings that *ftr1* is primarily expressed in iron-depleted environments.

Growth of the putative ftr1 disruption mutants were compared to *R. oryzae* wild-type or *R. oryzae* PyrF-complemented strain by growing on plates YNB+CSM-URA supplemented with 10 or 1000 μM of FeCl3, FeSO4, or with 100 μM of heme, or ferroxamine as a source of iron. Additionally, putative ftr1 null or RNAi mutants were compared to their corresponding control strains for their growth on YPD or chemically defined medium (i.e. YNB+CSM-URA). Briefly, ten microliters of 105 spores of *R. oryzae* spores were spotted in the center of plates and the mycelial diameter was measured after 48 h of growth for medium containing FeCl3, FeSO4, or ferroxamine or for 72 h for plates supplemented with heme. The experiment was repeated three times on different days and growth rate was expressed as increase in mycelial diameter of the fungus per hour.

Interestingly, growth of the putative *ftr1* null mutants increased to levels similar to the wild-type and *PyrF*-complemented strains after 96 h on iron-depleted media (data not shown). Furthermore, PCR analysis of these cultures after 96 h of growth confirmed that the *FTR1*

ORF was once again detectable in all of the putative *ftr1* null mutant transformants (Figure 23D). Similar results were obtained with several other putative *ftr1* null mutants and it was concluded that one round of sporulation and single colony isolation was not sufficient to purify an *ftr1* homokaryotic null allele strain.

R. oryzae is known to be coenocytic and it is generally presumed that sporangiospores are multinucleated, although the number of nuclei has not been previously described (Ma et al., *PLoS Genet* 5: e1000549 (2009)). Gene disruption appeared to be complicated by the presence of heterokaryotic nuclei in both the mycelium and sporangiospores, and the number of nuclei present in swollen spores using DAPI staining was determined. Briefly, to determine the number of nuclei present in *R. oryzae* spores, spores in YPD medium were pregerminated for 2 h at 37°C. Swollen spores were washed once with cold PBS then suspended at a concentration of 5 x 105/ ml in PBS. One µl of 50 µg/ml of 4'6-diamidino-2-phenylindole (DAPI, Sigma) were added and the cells were electroporated (BioRad) according to the manufacturer instructions. The swollen spores were washed five times using cold PBS prior to resuspending in 100 µl PBS. Ten µl sample was placed on a glass slide and covered with a coverslip. The stained cells were visualized using an epifluorescence microscope.

It was found that *R. oryzae* strain M16 had more than 10 nuclei per swollen spore (Figure 24A). Given the high number of nuclei present, 14 rounds of sporulation and single colony isolation of putative *ftr1* null mutants on iron-rich medium (i.e. medium containing 1000 µM of $FeCl_3$) were performed to segregate the null alleles by relieving the selective pressure for maintaining *FTR1* (since *FTR1* is poorly expressed in iron-rich conditions) (Fu et al., *FEMS Microbiol Lett* 235: 169-176 (2004)). PCR analysis of the putative null mutants after 14 rounds of selection demonstrated lack of amplification of *FTR1* ORF (Figure 24B). Similar to the results in Figure 23C, the null mutant had defective growth on iron limited sources for the first 48 h compared to wild-type or *PyrF*-complemented strains. However, after growth of the same putative null mutants in iron-depleted environment (100 µM ferrioxamine), the *FTR1* ORF was once again amplified by PCR. These results were confirmed with Southern blot analysis (Figure 24C). The Southern blot demonstrated almost complete elimination of the *FTR1* band (1960 kb) from gDNA of the putative *ftr1* null mutants grown on iron-rich medium, but return of the *FTR1* band after growth of the same strain on iron-depleted medium (Figure 24C). Additionally, Southern hybridization analysis confirmed the site-specific integration of the disruption cassette into the *ftr1* locus only when the putative *ftr1* null mutant was grown in iron-rich medium. Finally, there was no evidence of ectopic integration or

extrachromosomal replication, consistent with the fact that the relative copy number of the *ftr1* null allele was dependent on the ratio of heterokaryotic nuclei.

To compare the copy number of FTR1 in the putative ftr1 null mutant grown on iron-rich or iron-limited media or to those of PyrF-complemented strain, qPCR was used. Briefly, genomic DNA was extracted with the OmniPrep lysis buffer (GBiosciences) from PyrF-complemented *R. oryzae* grown in YNB+CSM-URA supplemented with 1mM FeCl3 or putative ftr1 null mutant grown in either YNB+CSM-URA supplemented with 1 mM FeCl3 or 100 μM ferrioxamine. FTR1 copy number in each sample was determined by qPCR using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and amplification products were detected with Power Sybr Green Cells-to-CTTM kit (Applied Biosystems). PCR conditions were as follows: denaturing at 95°C for 15 s min and amplification 40 cycles with annealing/extension carried out at 60°C for 1 min. FTR1 copy numbers were then normalized to *R. oryzae* ACT1, and relative copy number was estimated using the formula $2^{-\Delta\Delta CT}$, where $\Delta CT = [Ct_{\text{target gene}} - Ct_{ACT1}]$ and $\Delta\Delta CT$= [ΔCT of mutant-ΔCT of *PyrF*-complmented strain].

