Alternate lifestyles of *C. albicans*: Immunosensing through Efg1p promotes either colonization or pathogenesis

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Jessica V. Pierce

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Abstract

Candida albicans is a colonizing fungus of the human gastrointestinal tract. When a host becomes immunocompromised, *C. albicans* can cause significant mucosal and systemic infections. The ability of *C. albicans* to switch between commensal and pathogenic growth is not well understood. The transcription factor Efg1p regulates morphology and virulence, but the role of Efg1p during gastrointestinal colonization had not been previously characterized.

We proposed that phenotypic heterogeneity could alter the fitness of colonizing cells to promote either colonization or pathogenesis depending on the immune status of the host. We showed that Efg1p demonstrated phenotypic variation and is a major regulator of GI tract colonization. *efg1*$^{-}$ cells initially hyper-colonized the GI tract, but were preferentially eliminated by the host immune response. *EFG1* showed differential expression and activity between cells colonizing either a healthy or immunocompromised host. This shows that colonizing organisms have different gene expression patterns based on the host immune status.

Efg1p significantly altered the expression of genes involved in host interactions and metabolism by colonizing cells. Susceptibility of an *efg1*$^{-}$ strain to the immune response was influenced by the expression of *SOD5*, a superoxide dismutase, suggesting that reactive oxygen species are produced by the host during GI colonization. Altered metabolism of the *efg1*$^{-}$ mutant enabled an increase in population density, which was dependent on both carbon source and carnitine utilization. Efg1p negatively regulated genes involved in carnitine utilization in the gut, and this effect may contribute to hyper-colonization. Additionally, other regulators of *EFG1* and the yeast to hyphal transition

were observed to have unique effects on the ability of *C. albicans* to colonize the GI tract. By balancing the metabolic activity of *C. albicans* and defense against the host immune response, *EFG1* can act as a sensor of the host immune status. Production of cells with high or low Efg1p activity within a population through phenotypic variation of *EFG1* expression would allow *C. albicans* to quickly adapt to changes in the host immune system promoting either colonization or pathogenesis.

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Alternate lifestyles of *C. albicans*: Immunosensing through Efg1p promotes either colonization or pathogenesis

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Chapter 1:

Introduction

Candida albicans is an opportunistic fungal pathogen and colonizing organism of the human GI tract

Candida albicans is a polymorphic diploid yeast which colonizes a majority of the human population throughout the world. *C. albicans* has no known environmental reservoir, but has adapted to reside in the mammalian gastrointestinal tract, and can also be found on the skin and in the urogenital tract [1]. It is the most common fungal pathogen of humans and the fourth leading cause of nosocomial infections in the United States [2, 3]. *C. albicans* is able to exquisitely sense and respond to its environment using a range of tactics in order to exist in a delicate balance with its host. When that balance is upset and a host becomes immunocompromised, *C. albicans* is able to switch from a member of the commensal flora to an opportunistic human pathogen [1, 3]

C. albicans is able to cause a wide range of infections in the absence of an intact immune system. These include mucosal, oral, gastrointestinal, and systemic infections [1, 3]. The immune system of the host can become compromised due to other existing infections, such as HIV or septicemia, as well as from treatments such as organ transplants and chemotherapy [2]. Oral candidiasis, or thrush, is one of the major secondary infections among AIDS patients [1, 3]. Neutropenic patients and patients in intensive care units are also highly susceptible to systemic *C. albicans* infections [2]. In addition, the elderly and infant populations are susceptible to candidiasis due to their weakened immune responses and loss of colonization resistance [4, 5].

Systemic infections can occur when *C. albicans* escapes from the GI tract and disseminates through the bloodstream [1]. From there, the fungus may spread to other organs such as the liver, kidneys, and lungs, and this type of infection can be fatal [1, 2].

One of the major virulence factors which contributes to the pathogenesis of *C. albicans* is its ability to switch morphologies from a budding yeast to an elongated filamentous form, known as hyphae [3]. The ability to form hyphae is associated with tissue invasion: hyphae have been observed in both lesions in the GI tract and within deep tissues [6-9]. Mortality rates of systemic candidiasis range from 38% to 50%: even when patients are treated for systemic infections, complications from other illnesses can increase the mortality rate of candidiasis [1, 3]. Treatments for *C. albicans* infections include antifungals like amphotericin B, and azole compounds such as fluconazole. However, these drugs are not always effective treatments for candidiasis due to their toxicity and increasing drug resistance among *C. albicans* isolates [1, 2].

C. albicans/host interactions

As an opportunistic fungal pathogen, *C. albicans* interacts with its host through a variety of mechanisms. *C. albicans* adheres to host cells using pattern recognition receptors (PRR), which bind to components of the fungal cell wall, such as mannans and beta-glucans [10]. TLR2 and TLR4 are the major Toll-like receptors involved in the innate immune response to fungi, and Nod-like receptors have also been shown to contribute to the recognition of *C. albicans* through Nlrp3 [11-13]. C-type lectins such as dectin1 and 2, MMR1, and DC-SIGN are also involved in the recognition of *C. albicans* by host cells [14-18]. Some interactions with host receptors are dependent on either yeast or hyphal morphologies [19, 20]. Differences in morphology could therefore cause alterations in the immune response to *C. albicans* and be important for discriminating colonizing and invasive cells [21].

Interaction with host cells through PRRs leads to further activation of the cellular immune response. Cellular immunodeficiencies which predispose patients to the development of candidiasis have been studied to understand the interaction of these host cellular populations with *C. albicans*. T cells are an important component of the host immune response to *C. albicans* (Fig. 1-1) [22-24]. The activation of a T cell response occurs when naïve T cells differentiate in response to pathogen detection by antigen presenting cells, such as macrophages and dendritic cells. T cells also respond to TLR signaling and cytokine production, and can migrate to sites of mucosal infection after being produced in the thymus. The initiation of a Th1 response is protective against candidiasis and may promote colonization, while initiation of a Th2 response in the presence of *C. albicans* can result in excessive inflammation which hurts the host. The Th2 response can damage host tissues, and is associated with chronic and systemic disease. The Th1 response induces a pro-inflammatory cytokine response, including the production of IFN-γ, TNFα, TGF-β, IL-6, and IL-12, while a Th2 response produces cytokines such as IL-4 and IL-5. TNFα production is a major component of the protective Th1 immune response [25]. Regulating the balance between Th1 and Th2 responses is important for the host in order to limit inflammation and allow commensal growth [24]. $CD4^+$ $CD25^+$ regulatory T cells play a role in limiting the inflammatory response through the negative regulation of Th1 activity and induction of IL-10 [26]. Trif activation is necessary for the migration of Treg cells to sites of inflammation in the gut, and in the absence of Trif, *C. albicans* is capable of increased colonization and local dissemination at late times during infection [26].

Recently, Th17 cells have been shown to play a large role in gut inflammation.

