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# The high affinity iron permease is a key virulence factor required for *Rhizopus oryzae* pathogenesis

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## SUMMARY

*Rhizopus oryzae*is the most common cause of mucormycosis, an angioinvasive fungal infection that causes more then 50% mortality rate despite first-line therapy. Clinical and animal model data clearly demonstrate that the presence of elevated available serum iron predisposes the host to mucormycosis. The high affinity iron permease gene (*FTR1*) is required for *R. oryzae* iron transport in iron-depleted environments. Here we demonstrate that *FTR1* is required for full virulence of *R. oryzae* in mice. We show that *FTR1* is expressed during infection in diabetic ketoacidotic (DKA) mice. In addition, we disrupted *FTR1* by double cross-over homologous recombination, but multinucleated *R. oryzae* could not be forced to segregate to a homokaryotic null allele. Nevertheless, a reduction of the relative copy number of *FTR1* and inhibition of *FTR1* expression by RNAi compromised the ability of *R. oryzae* to acquire iron *in vitro* and reduced its virulence in DKA mice. Importantly, passive immunization with anti-Ftr1p immune sera protected DKA mice from infection with *R. oryzae*. Thus *FTR1* is a virulence factor for *R. oryzae*, and anti-Ftr1p passive immunotherapy deserves further evaluation as a strategy to improve outcomes of deadly mucormycosis.

### Keywords

Mucormycosis; *R. oryzae*; *FTR1*; virulence; iron; murine

## INTRODUCTION

Mucormycosis (zygomycosis) is a life-threatening infection which occurs in patients with diabetes, in patients with increased available serum iron (e.g. from systemic acidosis or deferoxamine therapy), and in patients immunocompromised by neutropenia or medications

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(e.g. corticosteroids) (Ibrahim *et al.*, 2003b, Kwon-Chung & Bennett, 1992, Spellberg *et al.*, 2005). *Rhizopus oryzae* is the most common cause of mucormycosis, and is responsible for approximately 70% of such infections (Reed *et al.*, 2008, Ribes *et al.*, 2000, Roden *et al.*, 2005, Spellberg et al., 2005). Due to the rising prevalence of diabetes, cancer, and organ transplantation in the aging United States population, the number of patients at risk for this deadly infection is significantly rising (Marr *et al.*, 2002). Unfortunately, despite disfiguring surgical debridement and adjunctive antifungal therapy, the overall mortality of mucormycosis remains approximately 50%, and it approaches 100% in patients with disseminated disease, or persistent neutropenia (Gleissner *et al.*, 2004, Kauffman, 2004, Kontoyiannis *et al.*, 2000, Marr et al., 2002, Siwek *et al.*, 2004, Spellberg et al., 2005). Clearly new strategies to prevent and treat mucormycosis are urgently needed.

The hypersusceptibility of patients with increased available serum iron to infection by *R. oryzae*, but not other pathogenic fungi (Ibrahim et al., 2003b, Sugar, 2005), highlights the central role of iron metabolism in the organism's virulence. For example, the fungi that cause mucormycosis are normally unable to grow in serum due to the sequestration of iron by iron binding proteins. However, diabetic ketoacidosis (DKA) causes proton-mediated dissociation of iron from iron-sequestering proteins, and the increased levels of available iron enable enhanced growth of these fungi in serum (Artis *et al.*, 1982, Ibrahim *et al.*, 2006, Spellberg et al., 2005). Moreover, patients treated with deferoxamine are uniquely susceptible to mucormycosis (Boelaert *et al.*, 1988, Boelaert *et al.*, 1987, Boelaert *et al.*, 1994). While deferoxamine is an iron-chelator from the perspective of the human host, *Rhizopus* spp. utilize deferoxamine as a xeno-siderophore to supply previously unavailable iron to the fungus (Boelaert *et al.*, 1993, de Locht *et al.*, 1994). Recently, we found that other iron chelators do not act as iron siderophores for *Rhizopus.* In fact, treatment of *Rhizopus*-infected mice with the iron chelators deferiprone (Ibrahim *et al.*, 2006) or deferasirox (Ibrahim *et al.*, 2007) markedly improved survival, reduced fungal burden in target organs, and enhanced the host inflammatory response to mucormycosis. These results further confirm the unique importance of iron in the pathogenesis of mucormycosis.

We have previously cloned the high-affinity iron permease of *R. oryzae* (*FTR1*), which is a primary effector of the organism's ability to acquire iron in iron-limited environments (akin to those found *in vivo* during infection) (Fu *et al.*, 2004). In the current study we sought to determine the role of *FTR1* in the pathogenicity of *R. oryzae* by studying the expression of the gene and its product in vivo, elucidating the effect of gene disruption and gene silencing on the virulence of *R. oryzae*, and finally by determining the role of anti-Ftr1p antibodies in protecting DKA mice from *R. oryzae* infection.

## RESULTS

### 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. We used quantitative real time PCR (qPCR) 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.*, 2005a). The brain was chosen for analysis because it is a primary target organ in this model (Ibrahim *et al.*, 2005a). 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*.

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 (strains described in

Table 1). 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 (Fig. 1A). 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., 2005a). 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 (Fig. 1B). 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 (Fig. 1B, DIC overlaid with fluorescence).

## 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. We sought to study the effect of FTR1 gene disruption on the ability of R. oryzae to take up iron in vitro and cause disease in vivo. 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 ≥ 350 μM of $FeCl_3$ (Fu et al., 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-P11/FTR1-P12 (expected 503 bp), which amplified a segment from the ORF of *FTR1* (Table 2 and Fig. 2A). PCR confirmed integration of the disruption cassette in the *FTR1* locus, and absence of *FTR1* ORF from several putative null mutant strains (Fig. 2B). 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).

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.*, 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) (Fig. 2C). 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 (Fig. 2C) consistent with our previous findings that *ftr1* is primarily expressed in iron-depleted environments.

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 (Fig. 2D). Similar results were obtained with five 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.*, 2009). We hypothesized that gene disruption was complicated by the presence of heterokaryotic nuclei in both the mycelium and sporangiospores, and sought to determine the number of nuclei present in swollen spores using DAPI staining. We found that *R. oryzae* strain M16 had at least > 10 nuclei per swollen spore (Fig. 3A). Given the high number of nuclei present, we performed 14 rounds of sporulation and single colony isolation on four independent putative *ftr1* null mutants on iron-rich medium (i.e. medium containing 1000 μM of $FeCl_3$) 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.*, 2004). PCR analysis of the putative null mutants after 14 rounds of selection demonstrated lack of amplification of *FTR1* ORF (Fig. 3B). Similar to the results in Fig. 2C, 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. Similar results were obtained with 12 other independent putative *ftr1* null transformants. Further, these results were confirmed with Southern blot analysis (Fig. 3C). 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 (Fig. 3C). 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.

We next used the more sensitive method of qPCR to quantify the copy numbers of wild-type *FTR1* or disrupted *ftr1* in the putative *ftr1* null mutant. The mutant was passaged through 14 rounds of sporulation and single colony isolation on iron-rich then grown on iron-depleted media. The putative *ftr1* null mutant strain grown in iron-rich media had a 60% reduction in the relative copy number of the wild-type functional *FTR1* (normalized to *ACT1* gene) compared to the same strain grown in iron-depleted media or to the *R. oryzae PyrF*-complemented strain (Fig. 4A and 4B). Thus, while it was possible to significantly decrease the relative copy number of functional *FTR1* in multinucleated *R. oryzae*, we were unable to obtain a homokaryotic isolate of this mutant allele. Additionally, the disrupted *ftr1* allele demonstrated a 90% decrease in copy number when the putative *ftr1* null mutant was grown on iron-depleted medium following the 14 rounds of sporulation and single colony isolation on iron-rich medium (Fig. 4C). These results are consistent with the Southern blot analysis which showed almost complete elimination of the disrupted allele (Fig 3C). Thus while it is possible to almost completely select for the wild-type phenotype (i.e. *pyrF/FTR1*) on iron-limited CSM-URA medium, small number of nuclei harboring *PyrF/ftr1* genotype remain, thereby explaining the growth of the these cells on CSM-URA medium.