qPCR was used to quantify the copy number of *FTR1* in a putative *ftr1* null mutant that was passed through 14 rounds of sporulation and single colony isolation on iron-rich media, as well as the same strain after growth in iron-depleted media, and the *R. oryzae PyrF*-complemented strain. The putative *ftr1* null mutant strain grown in iron-rich media had a 60% reduction in the relative copy number of *FTR1* (normalized to *ACT1* gene) compared to the same strain grown in iron-depleted media or to the *R. oryzae PyrF*-complemented strain (Figure 25A and 25B). Thus, while it was possible to significantly decrease the relative copy number of functional *FTR1* in multinucleated *R. oryzae*, a homokaryotic isolate of this mutant allele was not obtained.

Example 14

Reduction of the FTR1 copy number attenuates iron uptake *in vitro* and reduces virulence *in vivo*

As shown herein, reduction of the relative copy number of functional *FTR1* in *R. oryzae is* sufficient to decrease iron uptake and therefore reduce virulence. The putative *ftr1* null mutant had a ~35% reduction in ^{59}Fe uptake compared to wild-type or *R. oryzae PyrF*-complemented strain (Figure 25C).

To determine the *in vivo* relevance of the diminished *in vitro* iron uptake of the putative *ftr1* null mutant, its virulence was compared to *R. oryzae* wild-type or *PyrF*- complemented strains during infection in mice with DKA. Mice were infected intravenously (i.v.) or intranasally (i.n.) with strains that demonstrated similar growth *in vitro* on iron-rich environment of YPD or CSM-URA (0.185±0.005 or 0.257±0.003 cm/h for the putative *ftr1* null vs. 0.188 ± 0.008 or 0.260±0.0051cm/h for the *PyrF*-complemented on iron rich CSM-URA or YPD medium, respectively) (Figure 26A). In both models, the putative *ftr1* null mutant showed reduced virulence compared to the wild-type or *PyrF*-complemented strain (62% vs. 100% mortality for mutant vs. control strains in mice with disseminated infection, and 44% vs. 75% mortality for mutant vs. control strains in the intranasal model) (Figure 26B,C). As expected colonies retrieved from moribund animals infected with the putative *ftr1* null mutant strain demonstrated similar copy numbers of *FTR1* compared to the *pyrF*-complemented strain (data not shown), indicative of restoration of *FTR1* copy number as was seen after growth in iron-depleted environments *in vitro*. Additionally, in both models the *pyrF*-complemented strain had similar virulence to the wild-type *R. oryzae*, demonstrating that restoration of the *pyrF* gene in its original locus does not affect virulence.

Example 15

Inhibition of FTR1 gene expression by RNAi reduces iron uptake and diminishes virulence of *R. oryzae*

To confirm the phenotypes seen after gene disruption, RNA interference (RNAi) was used to diminish *FTR1* expression in *R. oryzae*.

RNA interference (RNAi) technology was utilized to inhibit the expression of FTR1 in *R. oryzae*. A 450 bp fragment of FTR1 ORF containing the REGLE motif believed to interact with iron during uptake (Stearman et al., *Science* 271: 1552-1557 (1996)) was PCR amplified and cloned as an inverted repeat under control of the Rhizopus expression vector pPdcA-Ex (Mertens et al., *Archives of microbiology* 186: 41-50 (2006)). Additionally, an intron from the Rhizopus pdcA gene (Skory, *Curr Microbiol* 47: 59-64 (2003)) was included between repeat to serve as a linker for stabilization of the intended dsRNA structure (Nakayashiki et al., *Fungal Genet Biol* 42: 275-283 (2005); Wesley et al. *Plant J* 27: 581-590 (2001)). The generated plasmid was transformed into *R. oryzae* pyrF mutant using the biolistic delivery system (BioRad) and transformants were selected on minimal medium lacking uracil.

Southern blot analysis (data not shown) revealed that all RNAi transformants maintained the transformed plasmid extrachromosomally, consistent with the fact that we did not linearize the plasmid during transformation (Skory, *Mol Genet Genomics* 268: 397-406 (2002)). *FTR1* expression was compared by end-point RT-PCR in five transformants vs. the control strain (*i.e. R. oryzae* pyrf null mutant [M16] transformed with empty-plasmid). *FTR1* expression was almost completely blocked in 4 transformants, while readily detected in the control strain (Figure 27A). Amplification of 18s *rDNA* with the same RT templates demonstrated the integrity of the starting sample and the lack of PCR inhibitors. Inhibition of *FTR1* expression by RNAi was specific, with no apparent reduction in off-site gene expression. A representative RNAi transformant demonstrated similar growth to control strain when grown on either iron rich YPD or CSM-URA media (0.193±0.082 or 0.205±0.016 cm/h for the transformant vs. 0.201 ± 0.087 or 0.211±0.011cm/h for the control strain on iron rich CSM-URA or YPD medium, respectively) (Figure 27B).