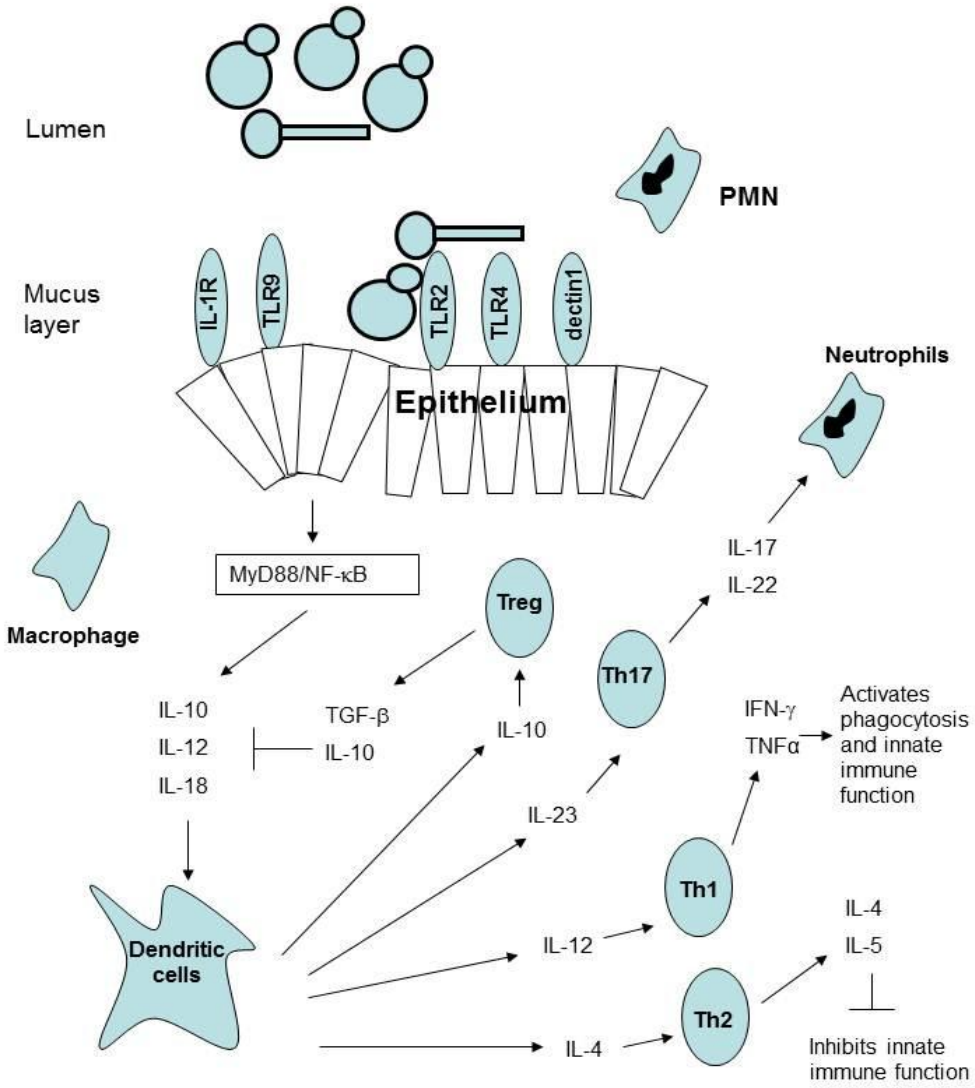


Figure 1-1. Mucosal immunity in the presence of *C. albicans*.

C. albicans yeast and hyphae bind to receptors on the surface of the epithelial layer which signal through MyD88 and NF-kB to produce inflammatory cytokines along with macrophages and neutrophils. Dendritic cells can initiate a differential T cell response by producing various cytokines. Regulatory T cells modulate the immune response, while a Th2 response inhibits innate immune function. Th1 and Th17 responses are pro-inflammatory and required for protection against *C. albicans* infections. PMN, polymorphonuclear leukocyte.

Th17 cells are induced by the production of cytokines such as IL-23, and are found in the lamina propria of the gut where they produce a pro-inflammatory response. This response includes the production of the cytokines IL-17 and IL-22, which recruit neutrophils to the site of infection [27, 28]. Th17 cells are also induced by commensal bacteria (such as SFB, segmented filamentous bacteria) and are involved in the production of autoimmune disorders through excessive inflammation [27, 29]. The Th17 response is also required for protection against candidiasis. Mice lacking the IL-17 receptor had increased mortality rates and fungal burdens during systemic candidiasis [30]. Chronic mucocutaneous candidiasis (CMC) occurs in patients carrying a rare mutation in *il17ra*, which causes a loss of expression of the IL-17 receptor [31]. STAT1 mutations affecting Th17 activity also predispose patients to CMC [32]. Th17 cells therefore play an important role in controlling mucosal candidiasis.

Although T cells are important for the course of infection, BALB/c *nu/nu* mice, which are athymic and therefore lack T cells, were readily colonized by wild type *C. albicans* when administered orally. In this model, there was no evidence of dissemination or systemic disease. This suggests that lack of T cells alone in these mice is not sufficient for *C. albicans* to escape from the GI tract [7]. Tgε26 germ-free mice, which lack natural killer cells and T-cells, however, are susceptible to *C. albicans* infections through oral inoculation. Infections with wild type *C. albicans* in these mice were lethal due to excessive gastric and oral inflammation. Infection resulted in filament formation and an increase in cytokine levels, including TNFα, IL-10, and Il-12 production [33]. Induction of a pro-inflammatory response involving the secretion of

cytokines occurs in the presence of hyphal invasion, and is critical to inducing clearance and protection against disease.

Neutrophils migrating to sites of infection are the major effector cells of the immune system during systemic *C. albicans* infections. Neutrophils are important for the production of reactive oxygen species (ROS) and phagocytosis [22]. Neutrophil depletion coupled with mucosal damage is sufficient for *C. albicans* to disseminate from the murine GI tract and cause disease [34]. In systemic models, lack of neutrophils significantly increases the mortality rate and fungal burden during an infection. Macrophages also play a role in *C. albicans* infections, but their ability to phagocytose fungi is variable [22]. In *phox*$^{-}$ (phagocyte oxidase) *nos2*$^{-}$ (nitric oxide synthase) mice, which have defects in the generation of reactive oxygen species, *C. albicans* was able to both colonize and disseminate from the GI tract to other organs, causing a lethal infection [35], showing that ROS production is major component of the host response controlling *C. albicans* infections.

IDO (indoleamine 2,3-dioxygenase) induction in human tissues occurs in response to the release of pro-inflammatory cytokines and has antimicrobial effects. It is also capable of regulating the immune response [36, 37]. IDO is an intracellular enzyme that catalyzes tryptophan catabolism. It is induced in response to *C. albicans* infection in both the kidney and stomach when mice are infected intravenously or intragastrically. Inhibition of IDO coincided with increased pathology and inflammation during infection. Additionally, it has been found that *C. albicans* expresses a fungal-specific IDO in both hyphal and yeast forms, and its inhibition is sufficient to induce hyphal formation [36]. Tryptophan metabolism through IDO activity was shown to inhibit the Th17 response to

C. albicans in human blood mononuclear cells [38]. IDO therefore seems to play a role in limiting inflammation and inhibiting hyphal growth, and may be one way in which *C. albicans* is able to influence the host while surviving as a commensal.

C. albicans is able to produce prostaglandins which are also capable of modulating the immune response [39-41]. *C. albicans* secretes prostaglandin E (PGE) through the utilization of arachidonic acid, which is produced by human cells. Synthetic PGE and fungal PGE are both capable of stimulating the yeast to hyphal transition and can mimic the function of mammalian prostaglandins [39]. *C. albicans* binds TLR2 receptors on macrophages and splenocytes, which induces PGE production through the activation of *COX2* [40]. Prostaglandins often inhibit Th1 dependent inflammation, chemokine production, and phagocytosis, while promoting a Th2 response [39, 41]. Two genes that contribute to prostaglandin production by *C. albicans* have been identified: *OLE2*, a fatty acid desaturase, and *FET3*, a polyphenol oxidase. Deletions in both genes showed a decrease in prostaglandin production, but neither abolished it completely [41]. Inhibiting the Th1 inflammatory response by PGE may allow for persistence and colonization by *C. albicans,* and in an immunocompromised host, further disable the immune response.

Colonization of the GI tract

Although pathogenesis is being increasingly studied in *C. albicans*, relatively little is known about colonization of the GI tract by this fungus and its relationship to the host immune system under these conditions. We have developed a model that allows us to identify factors involved in fungal commensal growth in the gastrointestinal tract in the

presence of an intact immune system. This environment poses a unique set of conditions that *C. albicans* must adapt to in order to first colonize a host, and then initiate an infection if the host becomes immunocompromised. In this model, mice are given antibiotics prior to inoculation and throughout colonization in order to decrease the overall gastrointestinal flora, providing a niche in which *C. albicans* can grow. *C. albicans* is administered intragastrically to mice, and colonization is monitored over time [42]. In this model, *C. albicans* is mostly present in the yeast form, although hyphae and pseudohyphae can be seen in the lumen. Therefore, yeast cells predominate in the population during colonization, while a small group of cells may switch to hyphal growth.

Several genes have been identified as important factors during gastrointestinal tract colonization with *Candida albicans*. One of these, the putative transcription factor *EFH1*, was highly expressed during colonization, but was not expressed during either mucosal infections or *in vitro* growth. An *efh1*$^{-}$ strain showed increased persistence in the GI tract, whereas a strain which overproduced *EFH1* from a strong promoter was defective for colonization [42]. These observations suggest that Efh1p is a repressor of colonization of the GI tract by *C. albicans*. Efh1p was not required for virulence using models of disseminated infection. Additionally, a strain that over-expresses Efh1p was defective for anaerobic growth *in vitro*, which may correlate with poor growth in the anaerobic sections of the gastrointestinal tract (unpublished observations). *C. albicans* regulates the level of colonization in the GI tract through the expression of Efh1p, which acts as a suppressor of colonization. Limiting its growth may be important for *C.*

albicans in order to maintain its lifestyle as a commensal organism and prevent excessive activation of the host immune system.