We hypothesized that *FTR1* is required for sporangiospore germination, since we could not isolate a homokaryon cell. If *FTR1* was required for germination, any sporangiospores

containing only homokaryotic *ftr1* null alleles would be incapable of growth. Therefore, we attempted to circumvent sporangiospore germination and relied on multiple hyphal tip transfers on medium containing 1 mM $FeCl_3$ (iron-rich conditions). Again, we were unable to obtain a homokaryotic isolate of the *FTR1* null allele (data not shown), suggesting the necessity of *FTR1* even in iron-rich growth conditions.

Finally, because gene disruption studies were performed in *R. oryzae* harboring a point mutation that renders the *PyrF* null, we hypothesized that lack of our ability to generate a homokaryotic *ftr1* null could have been complicated by correction of the point mutation of *pyrF* in nuclei that has *FTR1* intact. If this were the case, the selection pressure of growth on CSM-URA media would be lost, and there would be no way to select for growth of cells bearing nuclei with intact *PyrF/FTR1*, separating them from *PyrF/ftr1* null nuclei. To test this hypothesis, the wild-type allele of *PyrF* was amplified from transformants passaged 14 times on iron-rich medium without uracil or from transformants that have been grown on iron-depleted medium without uracil. The sequence of the *PyrF* from both was proven to still harbor the point mutation of G to A nucleotide transversion at nt +205 (Skory & Ibrahim, 2007) (data not shown). Therefore, it is clear that lack of obtaining a homokaryotic null is due to the inability of the homokaryotic null to grow under the conditions used in this study.

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

Although we could not achieve complete disruption of *FTR1* we hypothesized that reduction of the relative copy number of functional *FTR1* in *R. oryzae* would be 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 (Fig. 4D).

Uracil auxotrophy has been shown to affect *in vivo* virulence of several fungal infections (Kirsch & Whitney, 1991, Varma *et al.*, 1992, D'Enfert *et al.*, 1996). However, in contrast to other fungi, uracil auxotrophy did not affect the virulence of *R. oryzae* in the DKA mouse model (Fig. 5A) ($p=0.36$ by Log Rank test). These results indicate that the fungus can obtain uracil from infected mice and therefore, any defect in virulence of mice infected with putative *ftr1* null would be due to reduced *FTR1* copy number rather than a defect in uracil synthesis. Next, we compared the virulence of the putative *ftr1* null mutant to the *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) (Fig. 5B). In both models, the putative *ftr1* null mutant showed reduced virulence compared to the wild-type or *PyrF*-complemented strain (54% 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) (Fig. 5C,D).

By qPCR, brains from moribund mice (n = 5) had 40% fewer copies of the mutated *ftr1* and 78% increased copies of the wild-type *FTR1* alleles compared to surviving mice. Thus, reduction in copy number of wild type *FTR1* reduced virulence of the organism, and *in vivo* restoration of the wild type allele restored virulence. Additionally, in both models the *PyrF*-complemented strain had similar virulence to the wild-type *R. oryzae*, confirming that restoration of the *PyrF* gene in its original locus does not affect virulence.

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

To confirm the phenotypes seen after gene disruption, we used RNA interference (RNAi) to diminish *FTR1* expression in *R. oryzae* (Fig. 6A). 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, 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 (Fig. 6B). Amplification of 18s *rDNA* with the same RT templates demonstrated the integrity of the starting sample and the lack of PCR inhibitors. A representative RNAi transformant demonstrated similar growth to control strain when grown on CSM-URA media (0.193±0.082 cm/h for the transformant vs. 0.201 ± 0.087 cm/h for the control strain) (Fig. 6C).

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 (Fig. 6D).

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) (Fig. 7A and 7B). 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 ~ 6- and 3-fold reduction in kidney and brain fungal burden compared to mice infected with the control strain, respectively (Fig. 7C). These data demonstrate that the *FTR1* gene product is an important virulence factor for *R. oryzae* in DKA mice.

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

Because *FTR1* was required for full virulence of *R. oryzae*, we hypothesized 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. 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, $p<0.001$) (Fig. 7D).

## Discussion

Genetic manipulation of *R. oryzae* has long been hampered by the strong tendency of transformed plasmids to replicate in high-molecular-weight concatenated structures which rarely integrate into the chromosome (Skory, 2002). Even linearized plasmids tend to replicate autonomously following a non-homologous end joining event (Skory, 2005). It has been shown that introducing a double-strand break within the homologous regions on a linearized plasmid can significantly improve the likelihood of integration in *R. oryzae*, with up to 20% of the transformants showing homologous integration (Skory, 2002, Skory, 2005). However, this recombination of transformed plasmids happens only through a single cross over event or through a double strand break repair mechanism that corrects for point mutations (Skory, 2005). The standard double cross over event which is widely used to disrupt genes in other bacteria and fungi has not been described previously to occur in *R. oryzae*. We report for the first time that targeted gene disruption via a double cross over event is feasible in *R. oryzae.*

Previously, we unsuccessfully tried to disrupt genes through amplification of a gene disruption cassette in *E. coli.* This gene disruption cassette contained a 2.1 Kb *PyrF* fragment as a selectable marker flanked by up to 3 kb upstream and downstream sequences of *FTR1*. The disruption cassette was transformed into *R. oryzae* M16 after releasing it with restriction enzymes or as whole plasmid. In this study, we amplified a gene disruption cassette of ~1000 kb fragment of *pyrF* flanked by ~700 bp of upstream and downstream sequences of *FTR1.* The assembled disruption cassette was amplified by PCR instead of using *E. coli.* The current success in gene disruption may be due to trimming the selectable marker, or due to lack of methylation resulting from PCR amplification, in contrast to plasmid amplification in *E. coli* which results in DNA methylation. These possibilities are currently under active investigation.

We confirmed that the disruptant cassette was integrated into the appropriate locus and that growth in high iron concentrations selected for nuclei containing the *ftr1* null allele. However, we were unable to obtain a homokaryotic isolate even with 14 rounds of sporulation and single colony isolations or hyphae passaging on medium containing 1 mM $FeCl_3$, which exerted a diminished selective pressure to maintain *FTR1*. It is imperative to emphasize that this phenomenon of not obtaining a homokaryotic null is specific to *FTR1* since we were able to obtain a homokaryotic *his3* null in the background of *R. oryzae* M16 (unpublished data). Unlike other fungi, such as *Candida albicans* (Ramanan & Wang, 2000), *Saccharomyces cerevisiae* (Dix et al., 1994), *Cryptococcus neoformans* (Jung et al., 2008), and *Fusarium graminearum* (Park et al., 2006), *R. oryzae* lacks a low affinity iron permease and lacks a homolog for *FTR1*, which might support the hypothesis that *FTR1* is an essential gene for the organism. That a single gene can be essential for growth has been described in fungi (Pierson et al., 2004, Sanyal & Carbon, 2002). The possibility that *FTR1* is an essential gene in *R. oryzae* is currently under active investigation. These studies will include the possibility of using *FTR1* gene controlled by a regulatory promoter or by generating conditional mutants of *FTR1*.

Even though we were unable to obtain a homokaryotic isolate of the *FTR1* null allele, reduction in the relative copy number of functional *FTR1* significantly reduced iron uptake and significantly abrogated virulence *in vivo*. Although we could not construct an *FTR1*-complemented strain due to the undeveloped state of genetic manipulation of *R. oryzae* (e.g. lack of dominant selection marker and the absence of auxotrophic marker other than *pyrF*), we were able to use RNAi-mediated reduction of *FTR1* expression levels to confirm the role of *FTR1* in iron uptake and pathogenesis. Furthermore, anti-Ftr1p polyclonal antibody resulted in significant protection against mucormycosis infection in DKA mice. Finally,

several reports have demonstrated that altered *URA3* expression due to integration in different loci when used as a selection marker in *C. albicans* affects virulence when constructing null mutants (Brand *et al.*, 2004, Lay *et al.*, 1998, Sundstrom *et al.*, 2002). However, the diminished virulence of *R. oryzae ftr1* putative null mutant or RNAi transformant cannot be attributed to altered *PyrF* expression since *pyrF* auxotrophy had no effect on virulence of *R. oryzae* in the DKA mouse model. Collectively, these data confirm that *FTR1* is a virulence factor for the organism *in vivo*, and indicate that targeting of *FTR1* by immunotherapy is of great promise in improving outcomes of these infections.