The ability to take up iron was tested *in vitro* of the transformant with near complete inhibition of FTR1 expression and similar growth to the control strain. Interestingly, RNAi decreased ^{59}Fe uptake by *R. oryzae* more effectively than did gene disruption, with ~50% inhibition of iron uptake at all times tested (Figure 27C). Briefly, to characterize the effect of FTR1 manipulation on the ability of *R. oryzae* to take up iron *in vitro*, ftr1 putative disruption mutant or the RNAi mutant were compared to wild-type or *R. oryzae* PyrF-complemented strains in their ability to accumulate intracellular ^{59}FeCl3 (Amersham Pharmacia Biotech) using a modification of our published method (Fu et al., *FEMS Microbiol Lett* 235: 169-176 (2004)). Spores were pre-germinated for 3 h YPD medium supplemented with 1mM ferrozine and 1 mM ascorbic acid at 37°C with shaking. Cells were harvested by centrifugation, washed twice with ice cold assay buffer pH 6.1 (minimal medium + 10 mM 4-morpholinepropanesulfonic acid + 1 mM ferrozine), and then resuspended in assay buffer without any ferrozine to give a concentration of 108 cells per ml. To measure uptake of ^{59}Fe, 50 µl of the cell suspension was added to 450 µl of chilled assay buffer without ferrozine but supplemented with 0.1 lM 59FeCl3, and incubated in a shaking water bath at 30°C. After selected time points, the assay samples were chilled on ice, vortexed, vacuum filtered through Whatman GF/C filters and washed with 10 ml ice cold SSW (1 mM EDTA, 20 mM Na3-citrate pH 4.2, 1 mMKH2PO4, 1 mM CaCl2, 5 mM MgSO4, 1 mM NaCl). Filters were removed and placed in glass scintillation vials containing 10 ml scintillation fluid (Filter-count). Cell-associated ^{59}Fe was counted in a Packard 2200CA liquid scintillation counter (Packard Instrument Co., Downers Grove, Il). Nonspecific uptake due to cell surface adsorption was determined by preparing

parallel assays that were held on ice for 10 min before filtration and washing. The background levels of ^{59}Fe were subtracted before calculation of uptake rates. The experiment was carried out in triplicate and repeated three times on different days. The data is presented as specific uptake in pmole/ 5x 10^6 germinated spores.

Next, the virulence of the RNAi transformant was compared to the control strain in the DKA mouse models of hematogenously disseminated or intranasal mucormycosis. The RNAi-transformant demonstrated reduced virulence compared to the control strain in both models (75% vs. 100% mortality for RNAi transformant vs. control strain in mice with disseminated infection, and 11% vs. 67% mortality for RNAi transformant vs. control in the intranasal model, $p<0.02$ for both comparisons by Log Rand test) (Figure 28A,B). Interestingly, strains recovered from kidneys of mice that died of infection with the RNAi transformant had lost the RNAi plasmid as evident by growth of *R. oryzae* colonies on YPD plates and not YNB+CSM-URA (data not shown), and hence had regained ability to express *FTR1*. In contrast, strains recovered from kidneys of mice that survived the infection through day 25, when the experiment was terminated, had not lost their RNAi plasmid. Additionally, mice infected intravenously with the RNAi transformant had a ~ 6- and 3-fold reduction in kidney and brain fungal burden compared to mice infected with control strain, respectively (Figure 28C). These data demonstrate that the *FTR1* gene product is a pivotal virulence factor for *R. oryzae* in DKA mice.

Example 16

Passive immunization with sera collected from mice vaccinated with Ftr1p protects mice from *R. oryzae* infection

This example demonstrates that passive immunization targeting *FTR1* would protect against mucormycosis.