Environmental signaling during colonization of the GI tract

Filamentation by *C. albicans* is often associated with disease, and the environmental signals that *C. albicans* senses to induce hyphal formation have been well-studied *in vitro*. Nutrient availability, N-acetylglucosamine (GlcNAc), oxygen levels, temperature, pH, surface contact, and iron availability all contribute to the switch from yeast to hyphae [6, 43-46]. These *in vitro* conditions may mimic some of the environments in the host.

Oxygen levels are high in the blood, as well as on mucosal surfaces, but are limited in the gut, deep tissue, and within cells [46]. The concentration of oxygen decreases from the proximal to the distal regions of the GI tract [47]. pH also varies greatly throughout the GI tract, with the stomach at a pH ~2 and increasing to a pH of ~7.4 in the distal ileum, before dropping slightly in the cecum [48]. GlcNAc is present in serum, and is a component of both bacterial and fungal cell walls [49]. Iron is an essential nutrient for many microbes; iron scavenging by the host limits its availability in the environment to microbes [50-52]. Low iron levels in the environment can induce hyphal formation by *C. albicans* [44].

C. albicans growth and morphology is also regulated by nutrient availability [53, 54]. *C. albicans* preferentially metabolizes sugars, such as glucose, through the glycolysis pathway to produce ethanol and carbon dioxide by fermentation [55]. However, *C. albicans* can also grow solely on non-fermentable carbon sources, like

ethanol and acetate, using acetyl-CoA as an intermediate metabolite in a carnitine-dependent manner [56, 57]. Mutants in genes involved in carnitine metabolism, such as *CAT2* and *CTN1*, are defective in growth on non-fermentable carbon sources [56, 58]. The cecum has a high proportion of non-fermentable carbon sources such as acetate, propionate, and other short chain fatty acids [59]. Commensal bacteria are responsible for generating high levels of short chain fatty acids in the cecum from the breakdown of carbohydrates [60].

In the GI tract, *C. albicans* may grow either in the lumen or in association with mucosal tissue. Contact with either the contents of the lumen or the epithelial layer could provide pressure to the cell wall, similar to the effects seen during matrix embedding, and these effects could promote hyphal growth [61, 62]. *C. albicans* also forms hyphae in response to phagocytosis by macrophages: hyphae that form in the interior of cells can damage the host cell and help cells escape degradation [63]. Some of these signals may help *C. albicans* sense its location in the gastrointestinal tract and the immune status of its host, and therefore regulate its growth and morphology in these many environments.

Role of the microbiome in *C. albicans* and host interactions

In addition to interactions between *C. albicans* and its host, colonization is also affected by the microbiome. The presence of the bacterial commensal species promotes colonization resistance to both pathogens and *C. albicans* [64-66]. After antibiotic treatment, *C. albicans* altered the re-establishment of the bacterial flora through the inhibition of *Lactobacilli* and promotion of *Enterococci* colonization [67]. The presence of bacterial species in the gut also has significant effects on the production of secondary

metabolites, such as short chain fatty acids (SCFAs), during digestion [60, 68]. SCFAs like butyric acid have been shown to inhibit germination by *C. albicans* during laboratory growth [69]. The commensal flora thus alters both the metabolites available for consumption in the GI tract and prevents colonization of new microbial species.

Commensal bacteria are important for the development of the host immune response. *Bacteroides* altered the host immune response through the production of polysaccharide A, which induced a Treg response and IL-10 production in the gut [70, 71]. This is thought to contribute to immunotolerance by the host to commensal growth [72]. SFB have also been shown to induce a Th17 response in the gut mucosa [27]. These responses to *Bacteroides* and SFB by the host were protective against gut infections by *Helicobacter hepaticus* and *Citrobacter*, respectively [27, 73]. These responses exemplify the protective role commensal microbiota can play against invading pathogens.

C. albicans has been shown to interact with other microbes [74, 75]. Studies with *Acinetobacter* and *Pseudomonas* have shown that these microbes inhibit filamentation by *C. albicans* [76, 77]. Co-infections by *Pseudomonas* and *C. albicans* were observed in the lung of cystic fibrosis patients, and their interactions were mediated through quorum sensing [77, 78]. In the GI tract, studies of polymicrobial infections with *C. albicans* and *Salmonella typhimurium* have shown that *S. typhimurium* can both repress hyphal formation by *C. albicans* and decrease its viability through *sopB,* an effector of the type three secretion system [79, 80]. Therefore, the factors which affect colonization are multifactorial: host responses, the microbiota, and gene expression by colonizing organisms such as *C. albicans* all contribute to microbial colonization of the GI tract.

Virulence factors in *Candida albicans*

C. albicans expresses a wide array of virulence factors, some of which play a role in gastrointestinal colonization and dissemination. Multiple animal models have been used to study *C. albicans* pathogenesis *in vivo*. Intravenous infection of mice as a model for hematogenously dissemination candidiasis is typically used to assess the virulence of various *C. albicans* strains. *C. albicans* is unable to escape from the gastrointestinal tract of healthy mice when delivered orally or intragastrically, making the study of endogenous candidiasis challenging [6, 34, 81-83]. *C. albicans* is not a normal member of the murine gastrointestinal flora; therefore mice must be treated with antibiotics or immunosuppressed in order to decrease the normal flora and allow *C. albicans* to successfully colonize. In immunocompromised mice, hyphae can be visualized in association with the limiting ridge of the stomach, and *C. albicans* is thought to disseminate into the blood from this location [6, 7]. Multiple *C. albicans* mutants have been tested in these models to identify major virulence factors, but many remain to be tested.

Other models have been used to study the interactions between *C. albicans* and the host. The use of inbred mouse models has allowed for the study of the immune response during *C. albicans* infections [26, 33, 84]. In addition, a reconstituted human epithelial (RHE) tissue culture model has been used to model mucosal infections [25, 85]. Invertebrate models of candidiasis, including *Drosophila*, *C. elegans*, and *Galleria mellonella* (wax moth) models, have also been used to identify virulence factors and study the relationship between *C. albicans* and the immune system [86-89]. These invertebrate models are advantageous due to their high throughput capacity, and the

ability to study interactions between *C. albicans* and the innate immune response in these organisms.

One group of virulence factors expressed by *C. albicans* during infection includes the secreted aspartyl proteases (SAPs), which can promote tissue invasion through the degradation of host proteins [90]. Invasion of the oral epithelium was associated with expression of *SAP* genes [91]. However, deletion of individual or groups of *SAPs* did not alter the level of tissue damage to the host, suggesting redundancy in the function of these genes [92]. *SAPs1-6* were expressed in the GI tract of orally infected BALB/c mice treated with cyclophosphamide [93]. *SAPs1-3* appeared to be expressed at lower levels than *SAPs4-6*, while *SAP5* showed the greatest expression over time. There was no difference in the ability of strains lacking *SAPs1-3* or *4-6* in their ability to colonize the GI tract over time, and most infected mice showed dissemination through the bloodstream. The *sap4-6* mutant strain showed a slight decrease in the ability to invade the limiting ridge of the stomach. Both the *sap1-3* and *sap4-6* deletion strains were capable of forming hyphae. Despite increased expression of these genes during infection, the *SAP* family was not required for systemic candidiasis [94]. Although overall SAP production may increase the virulence of *C. albicans*, the proteins appear to have redundant roles during infection in the gastrointestinal tract, and are not necessary for colonization in an immunocompromised mouse model [93].