Fungi can acquire iron from the environment by three mechanisms (Ibrahim *et al.*, 2008). These include: 1) reduction of the ferric form of iron into the more soluble ferrous form, followed by subsequent transport of iron via a high or low affinity iron permeases (reductive/permease system) that function in iron-limited or iron-rich environments, respectively (Dix et al., 1994, Eck *et al.*, 1999, Knight *et al.*, 2002, Ramanan & Wang, 2000); 2) a siderophore permease that facilitates the uptake of siderophore-sequestered iron (Heymann *et al.*, 2002, Lesuisse *et al.*, 2002, Lesuisse *et al.*, 1998); and 3) release of ferric iron through degradation of heme by heme oxygenase (Santos *et al.*, 2003). Pathogenic fungi utilize one or more of these systems to acquire iron from the host during infection. For example, the reductive/permease system has been shown to be critical for pathogenicity of *C. albicans* and *C. neoformans* through mediating iron uptake from transferrin (Jung et al., 2008, Knight *et al.*, 2005). Additionally, heme oxygenase was shown to be involved in iron uptake from heme and hemoglobin by *C. albicans* (Pendrak *et al.*, 2004, Santos et al., 2003) through binding to the cell surface mannoproteins encoded by *RBT5* and *RBT51* (Weissman & Kornitzer, 2004, Weissman *et al.*, 2008). *C. neoformans* also utilizes heme and hemoglobin as a source of iron (Jung et al., 2008), however the receptor(s) that mediate binding of the fungus to these molecules are yet to be determined. In contrast, it was shown that the siderophore system and not the reductive/permease is needed for virulence in mice infected with *Aspergillus fumigatus* (Hissen *et al.*, 2004, Hissen *et al.*, 2005, Schrettl *et al.*, 2004). Recently the genome sequencing project of *R. oryzae* revealed the presence of a single copy of the high affinity iron permease, several copies of putative siderophore permeases, and two copies of heme oxygenases (Ma et al., 2009). We found that the putative *ftr1* null mutant had lagging ability to grow on $FeCl_3$ or $FeSO_4$ compared to wild-type or *PyrF*-complemented strains only in iron-depleted concentrations, confirming the importance of *FTR1* in iron uptake from iron-limited but not iron-rich environments. Additionally, previous studies on iron uptake from ferrioxamine by *Rhizopus* showed that the organism efficiently liberates ferric iron from the siderophore extracellularly through an energy dependent step that requires the reduction of $Fe^{3+}$ to $Fe^{2+}$ prior to transporting the iron intracellularly, suggesting the involvement of the reductase/high affinity permease system (de Locht et al., 1994). Concordant with this hypothesis is our finding of lagging growth of the *ftr1* putative null mutant compared to wild-type or *PyrF*-complemented strain when ferrioxamine is utilized as the source of iron. Moreover, we found the putative *ftr1* null mutant had lagging growth on media supplemented with heme as well. Similar to bacteria (Allen & Schmitt, 2009, Liu *et al.*, 2008, Stojiljkovic & Hantke, 1994, Zhu *et al.*, 2008), *FTR1* in *R. oryzae* may act as a cytoplasmic membrane permease that facilitates intracellular heme uptake which is followed by release of $Fe^{3+}$ iron through degradation with heme oxygenases intracellularly. In fact, a greater growth defect was found when the putative *ftr1* null mutant was grown on medium supplemented with ferrioxamine or heme compared to the putative *ftr1* null mutant grown on $FeCl_3$- or $FeSO_4$-depleted medium. These results suggest that *FTR1* is primarily responsible for the intracellular transport of iron from these two sources more than from iron salt sources.

*Rhizopus* is also known to secrete rhizoferrin, a siderophore that belongs to the polycarboxylate family (Thieken & Winkelmann, 1992). This siderophore supplies *Rhizopus*

with iron through a receptor-mediated, energy dependant process (de Locht *et al.*, 1994, Thieken & Winkelmann, 1992). However, it is not currently known whether rhizoferrin transports iron by release of iron extracellularly or if the siderophore is internalized prior to releasing iron in the cytoplasm. What is known is that rhizoferrin is inefficient in obtaining iron from serum (Boelaert *et al.*, 1993, de Locht *et al.*, 1994), and therefore the contribution of the organism's endogenous siderophores to its virulence in a mammalian host is likely minimal. The lack of rhizoferrin ability to take up iron from serum is also highlighted by the adaptation of the organism to utilize xeno-siderophores such as deferoxamine, which are more efficient in obtaining iron from the host. Instead rhizoferrin and siderophore permeases are possibly required for iron uptake in environments other than mammalian hosts and likely explain the lack of total abrogation of iron uptake in the putative null and RNAi mutants *in vitro* compared to control strains (Fig. 4D and 6D).

In summary, we report for the first time the successful use of a double cross over event to integrate a gene disruption cassette in *R. oryzae*. While we successfully targeted the disruption cassette to the chromosome, it was not possible to obtain a homokaryon of the *FTR1* null allele from multinucleated *R. oryzae* despite the fact that several independently constructed putative null mutants were tested. We found that *FTR1* was required for full pathogenesis *in vivo*. 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. These data contribute to the understanding of the critical role of iron uptake in mucormycosis pathogenesis. Finally, the use of passive immunotherapy against *FTR1* is worthy of investigation as a strategy to improve outcomes of these deadly infections.

## Experimental Procedures

### Organisms and culture conditions

*R. oryzae* strains used in this study are listed in Table 1. 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. We used an 815 bp partial *pyrF* PCR fragment (pyrF P11/P13) 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 & Ibrahim, 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.

### In vitro and in vivo expression of FTR1

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.*, 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-limited conditions) or without (iron-rich 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 $10^4$ events were calculated using CELLQUEST software.

For *in vivo* expression, BALB/c male (>20g) mice were rendered diabetic in ketoacidosis with a single i.p. injection of 210 mg/kg streptozotocin in 0.2 ml citrate buffer 10 days prior to infection with *R. oryzae* (Ibrahim *et al.*, 2003a, Ibrahim *et al.*, 2006, Ibrahim *et al.*, 2007). Glycosuria and ketonuria were confirmed in all mice 7 days after streptozotocin treatment. Mice were infected via the tail vein with $2 \times 10^6$ spores of *R. oryzae* GFP1. Mice were sacrificed 24 or 48 h post infection and infected organs were collected and fixed in 10% zinc formalin, embedded in paraffin, and 5 mm sections, were stained with hematoxylin and eosin (H&E) to detect *R. oryzae* hyphae (Ibrahim *et al.*, 2007). 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.*, 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, 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 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 $2(-\Delta\Delta C(T))$ method (Livak & Schmittgen, 2001). All reactions were performed in duplicate, and the mixture included a negative no-reverse transcription (RT) control in which reverse transcriptase was omitted.

## FTR1 disruption

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. 2A). 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. 2A). This was then used to transform *R. oryzae* M16 (*pyrF* mutant) with biolistic bombardment (Skory, 2002). The disruption cassette replaces the entire *FTR1* coding region from −16 to the stop codon, with the *PyrF* gene fragment. Isolates obtained from three separate transformations were purified with one round of sporulation and single colony isolation 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* expression in

this iron concentration is suppressed (Fu et al., 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-P11/FTR1-P12 to amplify 503 bp, primers FTR1-P5/FTR1-P6 to amplify the entire 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 $FeCl_3$.

## Nuclear staining

To determine the number of nuclei present in *R. oryzae* spores, we pregerminated spores in YPD medium for 2 h at 37°C. Swollen spores were washed once with cold PBS then suspended at a concentration of $5 \times 10^5$/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.