To generate immune serum for passive immunization, mice were immunized by SQ injection of Ftr1p mixed with complete Freund's adjuvant (CFA) followed by a boost with another dose of the antigen with incomplete Freund's adjuvant (IFA) at day 21, and bled for serum collection two weeks later. Another set of mice were vaccinated with supernatants collected from E. coli transformed with empty plasmid to generate non-immune control serum. Antibody titers were determined using ELISA plates coated with 5 μg of synthetic recombinant Ftr1p as we previously described (Ibrahim et al., *Infect Immun* 73: 999-1005 (2005)). Immune or control sera (0.25 ml) were administered i.p. to diabetic ketoacidosis

recipient mice 2 h before intranasal infection with 2.5 x 107 *R. oryzae* 99-880 spores. Sera doses were repeated 3 days post infection and survival of mice was followed for 35 days post infection. Pooled sera collected from vaccinated mice had anti-Ftr1p IgG titers of >1:800,000, whereas pooled sera collected from negative control mice vaccinated with purified supernatant from an empty plasmid transformed clone had an anti-Ftr1p IgG titer of 1:200. Administration of immune sera to DKA mice subsequently infected intranasally with *R. oryzae* significantly improved survival compared to mice treated with control serum (63% vs. 0% survival for immune sera vs. non-immune sera, $p<0.001$) (Figure 28D).

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific examples and studies detailed above are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A vaccine composition, comprising an *FTR* polypeptide, or an antigenic fragment of said polypeptide, and a pharmaceutically acceptable carrier.

2. The vaccine composition of claim 1, further comprising an adjuvant.

3. The vaccine composition of claim 1, wherein the antigenic fragment comprise an iron hinging domain or an extracellular region of *FTR*.

4. A vaccine composition, comprising a vector comprising a nucleotide sequence that is substantially complimentary to at least 18 contiguous nucleotides of *FTR* sequence encoding for *FTR* polypeptide according to claim 1, a transcription promoter, and a transcription terminator; wherein the promoter is operably linked to the *FTR* nucleotide sequence, and wherein the *FTR* nucleotide sequence is operably linked to the transcription terminator, and a pharmaceutically acceptable carrier.

5. The vaccine composition of claim 4, further comprising an adjuvant.

6. Use of an antisense, a small interfering RNA or an antibody inhibitor of *FTR* selected from the group consisting of a nucleotide sequence that is substantially complimentary to a portion of an *FTR* sequence encoding for *FTR* polypeptide according to claim 1; a nucleotide sequence that is substantially complimentary to at least 12 contiguous nucleotide bases of *FTR* sequence encoding for *FTR* polypeptide according to claim 1 and a pharmaceutically acceptable excipient or carrier in the manufacture of a vaccine for treating or preventing a fungal condition in a subject in need thereof.

7. Use of an immunogenic amount of an *FTR* polypeptide, or an immunogenic fragment thereof in the manufacture of a vaccine for treating or preventing a fungal condition, wherein said vaccine is to be administered to a subject having, or susceptible to having, a fungal condition, wherein said fungal condition comprises zygomycosis.

8. The use of claim 7, wherein the immunogenic amount of an *FTR* polypeptide is to be administered with a pharmaceutically acceptable medium or adjuvant.

9. The use of claim 7, wherein said zygomycosis further comprises mucormycosis.

10. The use of claim 9, wherein said mucormycosis comprises rhinocerebral mucormycosis, pulmonary mucormycosis, gastrointestinal mucormycosis, disseminated mucormycosis, bone mucormycosis, mediastinum mucormycosis, trachea mucormycosis, kidney mucormycosis, peritoneum mucormycosis, superior vena cava mucormycosis or external otitis mucormycosis.

11. The use of claim 10, wherein said mucormycosis is associated with an infectious agent within the order Mucorales.

12. The use of claim 11, wherein said agent within the order Mucorales is selected from the fungi families of *Choanephoraceae; Cunninghamellaceae; Mucoraceae; Mycotyphaceae; Phycomycetaceae; Pilobolaceae; Saksenaeaceae; Syncephalastraceae; or Umbelopsidaceae*.

13. The use of claim 11, wherein said agent within the order Mucorales is selected from the genera of *Rhizopus, Absidia, Apophysomyces, Mucor, or Cunninghamell.*

14. The use of claim 13, wherein said agent within the genera of *Rhizopus, Absidia, Apophysomyces, Mucor*, or *Cunninghamell* is selected from *Rhizopus oryzae, Rhizopus microsporus, rhizopodiformis, Absidia corymbifera, Apophysomyces elegans, or Rhizomucor pusillus.*

15. Use of an antisense, a small interfering RNA or an antibody (polyclonal or monoclonal) inhibitor of *FTR* nucleotide sequence encoding for *FTR* polypeptide according to claim 1 in the manufacture of a vaccine for treating or preventing a fungal condition in a subject in need thereof, wherein the use comprises exposing said fungi to said vaccine, wherein said fungal condition comprises zygomycosis.

16. The use of claim 15, wherein said antisense or antibody inhibitor of *FTR* comprises an inhibitor selected from the group consisting of a nucleotide sequence that is substantially complimentary to a portion of an *FTR* nucleotide sequence encoding for *FTR* polypeptide according to claim 1; an nucleotide sequence that is substantially complimentary to at least 12 contiguous nucleotide bases of *FTR* sequence encoding for *FTR* polypeptide according to claim 1.