In addition to the SAP family of proteases, other secreted factors may be necessary to cause infection [3]. In support of this, a phospholipase B (*plb1*$^{-}$) mutant of *C. albicans* was less virulent in the intravenous murine model of hematogenously disseminated candidiasis [82]. Despite a defect in virulence, the *plb1*$^{-}$ mutant was still

capable of forming hyphae. During intragastric colonization of infant mice, which have an immature immune system, colonization levels of the wild type and *plb1*$^{-}$ strains did not differ significantly in the stomach. Plb1p was secreted by *C. albicans* in the GI tract using immunogold staining of stomach sections. Deletion of the *PLB1* gene resulted in decreased dissemination from the stomach mucous to the liver and less tissue invasion [82]. Similar to *PLB1*, a second phospholipase, *PLD1*, was also required for systemic infections [95, 96]. After oral inoculation, a *pld1*$^{-}$ mutant colonized the GI tract at WT levels, but did not caused disseminated disease in an immunocompromised mouse model. In contrast to *PLB1*, *PLD1* was required for hyphal growth in laboratory conditions. Therefore, secretion of phospholipases is a major virulence factor during which promotes GI tract candidiasis.

The expression of adhesins also affects the ability of *C. albicans* to cause disease. *INT1* was identified as an adhesin encoded by *C. albicans* with homology to mammalian integrins [97]. Disruption of *INT1* caused a decrease in adhesion to epithelial cells by *C. albicans* and reduced its virulence in a systemic infection [98]. The *int1*$^{-}$ mutant was defective in hyphal growth in laboratory conditions [98]; however, these cells were capable of forming hyphae in the kidney [99]. Despite reduced virulence of the *int1*$^{-}$ mutant, this strain had increased persistence in the kidney of infection mice [99]. The *int1*$^{-}$ mutant was also defective in colonization of the GI tract after oral inoculation of antibiotic treated mice [100]. Therefore, the adhesin *INT1* is important both for colonization and virulence of *C. albicans*.

Iron sequestration by the host is one mechanism which inhibits microbial pathogenesis, and *C. albicans* expresses a number of proteins to acquire iron in the host

environment [44, 101-103]. Two transcription factors, Sef1p and Sfu1p, were shown to differentially regulate iron acquisition by *C. albicans* between the bloodstream and the gastrointestinal tract through a regulatory feedback loop [104, 105]. Disruption of Sef1p did not affect the morphology of *C. albicans*, suggesting that these regulators specifically respond to iron and do not contribute to hyphal growth [101]. A *sef1*$^{-}$ strain was attenuated in a systemic model of disease and had reduced fungal load in the kidney. Both Sef1p and Sfu1p were defective in colonization of the GI tract after 5 days, but the *sfu1*$^{-}$ strain had a much more severe defect in colonization. Sfu1p transcription was up-regulated in the GI tract compared to incubation with human plasma, while Sef1p had lower transcription in the GI tract. Sfu1p inhibited the expression of iron uptake genes (*RBT5, FET3*) during colonization, while Sef1p activated expression of these genes in plasma [104]. In the presence of iron, Sfu1p repressed Sef1p expression, while in low iron conditions, Sef1p repressed the activity Sfu1p indirectly through the regulator Hap34p [104]. Therefore, iron acquisition is differentially regulated between invasive cells present in the bloodstream and colonizing cells in the GI tract through the Sef1p-Sfu1p regulatory circuit. This type of transcriptional regulation suggests that *C. albicans* may have other mechanisms to promote either colonization of the GI tract or pathogenesis based on environmental signals.

Morphology is a major virulence factor of *C. albicans*

Several mechanisms have been proposed to contribute to the ability of *C. albicans* to cause disease: the expression of proteases and adhesins, altered metabolism in the host environment relative to laboratory growth, the ability to form biofilms, and the ability to

change morphology from yeast to hyphae [25, 83, 106-110]. Polymorphism in *C. albicans* has been well-studied: cells are able to grow as budding yeast, similar to *Saccharomyces cerevisiae*, but can also grow as pseudohyphae and hyphae in response to environmental signals [111]. Pseudohyphae exhibit constrictions at the septum and remain connected after cell division. True hyphae are comprised of a mother cell which divides in a polar manner to form rod-like segments which do not have constrictions at the septa [112]. Both pseudohyphae and true hyphae can revert to yeast growth depending on the growth conditions. Hyphal formation is often associated with tissue invasion: hyphae have been observed in the liver and kidneys of mice infected with *C. albicans* [6, 8]. Hyphae have also been observed in gastrointestinal lesions and may contribute to systemic infections by endogenous *C. albicans* cells [6, 113]. However, in some conditions, yeast or pseudohyphal cells are also capable of forming invasive lesions and disseminating from the GI tract [114, 115]. In an intravenous murine model of disease, strains locked in either the yeast or hyphal forms are both defective for virulence in an intravenous murine model of disease [83, 107, 116]. The ability to switch morphologies is one of the major mechanisms that *C. albicans* utilizes to cause disease; therefore, this process is highly regulated to allow *C. albicans* to colonize the GI tract and invade tissue during infection. While polymorphism has been well-studied in disease models of systemic candidiasis, how *C. albicans* regulates its growth and morphology during colonization of the gastrointestinal tract is unknown.

The ability to form hyphae that invade host tissue is one of the major virulence mechanisms of *C. albicans.* The transcription factor Efg1p, a major regulator of hyphal growth, is required for systemic virulence of *C. albicans* [107, 111]. An *efg1*$^{-}$ strain,

which does not form hyphae under most conditions, had reduced virulence in an intravenous infection, and the additional deletion of *CPH1*, a second transcription factor, rendered the strain completely avirulent [81, 107]. In a model of colonization using neutrophil depletion and mucosal disruption, an *efg1*$^{-}$ *cph1*$^{-}$ strain colonized the GI mucosa equally as well as WT, but exhibited decreased virulence [34]. After oral inoculation of mice lacking natural killer cells and T cells, an *efg1*$^{-}$ mutant was capable of colonization, but had decreased virulence compared to a WT strain. In this host, both WT and the *efg1*$^{-}$ mutant were capable of filamentation and induced an inflammatory response, even though the strains exhibited different levels of virulence. Therefore, *efg1*$^{-}$ cells are capable of GI tract colonization, but have decreased virulence in specific host environments.

Other host environments promote differing degrees of disease by the *efg1*$^{-}$ mutant. For example, in BALB/c *nu/nu* mice which lack T cells, *efg1*$^{-}$ cells colonized at WT levels after oral inoculation, but in this case neither WT nor *efg1*$^{-}$ cells showed evidence of disseminated disease. Studies using orally inoculated mice exposed to hypoxic conditions showed that the *efg1*$^{-}$ mutant had increased dissemination from the GI tract, but caused non-lethal infections [114]. In mice deficient in ROS production, the *efg1*$^{-}$ mutant colonized the GI tract equally well as a WT strain, and both the mutant and the WT strain were able to disseminate to the internal organs and cause lethal infections [35]. These studies support the finding that that Efg1p is required for virulence, but also show that *efg1*$^{-}$ cells are capable of dissemination in certain environments.

Efg1p is necessary for infection in an immunocompromised gnotobiotic (IGB) piglet model of oral infection [115, 117]. Wild type *C. albicans* in the IGB piglet model

colonized the GI tract and produced intestinal lesions after immunosuppression [117]. Oral administration of the *efg1*$^{-}$ *cph1*$^{-}$ strain to IGB piglets resulted in colonization of the GI tract, but this mutant was incapable of forming intestinal lesions and may be defective for dissemination from the GI tract. However, the *efg1*$^{-}$ *cph1*$^{-}$ strain caused low level formation of oral and ocular lesions, and these lesions were shown to contain filamentous growth [115]. Similar to growth under embedded conditions, Efg1p may act as a repressor of filamentous growth under these conditions, allowing those cells which adhere to become invasive.

Using a reconstituted human epithelium (RHE) model and mucosal patient samples, the *efg1*$^{-}$ strain was significantly less able to cause tissue damage and invasion compared to WT *C. albicans* [85]. However, *efg1*$^{-}$ yeast cells were capable of adhering and proliferating on the surface [85]. During invasion of the oral epithelium, tissue damage was reduced in the absence of Efg1p [85]. Therefore, Efgp1 is important for tissue damage and oral invasion, but in the absence of Efg1p, *C. albicans* is still capable of adhesion and proliferation on a mucosal surface.

Other transcription factors involved in morphogenesis have also been studied in the context of systemic infection and colonization. A *tup1*$^{-}$ strain, which is constitutively hyphal, was avirulent when given to mice intravenously [1, 81, 116]. When inoculated orally, *tup1*$^{-}$ cells had a decreased ability to colonize the GI tract of mice. Despite this, when mice were depleted for neutrophils and had mucosal disruption, *tup1*$^{-}$ cells were capable of dissemination and were as virulent as wild type cells [34]. In a model in which mice were orally inoculated and immunosuppressed, *tup1*$^{-}$ cells also exhibited decreased colonization, but did not disseminate from the GI tract. Constitutive

filamentation therefore leads to poor colonization, yet the ability to form hyphae is required for dissemination in some host environments.