## Determination of the FTR1 wild-type or ftr1 mutant allele copy number

To compare the copy number of *FTR1* wild-type or *ftr1* mutant allele in the putative *ftr1* null mutant grown on iron-rich or iron-limited media or to those of *PyrF*-complemented strain, we used qPCR. Briefly, genomic DNA was extracted with the OmniPrep lysis buffer (GBiosciences) from *PyrF*-complemented *R. oryzae* grown in YNB+CSM-URA supplemented with 1mM $FeCl_3$ or putative *ftr1* null mutant grown in either YNB+CSM-URA supplemented with 1 mM $FeCl_3$ or 100 μM ferrioxamine. *FTR1* wild-type or *ftr1* mutant allele 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-CT™ 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* wild-type or *ftr1* mutant allele 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*-complemented strain].

## Growth comparisons

Growth of the putative *ftr1* disruption mutants were compared to *R. oryzae* wild-type or *R. oryzae PyrF*-complemented strains by growing on plates YNB+CSM-URA supplemented with 10 or 1000 μM of $FeCl_3$, $FeSO_4$, or with 100 μM of heme, or ferrioxamine 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, 10 μl of $10^5$ 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 $FeCl_3$, $FeSO_4$, or ferrioxamine or for 72 h for plates supplemented with heme. The experiment was repeated three times on different days and growth rate was expressed as average increase in mycelial diameter of the fungus per hour.

## RNAi of FTR1

We used RNAi technology to inhibit the expression of *FTR1* in *R. oryzae*. The RNAi construct was established in plasmid pRNAi-pdc intron (Fig 6A) which was generated using

expression vector pPdcA-Ex (Mertens *et al.*, 2006) as a backbone plasmid. Approximately a 2.0 kb fragment representing the *PyrG* was excised from plasmid pRNAi-pdc intron and replaced by 1641 bp of *PyrF* by digesting with *XhoI* and *XmaI*. The generated plasmid contained a pdc promoter for constitutive expression and an intron segment from *Rhizopus* pdcA gene (Skory, 2003) to serve as a linker for stabilization of the intended dsRNA structure (Nakayashiki *et al.*, 2005,Wesley *et al.*, 2001). We PCR amplified the first 450 bp fragment of *FTR1* ORF containing the REGLE motif believed to interact with iron during uptake (Stearman *et al.*, 1996). The amplified fragment was cloned in plasmid pRNAi-pdc intron down stream of the pdc promoter following digestion with *SphI* and *AscI* to yield pRNAi-FTR1partial. A reverse complement sequence of the 450 bp fragment was PCR amplified and cloned down stream of the intron segment following digestion with *SacII* and *NheI* to yield pRNAi-FTR1complete. 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 (Skory, 2003).

To detect the effect of the RNAi construct on the expression of *FTR1* in vitro, *R. oryzae* transformed with pRNAi-FTR1 complete or empty plasmid were grown in potato dextrose broth (PDB) at 37°C for 18 h. The mycelia were collected by filtration and transformed to fresh PDB containing 2 mM deferasirox dissolved in 2% DMSO for 1 h to induce iron starvation and *FTR1* expression (Ibrahim *et al.*, 2007). RNA samples were extracted using the RNeasy Plant Mini kit (Qiagen) and first-strand cDNA were generated with oligo(dT) primer using the SuperScript First-Strand Synthesis System (Invitrogen). The products were diluted and used in PCR to detect the expression of *FTR1* of *R. oryzae* and 18S *rDNA*. The PCR products were separated on a 2% agarose gel containing 0.1 μg/ml ethidium bromide.

## Iron uptake assay

To characterize the effect of *FTR1* manipulation on the ability of *R. oryzae* to take up iron in vitro, *ftr1* putative disruption mutants or RNAi mutants were compared to wild-type or *R. oryzae PyrF*-complemented strains in their ability to accumulate intracellular $^{59}FeCl_3$ (Amersham Pharmacia Biotech) using a modification of our published method (Fu *et al.*, 2004). Briefly, spores were pre-germinated for 3 h in 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 $10^8$ 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 0.1μM $^{59}FeCl_3$, 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 $Na_3$-citrate pH 4.2, 1 mM$KH_2PO_4$, 1 mM $CaCl_2$, 5 mM $MgSO_4$, and 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/5× $10^6$ germinated spores.

## Pathogenesis studies

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.*, 2003a) Glycosuria and ketonuria were confirmed in all mice 7 days after streptozotocin treatment. DKA mice were infected with fungal spores by tail vein injection with a target inoculum of $5 \times 10^3$ 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, $10^7$ 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.*, 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.*, 2005a) 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 $\log_{10}$ cfu $g^{-1}$ tissue.

### Expression and purification of a synthetic recombinant Ftr1p

Initially we expressed Ftr1p in *E. coli* using a Qiagen expression system (QIAexpressionist), however the yield was low. To enhance protein production we synthesized (Genscript) a gene encoding a more hydrophilic protein by removing the signal peptide and 6 transmembrane domains (Stearman *et al.*, 1996, Fu *et al.*, 2004) which 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-agarose affinity column according to the manufacturer instructions (Qiagen). The generated protein was used to raise murine antibodies as described below.

### Passive immunization

To generate immune serum for passive immunization, BALB/c mice were immunized by SQ injection of synthetic recombinant Ftr1p (20 μg) mixed with CFA at day 0, boosted with another dose of the antigen with 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.*, 2005b). Immune or control sera (0.25 ml) were administered i.p. to DKA recipient mice 2 h before intranasal infection with $2.5 \times 10^7$ *R. oryzae* 99–880 spores. Sera doses were repeated 3 days post infection and survival of mice was followed for 35 days post infection.

All animal experiments described in this study were approved by the institutional animal use and care committee, following the National Institutes of Health guidelines for animal housing and care.

### Statistical analysis

The non-parametric log-rank test was used to determine differences in survival time, 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.

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## References

Allen CE, Schmitt MP. HtaA is an iron-regulated hemin binding protein involved in the utilization of heme iron in Corynebacterium diphtheriae. J Bacteriol. 2009; 191:2638–2648. [PubMed: 19201805]

Artis WM, Fountain JA, Delcher HK, Jones HE. A mechanism of susceptibility to mucormycosis in diabetic ketoacidosis: transferrin and iron availability. Diabetes. 1982; 31:1109–1114. [PubMed: 6816646]

Boelaert JR, de Locht M, Van Cutsem J, Kerrels V, Cantinieaux B, Verdonck A, Van Landuyt HW, Schneider YJ. Mucormycosis during deferoxamine therapy is a siderophore-mediated infection. In vitro and in vivo animal studies. Journal of Clinical Investigation. 1993; 91:1979–1986. [PubMed: 8486769]

Boelaert JR, Van Cutsem J, de Locht M, Schneider YJ, Crichton RR. Deferoxamine augments growth and pathogenicity of Rhizopus, while hydroxypyridinone chelators have no effect. Kidney International. 1994; 45:667–671. [PubMed: 8196268]

Boelaert JR, van Roost GF, Vergauwe PL, Verbanck JJ, de Vroey C, Segaert MF. The role of desferrioxamine in dialysis-associated mucormycosis: report of three cases and review of the literature. Clinical Nephrology. 1988; 29:261–266. [PubMed: 3293856]

Boelaert JR, Vergauwe PL, Vandepitte JM. Mucormycosis infection in dialysis patients [letter]. Annals of Internal Medicine. 1987; 107:782–783. [PubMed: 3662297]

Brand A, MacCallum DM, Brown AJ, Gow NA, Odds FC. Ectopic expression of URA3 can influence the virulence phenotypes and proteome of Candida albicans but can be overcome by targeted reintegration of URA3 at the RPS10 locus. Eukaryot Cell. 2004; 3:900–909. [PubMed: 15302823]

D'Enfert C, Diaquin M, Delit A, Wuscher N, Debeaupuis JP, Huerre M, Latge JP. Attenuated virulence of uridine-uracil auxotrophs of Aspergillus fumigatus. Infect Immun. 1996; 64:4401–4405. [PubMed: 8926121]

de Locht M, Boelaert JR, Schneider YJ. Iron uptake from ferrioxamine and from ferrirhizoferrin by germinating spores of Rhizopus microsporus. Biochemical Pharmacology. 1994; 47:1843–1850. [PubMed: 8204101]

Dix DR, Bridgham JT, Broderius MA, Byersdorfer CA, Eide DJ. The FET4 gene encodes the low affinity Fe (II) transport protein of Saccharomyces cerevisiae. J Biol Chem. 1994; 269:26092–26099. [PubMed: 7929320]

Eck R, Hundt S, Hartl A, Roemer E, Kunkel W. A multicopper oxidase gene from Candida albicans: cloning, characterization and disruption. Microbiology. 1999; 145(Pt 9):2415–2422. [PubMed: 10517594]

Fu Y, Lee H, Collins M, Tsai HF, Spellberg B, Edwards JE Jr, Kwon-Chung KJ, Ibrahim AS. Cloning and functional characterization of the Rhizopus oryzae high affinity iron permease (rFTR1) gene. FEMS Microbiol Lett. 2004; 235:169–176. [PubMed: 15158278]

Gleissner B, Schilling A, Anagnostopolous I, Siehl I, Thiel E. Improved outcome of zygomycosis in patients with hematological diseases? Leuk Lymphoma. 2004; 45:1351–1360. [PubMed: 15359632]

Gravelat FN, Doedt T, Chiang LY, Liu H, Filler SG, Patterson TF, Sheppard DC. In vivo analysis of Aspergillus fumigatus developmental gene expression determined by real-time reverse transcription-PCR. Infect Immun. 2008; 76:3632–3639. [PubMed: 18490465]

Heymann P, Gerads M, Schaller M, Dromer F, Winkelmann G, Ernst JF. The siderophore iron transporter of Candida albicans (Sit1p/Arn1p) mediates uptake of ferrichrome-type siderophores and is required for epithelial invasion. Infect Immun. 2002; 70:5246–5255. [PubMed: 12183576]

Hissen AH, Chow JM, Pinto LJ, Moore MM. Survival of Aspergillus fumigatus in serum involves removal of iron from transferrin: the role of siderophores. Infect Immun. 2004; 72:1402–1408. [PubMed: 14977945]

Hissen AH, Wan AN, Warwas ML, Pinto LJ, Moore MM. The Aspergillus fumigatus siderophore biosynthetic gene sidA, encoding L-ornithine N5-oxygenase, is required for virulence. Infect Immun. 2005; 73:5493–5503. [PubMed: 16113265]

Ibrahim AS, Avanessian V, Spellberg B, Edwards JE Jr. Liposomal amphotericin B, and not amphotericin B deoxycholate, improves survival of diabetic mice infected with Rhizopus oryzae. Antimicrob Agents Chemother. 2003a; 47:3343–3344. [PubMed: 14506054]

Ibrahim AS, Bowman JC, Avanessian V, Brown K, Spellberg B, Edwards JE Jr, Douglas CM. Caspofungin inhibits Rhizopus oryzae 1,3-beta-D-glucan synthase, lowers burden in brain measured by quantitative PCR, and improves survival at a low but not a high dose during murine disseminated zygomycosis. Antimicrob Agents Chemother. 2005a; 49:721–727. [PubMed: 15673756]

Ibrahim AS, Edwards JE Jr, Fu Y, Spellberg B. Deferiprone iron chelation as a novel therapy for experimental mucormycosis. J Antimicrob Chemother. 2006; 58:1070–1073. [PubMed: 16928702]

Ibrahim, AS.; Edwards, JEJ.; Filler, SG. Zygomycosis. In: Dismukes, WE.; Pappas, PG.; Sobel, JD., editors. Clinical mycology. New York: Oxford University Press; 2003b. p. 241-251.

Ibrahim AS, Gebermariam T, Fu Y, Lin L, Husseiny MI, French SW, Schwartz J, Skory CD, Edwards JE, Spellberg BJ. The iron chelator deferasirox protects mice from mucormycosis through iron starvation. J Clin Invest. 2007; 117:2649–2657. [PubMed: 17786247]

Ibrahim AS, Spellberg B, Edwards J Jr. Iron acquisition: a novel perspective on mucormycosis pathogenesis and treatment. Curr Opin Infect Dis. 2008; 21:620–625. [PubMed: 18978530]

Ibrahim AS, Spellberg BJ, Avenissian V, Fu Y, Filler SG, Edwards JE Jr. Vaccination with recombinant N-terminal domain of Als1p improves survival during murine disseminated candidiasis by enhancing cell-mediated, not humoral, immunity. Infect Immun. 2005b; 73:999–1005. [PubMed: 15664943]

Jung WH, Sham A, Lian T, Singh A, Kosman DJ, Kronstad JW. Iron source preference and regulation of iron uptake in Cryptococcus neoformans. PLoS Pathog. 2008; 4:e45. [PubMed: 18282105]

Kauffman CA. Zygomycosis: reemergence of an old pathogen. Clin Infect Dis. 2004; 39:588–590. [PubMed: 15356828]

Kirsch DR, Whitney RR. Pathogenicity of Candida albicans auxotrophic mutants in experimental infections. Infect Immun. 1991; 59:3297–3300. [PubMed: 1879944]

Knight SA, Lesuisse E, Stearman R, Klausner RD, Dancis A. Reductive iron uptake by Candida albicans: role of copper, iron and the TUP1 regulator. Microbiology. 2002; 148:29–40. [PubMed: 11782496]

Knight SA, Vilaire G, Lesuisse E, Dancis A. Iron acquisition from transferrin by Candida albicans depends on the reductive pathway. Infect Immun. 2005; 73:5482–5492. [PubMed: 16113264]

Kontoyiannis DP V, Wessel C, Bodey GP, Rolston KV. Zygomycosis in the 1990s in a tertiary-care cancer center. Clin Infect Dis. 2000; 30:851–856. [PubMed: 10852735]

Kwon-Chung, KJ.; Bennett, JE. Medical Mycology. Philadelphia: Lea & Febiger; 1992. Mucormycosis; p. 524-559.

Lay J, Henry LK, Clifford J, Koltin Y, Bulawa CE, Becker JM. Altered expression of selectable marker URA3 in gene-disrupted Candida albicans strains complicates interpretation of virulence studies. Infect Immun. 1998; 66:5301–5306. [PubMed: 9784536]

Lesuisse E, Knight SA, Camadro JM, Dancis A. Siderophore uptake by Candida albicans: effect of serum treatment and comparison with Saccharomyces cerevisiae. Yeast. 2002; 19:329–340. [PubMed: 11870856]

Lesuisse E, Simon-Casteras M, Labbe P. Siderophore-mediated iron uptake in Saccharomyces cerevisiae: the SIT1 gene encodes a ferrioxamine B permease that belongs to the major facilitator superfamily. Microbiology. 1998; 144(Pt 12):3455–3462. [PubMed: 9884238]

Liu M, Tanaka WN, Zhu H, Xie G, Dooley DM, Lei B. Direct hemin transfer from IsdA to IsdC in the iron-regulated surface determinant (Isd) heme acquisition system of Staphylococcus aureus. J Biol Chem. 2008; 283:6668–6676. [PubMed: 18184657]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. Methods. 2001; 25:402–408. [PubMed: 11846609]

Ma LJ, Ibrahim AS, Skory C, Grabherr MG, Burger G, Butler M, Elias M, Idnurm A, Lang BF, Sone T, Abe A, Calvo SE, Corrochano LM, Engels R, Fu J, Hansberg W, Kim JM, Kodira CD, Koehrsen MJ, Liu B, Miranda-Saavedra D, O'Leary S, Ortiz-Castellanos L, Poulter R, Rodriguez-Romero J, Ruiz-Herrera J, Shen YQ, Zeng Q, Galagan J, Birren BW, Cuomo CA, Wickes BL. Genomic analysis of the basal lineage fungus Rhizopus oryzae reveals a whole-genome duplication. PLoS Genet. 2009; 5:e1000549. [PubMed: 19578406]