To further investigate the importance of hyphal formation *in vivo*, a strain was constructed in which filamentation was blocked in the presence of doxycycline by placing the *NRG1* gene under control of the tetracycline promoter [83, 118]. Nrg1p is a repressor of filamentation, and in the presence of doxycycline, this construct inhibits hyphal growth [119]. Yeast cells and hyphal cells of this strain were able to colonize mice at equal levels. *C. albicans* that were locked in the yeast form were able to disseminate to distal sites from the bloodstream equally as well as cells capable of forming hyphae. However, only cells able to form hyphae caused killing of the host in the intravenous infection model [83]. Thus, hyphae appear to be necessary for lethal damage to the host, but in this model both and yeast and hyphae colonized and disseminated from the GI tract.

The Sfl2p transcription factor promotes hyphal growth in response to body temperature [120, 121]. A *sfl2*$^{-}$ mutant was able to colonize the GI tract at WT levels; however, after treating mice with the chemotherapy agent cyclophosphamide, the *sfl2*$^{-}$ mutant had reduced dissemination from the GI tract to the liver, as well as reduced virulence [120]. Based on these results, Sfl2p is important for initiating dissemination from the GI tract. In a systemic model of infection, Sfl2p was not required for virulence [121]. Studies with these mutants show that the ability to switch between yeast and hyphae forms is necessary for *C. albicans* to cause disease. Comparisons between models using different methods of inoculation or immune suppression highlight the differential requirements for these morphological forms during the disease process.

A network of transcription factors regulates the yeast to hyphal transition

Efg1p, a major virulence factor of *C. albicans*, is part of a complex regulatory network that controls morphogenesis, and is required for the yeast to hyphal transition (Fig. 1-2) [111, 122, 123]. Multiple transcription factors in this network allow *C. albicans* to assimilate various environmental signals and enact a finely controlled response. Efg1p is activated through the cAMP/PKA pathway to regulate hyphal growth in response to a wide array of signals [124]. The alternate transcription factor Cph1p controls a second pathway which is partially redundant with Efg1p [125]. Cph1p activated a MAPK-dependent filamentation pathway when cells were grown on nutrient poor medium [111, 126, 127].

Other transcription factors regulate hyphal growth in response to various environmental signals. Rim101p induced filamentation in response to alkaline pH [128]. Sfl2p was induced at increased temperatures and positively regulated hyphal growth under these conditions [121]. Several genes also negatively regulate hyphal growth, such as Tup1p, Sfl1p, and Nrg1p. Deletions in these repressors of hyphal growth caused constitutive filamentation [83, 116, 129].

Cph2p and Tec1p control another pathway that regulates hyphal development: Cph2p was required for filamentation during growth in Lee's medium, and therefore may respond to media-specific growth signals [130]. Tec1p was required for hyphal growth after serum induction [131]. Cph2p recognizes SRE-elements in the promoters of downstream genes, while Tec1p binds to CATTCY sequences [130]. Tec1p is regulated by Cph2p, which binds to SRE elements in the *TEC1* promoter, and also by Efg1p [125, 130]. Both Efg1p and Cph2p, through Tec1p, regulate the expression of hyphal genes

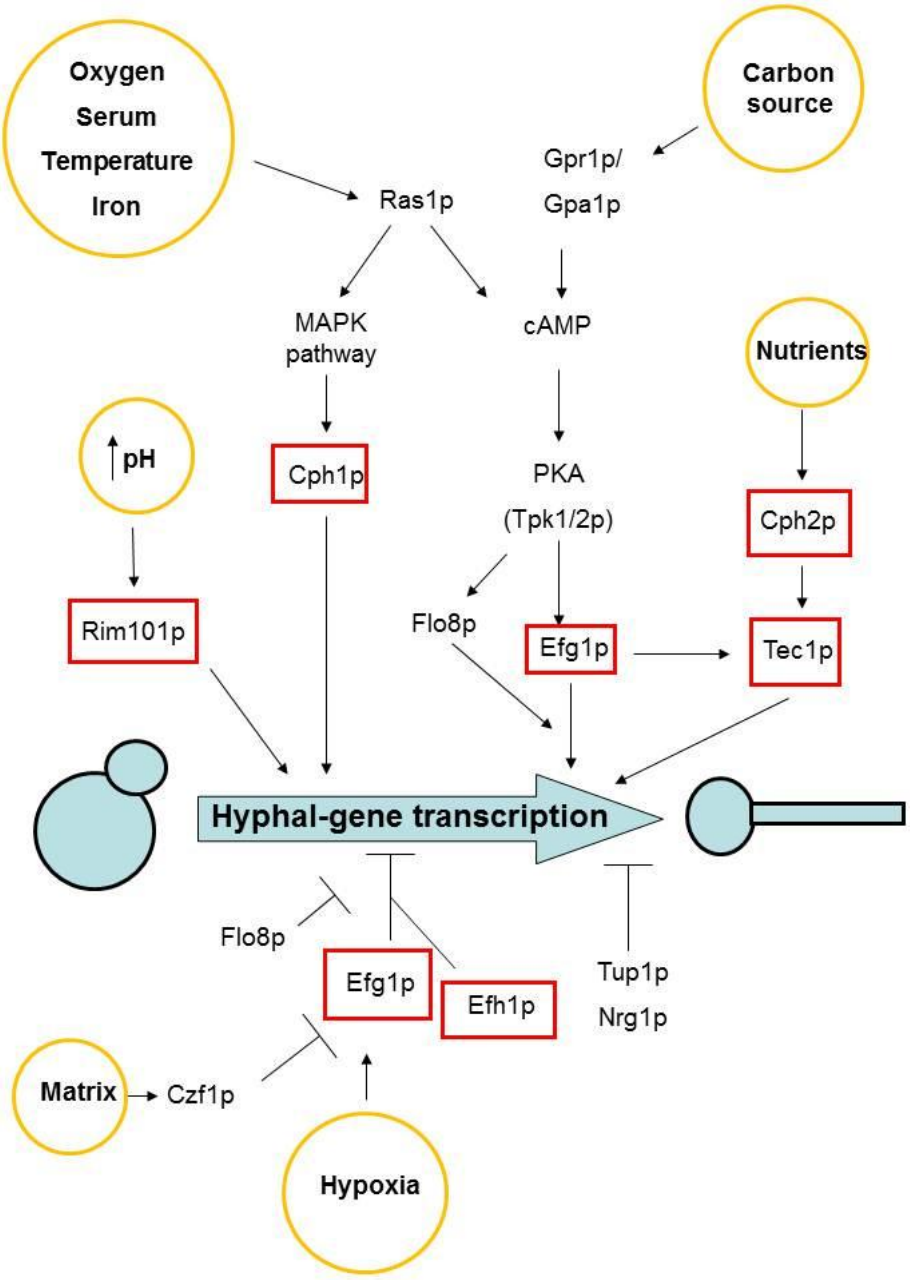


Figure 1-2. Transcriptional regulation of hyphal formation in *C. albicans.*

Known environmental signals which promote filamentation are circled, and signaling pathways leading to the transcription factors they influence are shown. Transcription factors discussed in these studies are boxed in red.

such as *ECE1*, *HWP1*, and *HYR1* [125]. Regulation of a core set of hyphal genes through the Efg1p and Cph2p pathways allows *C. albicans* to alter its gene expression in response to multiple environmental signals.

Several proteins directly interact with Efg1p to regulate gene expression and morphology. Czf1p is a transcription factor which antagonized Efgp1 activity under embedded and hypoxic conditions, and is capable of binding to Efg1p (Fig. 1-3) [62, 132]. Efg1p has also been shown to bind Flo8p using a two-hybrid assay and co-immunoprecipitation [132, 133]. Flo8p, like Efg1p, activated hyphal growth in response to serum, and repressed this response under low oxygen and embedded conditions. Flo8p has been shown to regulate a subset of genes that are controlled by Efg1p under these conditions [132, 133]. Interactions with Czf1p and Flo8p occur at multiple regions of the Efg1 protein; these regions are distinct from the regions involved in DNA binding [132]. Hyphal growth in response to Sfl2p activation also requires Efg1p and Flo8p, suggesting that Sfl2p acts upstream of these regulators [120]. Multiple layers of regulation allow *C. albicans* to precisely control morphogenesis in response to a changing environment.