Marr KA, Carter RA, Crippa F, Wald A, Corey L. Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin Infect Dis. 2002; 34:909–917. [PubMed: 11880955]

Mertens JA, Skory CD, Ibrahim AS. Plasmids for expression of heterologous proteins in Rhizopus oryzae. Archives of microbiology. 2006; 186:41–50. [PubMed: 16804680]

Nakayashiki H, Hanada S, Nguyen BQ, Kadotani N, Tosa Y, Mayama S. RNA silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genet Biol. 2005; 42:275–283. [PubMed: 15749047]

Park YS I, Choi D, Kang CM, Ham MS, Kim JH, Kim TH, Yun SH, Lee YW, Chang HI, Sung HC, Yun CW. Functional identification of high-affinity iron permeases from Fusarium graminearum. Fungal Genet Biol. 2006; 43:273–282. [PubMed: 16464625]

Pendrak ML, Chao MP, Yan SS, Roberts DD. Heme oxygenase in Candida albicans is regulated by hemoglobin and is necessary for metabolism of exogenous heme and hemoglobin to alpha-biliverdin. J Biol Chem. 2004; 279:3426–3433. [PubMed: 14615478]

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:e45. [PubMed: 11328886]

Pierson CA, Jia N, Mo C, Lees ND, Sturm AM, Eckstein J, Barbuct R, Bard M. Isolation, characterization, and regulation of the Candida albicans ERG27 gene encoding the sterol 3-keto reductase. Med Mycol. 2004; 42:461–473. [PubMed: 15552648]

Ramanan N, Wang Y. A high-affinity iron permease essential for Candida albicans virulence. Science. 2000; 288:1062–1064. [PubMed: 10807578]

Reed C, Bryant R, Ibrahim AS, Edwards J Jr, Filler SG, Goldberg R, Spellberg B. Combination polyene-caspofungin treatment of rhino-orbital-cerebral mucormycosis. Clin Infect Dis. 2008; 47:364–371. [PubMed: 18558882]

Ribes JA, Vanover-Sams CL, Baker DJ. Zygomycetes in human disease. Clin Microbiol Rev. 2000; 13:236–301. [PubMed: 10756000]

Roden MM, Zaoutis TE, Buchanan WL, Knudsen TA, Sarkisova TA, Schaufele RL, Sein M, Sein T, Chiou CC, Chu JH, Kontoyiannis DP, Walsh TJ. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. Clin Infect Dis. 2005; 41:634–653. [PubMed: 16080086]

Santos R, Buisson N, Knight S, Dancis A, Camadro JM, Lesuisse E. Haemin uptake and use as an iron source by Candida albicans: role of CaHMX1-encoded haem oxygenase. Microbiology. 2003; 149:579–588. [PubMed: 12634327]

Sanyal K, Carbon J. The CENP-A homolog CaCse4p in the pathogenic yeast Candida albicans is a centromere protein essential for chromosome transmission. Proc Natl Acad Sci U S A. 2002; 99:12969–12974. [PubMed: 12271118]

Schrettl M, Bignell E, Kragl C, Joechl C, Rogers T, Arst HN Jr, Haynes K, Haas H. Siderophore biosynthesis but not reductive iron assimilation is essential for Aspergillus fumigatus virulence. J Exp Med. 2004; 200:1213–1219. [PubMed: 15504822]

Siwek GT, Dodgson KJ, de Magalhaes-Silverman M, Bartelt LA, Kilborn SB, Hoth PL, Diekema DJ, Pfaller MA. Invasive zygomycosis in hematopoietic stem cell transplant recipients receiving voriconazole prophylaxis. Clin Infect Dis. 2004; 39:584–587. [PubMed: 15356827]

Skory CD. Homologous recombination and double-strand break repair in the transformation of Rhizopus oryzae. Mol Genet Genomics. 2002; 268:397–406. [PubMed: 12436261]

Skory CD. Induction of Rhizopus oryzae pyruvate decarboxylase genes. Curr Microbiol. 2003; 47:59–64. [PubMed: 12783195]

Skory CD. Inhibition of Non-Homologous End Joining and integration of DNA upon transformation of Rhizopus oryzae. Mol Genet Genomics. 2005; 274:373–383. [PubMed: 16133163]

Skory CD, Ibrahim AS. Native and modified lactate dehydrogenase expression in a fumaric acid producing isolate Rhizopus oryzae 99–880. Curr Genet. 2007; 52:23–33. [PubMed: 17551728]

Spellberg B, Edwards J Jr, Ibrahim A. Novel perspectives on mucormycosis: pathophysiology, presentation, and management. Clin Microbiol Rev. 2005; 18:556–569. [PubMed: 16020690]

Stearman R, Yuan DS, Yamaguchi-Iwai Y, Klausner RD, Dancis A. A permease-oxidase complex involved in high-affinity iron uptake in yeast. Science. 1996; 271:1552–1557. [PubMed: 8599111]

Stojiljkovic I, Hantke K. Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding-protein-dependent transport system in Yersinia enterocolitica. Mol Microbiol. 1994; 13:719–732. [PubMed: 7997183]

Sugar, AM. Agents of Mucormycosis and Related Species. In: Mandell, GL.; Bennett, JE.; Dolin, R., editors. Principles and Practice of Infectious Diseases. Philadelphia, PA: Elsevier; 2005. p. 2979

Sundstrom P, Cutler JE, Staab JF. Reevaluation of the role of HWP1 in systemic candidiasis by use of Candida albicans strains with selectable marker URA3 targeted to the ENO1 locus. Infect Immun. 2002; 70:3281–3283. [PubMed: 12011025]

Thieken A, Winkelmann G. Rhizoferrin: a complexone type siderophore of the Mucorales and entomophthorales (Zygomycetes). FEMS Microbiol Lett. 1992; 73:37–41. [PubMed: 1387861]

Varma A, Edman JC, Kwon-Chung KJ. Molecular and genetic analysis of URA5 transformants of Cryptococcus neoformans. Infect Immun. 1992; 60:1101–1108. [PubMed: 1541525]

Waldorf AR, Ruderman N, Diamond RD. Specific susceptibility to mucormycosis in murine diabetes and bronchoalveolar macrophage defense against Rhizopus. Journal of Clinical Investigation. 1984; 74:150–160. [PubMed: 6736246]

Weissman Z, Kornitzer D. A family of Candida cell surface haem-binding proteins involved in haemin and haemoglobin-iron utilization. Mol Microbiol. 2004; 53:1209–1220. [PubMed: 15306022]

Weissman Z, Shemer R, Conibear E, Kornitzer D. An endocytic mechanism for haemoglobin-iron acquisition in Candida albicans. Mol Microbiol. 2008; 69:201–217. [PubMed: 18466294]

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM. Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. 2001; 27:581–590. [PubMed: 11576441]

Zhu H, Xie G, Liu M, Olson JS, Fabian M, Dooley DM, Lei B. Pathway for heme uptake from human methemoglobin by the iron-regulated surface determinants system of Staphylococcus aureus. J Biol Chem. 2008; 283:18450–18460. [PubMed: 18467329]

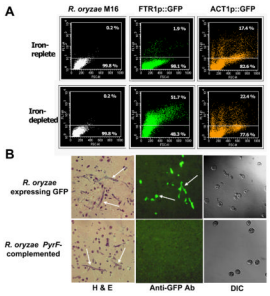


**Fig. 1.**

*FTR1* is expressed in DKA mice infected intravenously with *R. oryzae*. (A) 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. (B) *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.

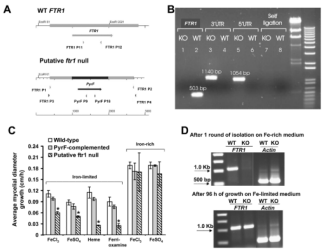

**Fig. 2.**

Disruption cassette integrates in *FTR1* locus but complete elimination of *FTR1* could not be achieved. (A) A diagram summarizing the strategy 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. (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). (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 of $FeCl_3$ or $FeSO_4$ or 100 μM of ferrioxamine, while growth was measured after 72 h for medium supplemented with 100 μM heme. Values (n=12 from four independent transformants with their growth measured in three experiments with similar results) 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. (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 $FeCl_3$) and amplification of the *FTR1* from the same isolate following growth on iron-depleted medium (i.e. 100μM ferrioxamine) for 96 h. Amplification of actin (600 bp) was used to control for DNA loading.

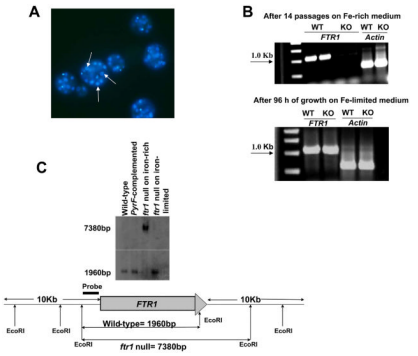


**Fig. 3.**

Confirmation of the lack of complete disruption of *FTR1* in the multinucleated *R. oryzae*. (A) DAPI stain of swollen *R. oryzae* spores showing the presence of multiple nuclei with a single spore. Arrows denote nuclei. Original magnification, x1000. (B) Gel electrophoresis showing lack of amplification of *FTR1* after 14 passages of the putative null mutants on iron-rich medium (1000 μM $FeCl_3$) 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. (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).

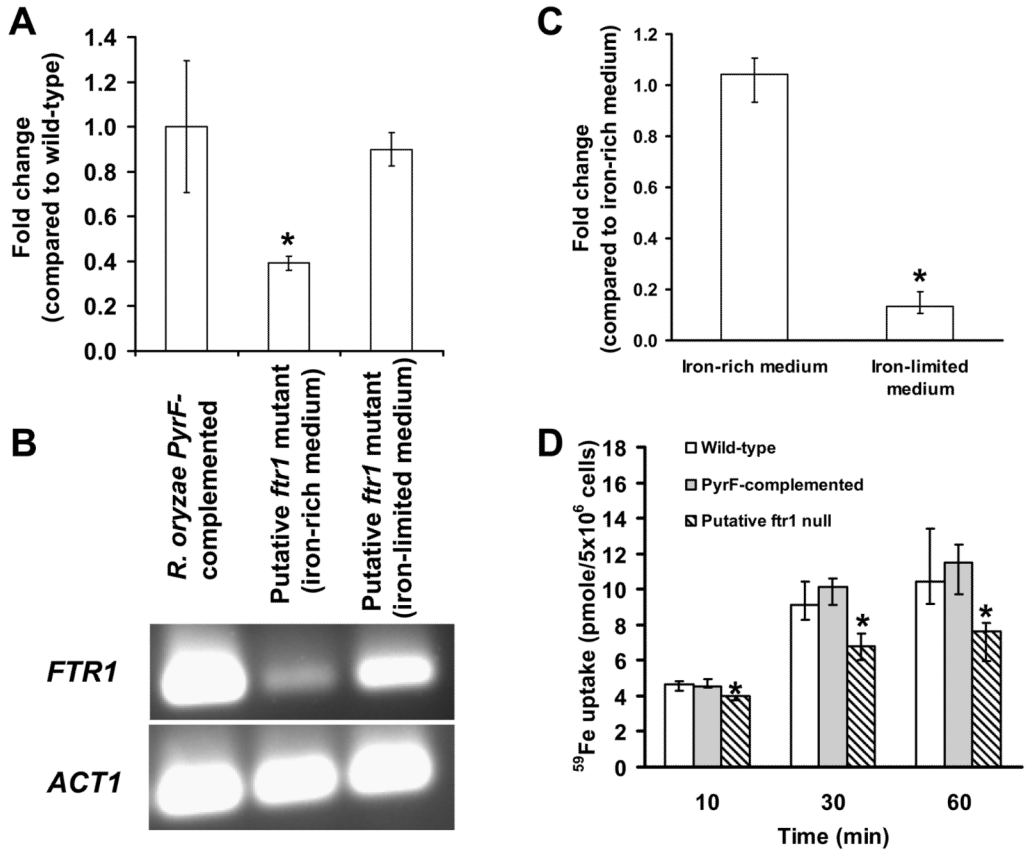


**Fig. 4.**

Reduced copy number results in compromised ability of *R. oryzae* to take up iron. (A) qPCR demonstrating reduced copy number of the wild-type *FTR1* in the putative *ftr1* null mutant compared to *R. oryzae PyrF*-complemented strain or to the same mutant grown in iron-depleted medium. (B) Gel electrophoresis of samples taken from the qPCR tube showing the amplification specificity for the *FTR1* product. (C) qPCR demonstrating reduced copy number of the disrupted *ftr1* allele in the putative *ftr1* null when grown in iron-limited medium following passing in iron-rich medium (n=9 samples from two independent transformants). (D) The putative *ftr1* mutant demonstrated reduced ability to acquire $^{59}Fe$ compared to *R. oryzae* wild-type or *R. oryzae PyrF*-complemented strains. $^{59}Fe$ uptake by wild-type, *R. oryzae PyrF*-complemented, or putative *ftr1* mutant. Germinated spores were incubated with 0.1 μM $^{59}FeCl_3$ (a concentration in which high-affinity iron permeases are induced (Fu *et al.*, 2004)). **P* <0.05 when compared with *R. oryzae* wild-type, *R. oryzae PyrF*-complemented strains, or the same strain grown on different concentrations of iron. Data (n= 9 from three separate experiments using three independent transformants) are expressed as medians + interquartile ranges.