Efg1p is a DNA binding protein involved in the regulation of transcription

Efg1p and Efh1p were discovered in *C. albicans* as homologues of the APSES family of transcription factors (Asm1, PhD1, Sok2, Efg1, and StuA) which regulate cellular morphology in other fungi [111, 122, 134]. Efg1p is a homologue of Phd1p in *Saccharomyces cerevisiae*, which induces pseudohyphal growth when over-expressed [134]. In other fungi, pairs of APSES proteins tend to have opposing regulatory

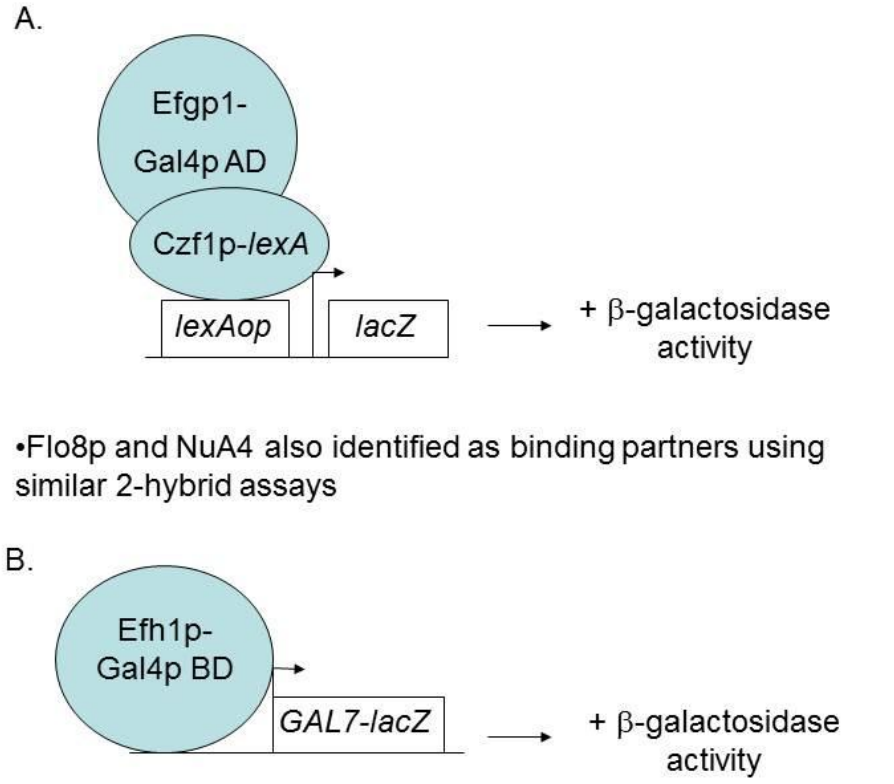


Figure 1-3. Two-hybrid assays in *S. cerevisiae* with Efg1p and Efh1p.

(A) Efg1p has been shown to bind to Czf1p, Flo8p, and NuA4p using a two-hybrid assay. β-galactosidase activity is detected when the target protein, fused to a DNA-binding domain (l*exA*) and Efg1p, fused to an activation domain (Gal4 AD), interact to initiate transcription of the *lacZ* reporter gene. (B) An Efh1p-Gal4p binding domain fusion initiates transcription of a *GAL7*-lacZ reporter gene, and its activity doubles in the presence of an Efh1p-Gal4p activating domain fusion.

functions: Sok2p, the partner of Phd1p, acts as a repressor of pseudohyphal growth [111]. The *Candida albicans* APSES proteins appear to have evolved differently from other fungi: this may be a result of its adaptation to growth as a commensal organism. In *C. albicans*, Efg1p is a major regulator or transcription and morphogenesis, while Efh1p is important for persistence in the GI tract [42, 111, 122].

Efg1p, like other APSES proteins, has a conserved domain of about 100 amino acid residues in length which contains a basic helix-loop-helix motif (bHLH). This motif is usually involved in DNA binding and may induce dimerization with other similar proteins [111, 122]. This region of Efg1p has been shown to be necessary *in vitro* for binding to several DNA sequences, including E-boxes (CATTTG) and MCB sequences (ACGCGT) [132, 135]. The APSES domain alone was sufficient to promote binding to an MCB sequence. *In vitro*, Efg1p binds to synthetic E-boxes and to the promoter of the *ALS8* gene, which encodes an adhesin [135]. The relevance of these binding sequences within the cell is unknown. Chromosome immunoprecipitation identified a novel binding domain for Efg1p (EGR-box) during yeast phase growth [136]. During hyphal growth, Efg1p no longer bound these domains and may have altered binding specificity under these conditions. Efg1p has been shown to interact with multiple DNA sequences at the promoters of both yeast and hyphal-regulated genes, consistent with its function as a transcription factor.

Using one-hybrid assays in *C. albicans* with an Efg1p-*lexA* fusion, Efg1p was shown to be a transcriptional repressor [111]. There was a decrease in β-galactosidase activity in the presence of Efg1p when compared to the basal level of activation of the *lacZ* gene from the *ADH1* promoter alone (Fig. 1-4). Two-hybrid assays in

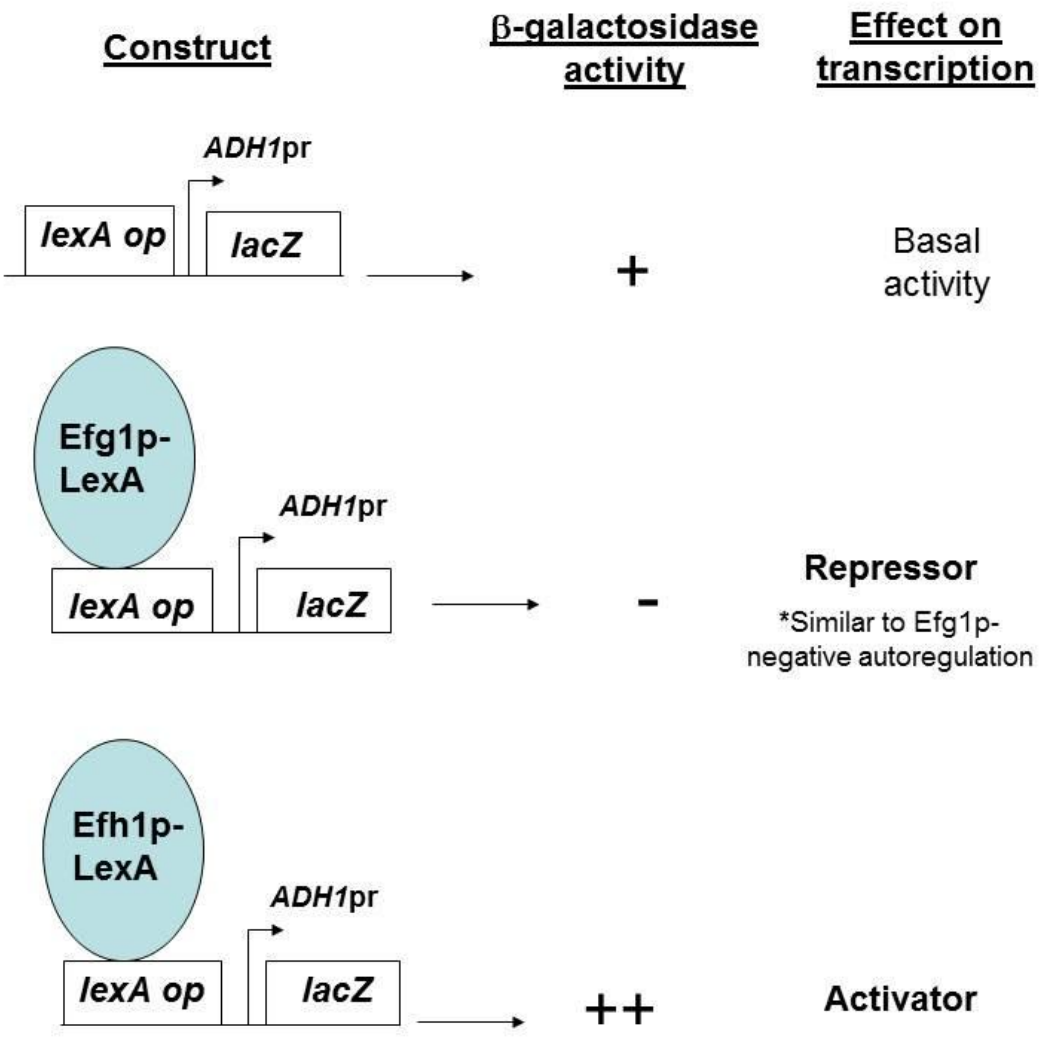


Figure 1-4. One-hybrid assays in *Candida albicans* with Efg1p and Efh1p.

Efg1p or Efh1p-*lexA* fusions bind to the *lexA* operator upstream of the *lacZ* reporter gene under control of the *ADH1* promoter. In the absence of any fusion protein, low levels of β-galactosidase activity were detected. The Efh1p-*lexA* fusion activated transcription and increased β-galactosidase levels were detected. This activity was abolished in the presence of the Efg1p-*lexA* fusion.

Saccharomyces suggest that Efg1p requires additional *C. albicans*-specific proteins in order to act as a repressor because in this organism, no effect on *EFG1* transcription was observed (Fig. 1-3). An Efh1p-*lexA* fusion activated transcription and increased β-galactosidase levels in a one-hybrid assay in *C. albicans* (Fig. 1-4). Similarly, an Efh1p-Gal4 binding domain (BD) fusion was able to activate transcription in a two-hybrid assay in *S. cerevisiae* (Fig. 1-3). Twice the level of activity was detected when both an Efh1p-Gal4 activation domain fusion and an Efh1p-Gal4 BD fusion were present. This observation suggests that Efh1p may be able to form homodimers and activate transcription. Efg1p did not appear to dimerize with itself or with Efh1p under these conditions, and was active as a transcriptional repressor [111].

Control of morphogenesis by Efg1p

Efg1p has many effects on the biology of *C. albicans*, and one of the best studied is its role in morphogenesis. Efg1p has direct effects on cellular morphology that can be observed under a number of laboratory conditions and reflect its role in the control of hyphal gene expression in response to various environmental stimuli [107, 111, 126]. Efg1p is a positive regulator of hyphal formation under most inducing conditions (temperature, serum, low oxygen, and low iron) during laboratory growth [44, 111, 122, 137]. Over-expression of Efg1p caused some of the cells in a culture to undergo pseudohyphal formation, while deletion of the gene caused *C. albicans* to grow as elongated yeast cells [122]. Unlike *EFG1*, its homologue *EFH1* is only expressed at low levels *in vitro*, and an *efh1*$^{-}$ deletion has no major effect on filamentation in most standard

lab conditions. Over-expression of Efh1p led to pseudohyphal formation by some of cells in a culture, and this phenotype was dependent on the presence of Efg1p [111].

During hypoxic and embedded growth conditions, Efg1p and Efh1p together repress filamentation. An *efg1*$^{-}$ strain was unable to filament under low oxygen conditions, but an *efg1*$^{-}$ *efh1*$^{-}$ mutant strain showed increased filamentation under these conditions compared to wild type cells. The *efg1*$^{-}$ *efh1*$^{-}$ mutant was also hyper-filamentous under embedded conditions [111]. These data show that Efg1p is a major transcriptional regulator of morphogenesis in *C. albicans* in response to changing environmental signals, while Efh1p plays a minor role controlling filamentation *in vitro*.

Efg1p may regulate hyphal growth by altering cell division [138]. During yeast growth, cell separation occurs through the degradation of the septum between the mother cell and the bud. In hyphae, the septum is not degraded, and cells remain attached as they continue to bud in a polarized manner. During hyphal growth, decreased expression of genes encoding proteins that are involved in cell separation, such as the chitinase *CHT3*, required Efg1p [138]. This activity was dependent on phosphorylation of Efg1p by the cyclin-dependent kinase Cdc28-Hgc1 [138]. Therefore, Efg1p activity may contribute to hyphal growth through the down-regulation of genes required for cell separation.

Efg1p-dependent gene expression

In addition to altering cellular morphology, Efg1p has been shown to regulate the expression of multiple cell surface proteins associated with hyphal cell growth (Fig. 1-5) [111, 137, 139]. Efg1p is required for the expression of a number of genes during hyphal growth, including the *ALS* family, the *RBT* genes, *ECE1*, and *HWP1*, all of which encode

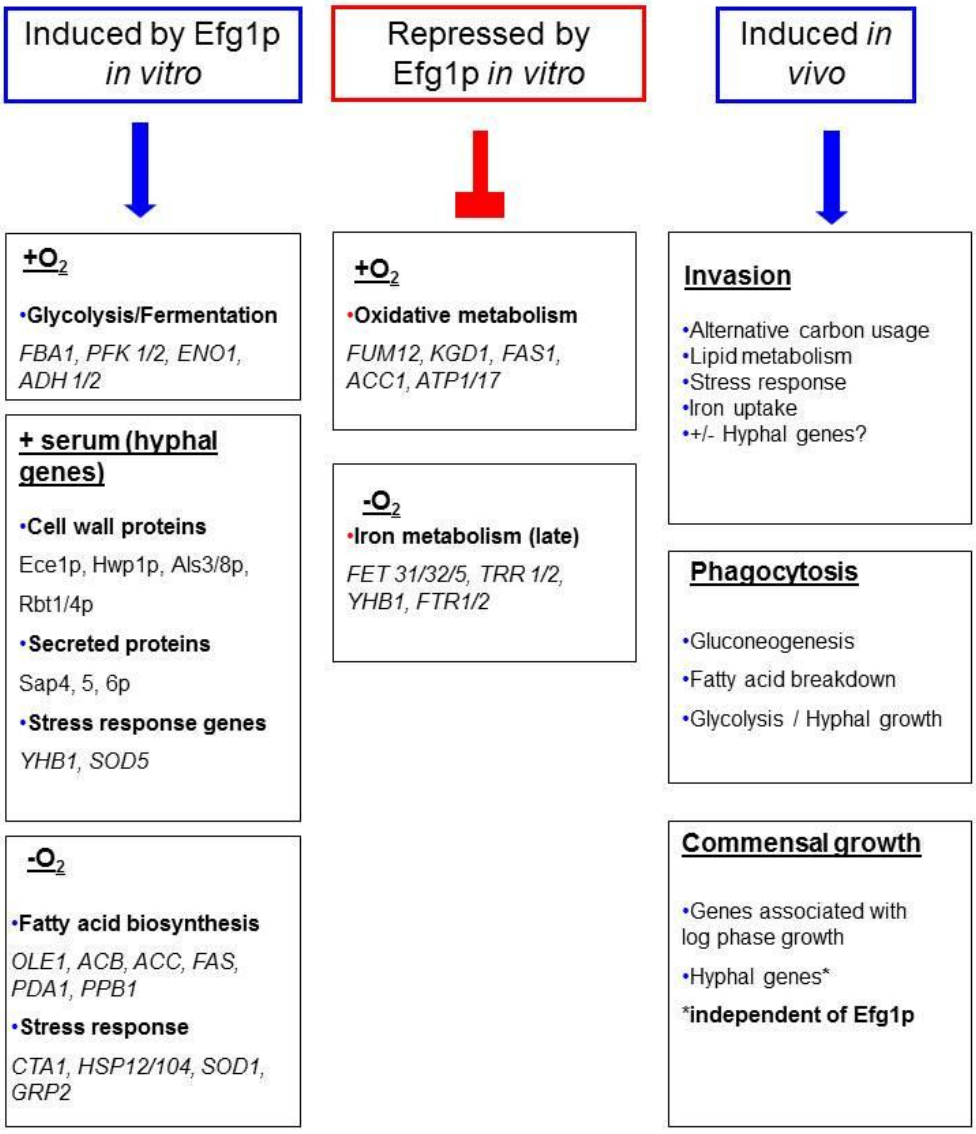


Figure 1-5. Regulation of gene expression by Efg1p.

Microarrays were done in normal or hypoxic growth conditions (+/- O_2), in the presence of serum, from kidney/liver lesions, during phagocytosis by macrophages, and during commensal growth to identify global changes in gene expression by *C. albicans*. Genes regulated by Efg1p were determined in multiple *in vitro* conditions. For comparison, WT gene expression in association with a host is shown.

either cell wall or secreted proteins [116]. Most genes which require Efg1p for their expression are positively regulated by Efg1p, and relatively few genes have been characterized as negatively regulated by Efg1p.