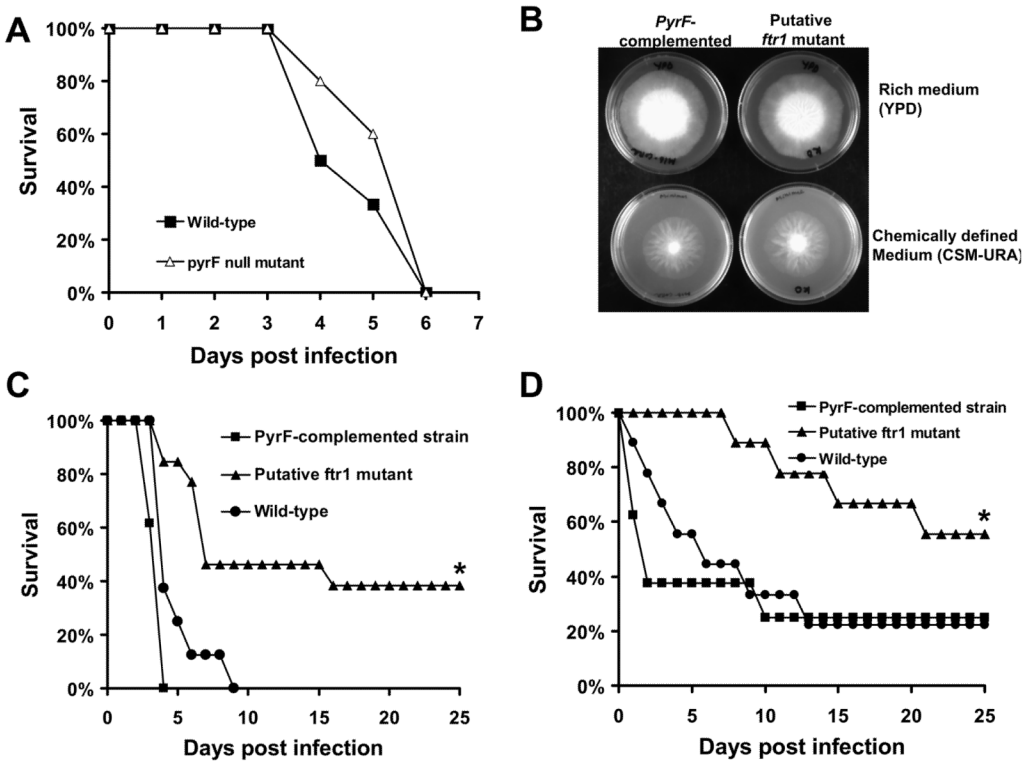


**Fig. 5.**

Reduction of *FTR1* copy number reduces *R. oryzae* virulence in the DKA mouse models. (A) Survival of DKA mice (n=6 for wild-type and 5 for *pyrF* null mutant) infected with *R. oryzae* wild-type ($3.3 \times 10^3$) or *pyrF* null mutant ($2.6 \times 10^3$). (B) A representative of the putative *ftr1* null mutant demonstrated comparable growth to *R. oryzae PyrF*-complemented strain on YPD or CSM-URA media. (C) Survival of mice (n=13 per group from two experiments with similar results) infected i.v. with *R. oryzae* wild-type (average inoculum of $4.3 \times 10^3$ spores), *R. oryzae PyrF*-complemented strain (average inoculum of $4.2 \times 10^3$ spores) or with putative *ftr1* null mutant (average inoculum of $3.2 \times 10^3$ spores). *, $P<0.0005$ compared to wild-type or *PyrF*-complemented strains. (D) Survival of mice (n= 9) infected intranasally with *R. oryzae* wild-type ($4.3 \times 10^3$ spores), *R. oryzae PyrF*-complemented strain ($5.1 \times 10^3$ spores) or putative *ftr1* null mutant ($5.3 \times 10^3$ spores). *, $P=0.04$ compared to wild-type or *PyrF*-complemented strains.

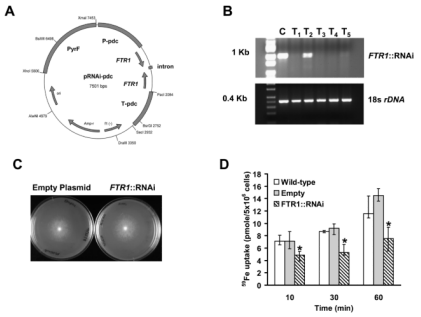


**Fig. 6.**

Inhibition of *FTR1* expression reduces *R. oryzae* ability to take up $^{59}$Fe *in vitro*. (A) Plasmid pRNAi-pdc intron used to generate FTR1::RNAi strains. (B) RT-PCR showing lack of expression of *FTR1* in *R. oryzae* transformed with RNAi 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. (C) A representative of the RNAi transformants demonstrated comparable growth to the *R. oryzae* M16 transformed with empty plasmid on CSM-URA media. (D) $^{59}$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., 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.

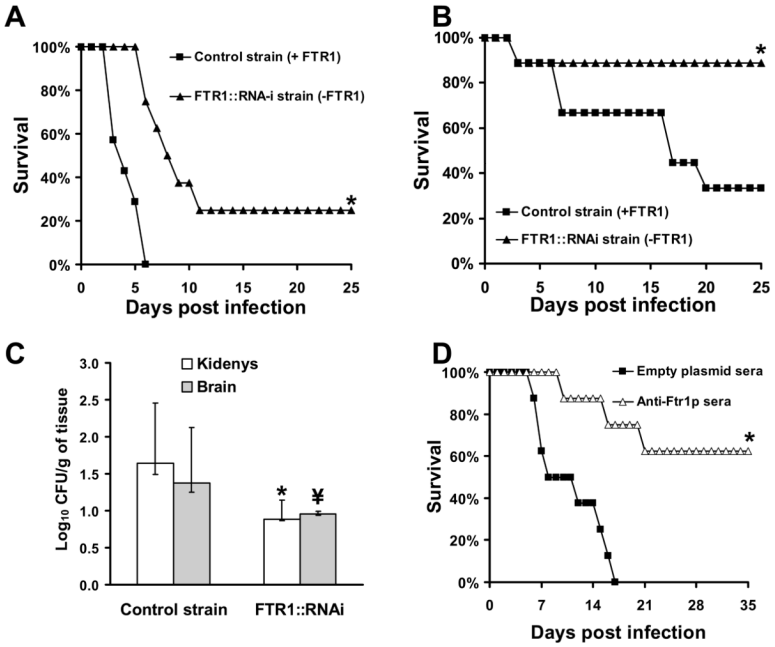


**Fig. 7.**

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. (A) Survival of mice (n=8) infected i.v. with *R. oryzae* transformed with empty plasmid (control strain, $2.9 \times 10^3$ spores) or with RNAi plasmid targeting expression of *FTR1* (FTR1-i, $4.1 \times 10^3$ spores). *, $P<0.001$. (B) Survival of mice (n=9) infected intranasally with *R. oryzae* transformed with empty plasmid (control strain, $2.8 \times 10^3$ spores) or with RNAi plasmid targeting expression of *FTR1* (FTR1-i, $7.6 \times 10^3$ spores). *, $P<0.02$. (C) Kidney or brain Fungal burden of mice (n=8) infected i.v. with *R. oryzae* transformed with empty plasmid (control strain, $4.2 \times 10^3$ spores) or with RNAi plasmid targeting expression of *FTR1* (FTR1-i, $5.1 \times 10^3$ 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 $2.5 \times 10^7$ spores and actual inhaled inoculum of $9 \times 10^3$ spores) and treated with serum collected from mice immunized with either Ftr1p or proteins collected form empty plasmid clone. *, $P<0.007$.

## Table 1

Strains used in this study

| Strain | Genotype | Description and Source |
|---|---|---|
| *R. oryzae* 99–880 | Wild-type | Clinical isolate (Ibrahim et al., 2007) |
| *R. oryzae* M16 | *pyrF205* | Uracil deficient (Skory & Ibrahim, 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 (p$P_{Ftr1}$-*GFP*) | M16 transformed with a plasmid containing an *FTR1* promoter driven *GFP* (Ibrahim et al., 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 |

## 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* wild-type or *ftr1* mutant copy number | | |
| FTR1 P9 | CCAACAGTGAAAAGTCATCCTTT | 5′ primer to amplify *FTR1* (150 bp) |
| FTR1 P10 | GCAATAGGAATTGATTTTCCTTG | 3′ primer to amplify *FTR1* (150 bp) |
| *ftr1* P1 | TTCAACCCTTTTCTCCAAGG | 5′ primer annealing to UTR of *FTR1* to amplify *ftr1* mutant allele (100 bp) |
| *ftr1* P2 | TTGATCAAGGGCACAATGA | 3′ primer annealing to *PyrF* to amplify *ftr1* mutant allele (100 bp) |
| ACT1 P3 | TATCGTTCTTGACTCTGGTGATG | 5′ primer to amplify actin (150 bp) |
| ACT1 P4 | GAAAGAGTGACCACGTTCAGC | 3′ primer to amplify actin (150 bp) |
| Primers used for making RNAi strain | | |

| Primers | Sequence | Description |
|---|---|---|
| PyrF14 | CTCGAGGCTTTAGGTCAAATTGTGG | 5′ primer to amplify 1641bp of *PyrF* to clone in pRNAi-pdc intron |
| PyrF15 | CCCGGGTTATTGCTTGATACCATATTGTG | 3′ primer to amplify 1641bp of *PyrF* to clone in pRNAi-pdc intron |
| FTR1 P7 | GCGGCCGCGCTAGCGCATGCATGTCTCAAGATCTCTTCAACGTACCGATC | 5′ primer to amplify 450bp of *FTR1*to clone in pRNAi-pdc |
| FTR1 P8 | GACGTCCCGCGGGGCGCGCCGG TGATAAAAGGCAAGACAAAGAACGCGTA | 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 | CATCACCATGGGATCAAAAGAATGTTTAATACTGAATCTCCA | 5′ primer to amplify synthetic Ftr1p |
| SynFtr1p P6 | CTAATTAAGCTTGGCTTAAGCTTTAATAGCATCAGATTCAATTTTTTC | 3′ primer to amplify synthetic Ftr1p |