Several genes positively regulated by Efg1p during hyphal growth are involved in adhesion. Hwp1p is a glycoprotein that is a target of a mammalian transglutaminase that cross-links the yeast to the mammalian cell surface [108]. Cells lacking *HWP1* did not stably associate with epithelial cells [108]. Efg1p, along with several other transcriptional regulators, was responsible for the expression of *ALS1* and *ALS3*, hyphal-specific genes that encode cell-surface adhesins [116, 140]. Expression of Als3p and other adhesins promoted attachment to host cells and biofilm formation in an Efg1p-dependent manner [140]. An *efg1*$^{-}$ mutant was defective in forming robust biofilms and binding to components of the extra-cellular matrix [44, 141]. Proteomic analysis also identified 30 potential surface proteins that showed altered expression in an *efg1*$^{-}$ mutant. These proteins may represent additional adhesins [141]. Defects in adhesion and biofilm development may be related to altered expression of cell surface proteins that are associated with hyphal growth.

In addition to adhesins, Efg1p was required for the expression of genes involved in defense against reactive oxygen species, such as the superoxide dismutase Sod5p [142, 143]. Production of reactive oxygen species by neutrophils is a major host response to *C. albicans* infections. The *efg1*$^{-}$ mutant is more susceptible to phagocytosis by neutrophils compared to WT *C. albicans* [107]. Therefore, sensitivity to ROS response may contribute to the susceptibility of the *efg1*$^{-}$ strain to the host immune response.

Several of the *SAP* genes, encoding secrete aspartyl proteases, are regulated by Efg1p during hyphal growth [125, 144]. *SAP5* expression was regulated by both Efg1p and Cph1p during invasion of oral tissue, and the reduced expression of *SAP5* coincided with decreased invasion by the *efg1*$^{-}$ mutant in this model [85]. In a systemic infection, Efg1p was also required for the expression of *SAP5* [145]. Increased expression of secreted aspartyl proteases contributes to the virulence of *C. albicans* in an Efg1p-dependent manner.

Genome-wide transcription analysis has been done on *efg1*$^{-}$ single null, *efh1*$^{-}$ single null, and *efg1*$^{-}$ *efh1*$^{-}$ double null strains of *C. albicans* grown in a variety of *in vitro* conditions. These include normal yeast growth, hyphal-inducing conditions, and hypoxic growth [111, 137, 146]. During normal growth, an *efg1*$^{-}$ strain showed large changes in overall gene expression compared to wild type cells. In particular, genes involved in glycolysis, fermentation, stress response, and cell wall proteins were affected [111, 139]. Under normal growth conditions, Efg1p activated glycolysis genes (*PFK1*, *FBA1*) and repressed oxidative metabolism through down-regulation of the citric acid cycle enzymes (*FUM12, KGD1*), which suggests that Efg1p is a major regulator of metabolism as well as morphogenesis. The *efh1*$^{-}$ strain showed relatively small changes in gene expression under these conditions, and the genes that showed differential regulation in the *efg1*$^{-}$ *efh1*$^{-}$ double null strain were similar to those seen in the *efg1*$^{-}$ single null strain [111].

Under hyphal-inducing conditions, there were large differences between the wild type and *efg1*$^{-}$ strains, although these differences were similar to those observed under normal conditions when comparing the strains. Glycolysis was reduced in the *efg1*$^{-}$ strain, but oxidative metabolism was unaffected. However, this effect may be due to the

lack of glucose in the medium used in this study [111]. Efg1p over-expression induced higher expression of cell wall proteins compared to a wild type strain, as expected in hyphal-inducing conditions. Additionally, increased expression of stress response genes such as *YHB1*, a flavohemoglobin, and *SOD5*, a superoxide dismutase, was observed when Efg1p was over-expressed [6, 137]. The genomic expression profiles of *EFH1* and *EFG1* over-expressing strains were highly similar, and different from those seen during true hyphal formation. This suggests that pseudohyphal formation in these two strains is similar and that both are distinct from true hyphal formation [111]. Efg1p is a central regulator of metabolism, as well as morphogenesis, while Efh1p alone has little effect on transcription under these conditions.

During hypoxic growth, glycolysis, fermentation, and stress response genes were up-regulated by wild type *C. albicans*, while genes involved in oxidative metabolism were down-regulated [146]. Hyphal genes were also up-regulated by the wild type strain under hypoxic conditions. In the presence of oxygen, Efg1p positively regulated glycolysis and fermentation and repressed oxidative metabolism, but differential regulation of these pathways did not require Efg1p during hypoxia. Fewer genes overall were regulated by Efg1p during hypoxic growth. Of these, the majority of Efg1p-dependent genes were involved in fatty acid metabolism. *OLE1* (a fatty acid desaturase) and *PDB1* (pyruvate dehydrogenase) were up-regulated under hypoxic growth conditions in an *EFG1* over-expressing strain [46, 146]. Therefore, Efg1p regulates different pathways in response to either normoxia or hypoxia.

Transcriptional profiling has been used to identify changes in gene expression in *C. albicans* cells in association with a host. Genes up-regulated in invasive infections

corresponded to alternative carbon and lipid usage, as well as stress response genes [8, 9, 42, 63]. During phagocytosis by macrophages, *C. albicans* initially up-regulated gluconeogenesis and fatty acid breakdown, but resumed glycolysis at later time points coinciding with hyphal growth [63]. In invasive liver infections, genes involved in iron uptake were up-regulated [8]. Strains of *C. albicans* with defects in iron uptake and metabolism have also been shown to have defects in virulence [110, 147].

The expression of genes associated with hyphal formation has also been observed during invasion of tissues. Hyphal genes were up-regulated in liver infections compared to *in vitro* growth in rich media, and abundant hyphae were observed in both kidney and liver lesions [8, 9]. We have observed that during GI tract colonization the majority of cells present are in the yeast form, yet hyphal genes are up-regulated [42]. Therefore, gene expression during *in vivo* growth differs from the patterns observed *in vitro*, and may be independent of morphology.

Although the genes affected by Efg1p *in vitro* have been well characterized, genes regulated by Efg1p during commensal growth have not been. Some genes normally controlled by Efg1p during laboratory growth are regulated in an Efg1p-independent fashion in the murine GI tract [42]. *In vitro*, Efg1p has been shown to influence the expression of *ECE1*, *RBT1* and *RBT4*; however, in the cecum of mice during colonization, the level of *RBT1* and *RBT4* expression was unaffected by an *efg1*$^{-}$ *cph1*$^{-}$ mutant. *ECE1* levels were only slightly lower in *efg1*$^{-}$ *cph1*$^{-}$ cells than in wild type cells. While Rbt1p and Rbt4p are required for virulence in the disseminated model of candidiasis, they are not necessary for colonization of the GI tract [42, 116].

Regulation of Efg1p activity

In addition to regulation by multiple transcription factors, hyphal gene transcription is also regulated by chromatin structure remodeling and histone modification. Using a two-hybrid assay, Efg1p was shown to interact with NuA4p (nucleosome acetyltransferase), which is part of a complex involved in histone acetylation and activation of transcription [148]. It was observed using chromatin immunoprecipitation (ChIP) that Efg1p and NuA4p both bind to the promoters of hyphal genes in yeast and hyphal cells. This is necessary for the recruitment of the Swi-Snf chromatin remodeling complex to hyphal gene promoters under inducing conditions and the induction of transcription [148].

To add another level of complexity, Efg1p also undergoes negative auto-regulation quickly after hyphal induction [124]. Efg1p was shown to bind to its own promoter using ChIP, and over-expression of *EFG1* from a plasmid reduced the expression of the native *EFG1* transcript [124, 136]. Native transcript levels decreased immediately after the induction of hyphal growth, and then rose slowly over time. *EFG1* negative regulation also required the Sin3p histone deacetylase complex, which was shown by ChIP to bind the *EFG1* promoter [122, 124]. Thus, morphogenesis in *C. albicans* is highly regulated: multiple transcription factors, chromatin remodeling/histone modifications, and post-translational modification (described below) all regulate the activity of Efg1p and morphogenesis.

Activation of Efg1p through the cAMP/PKA pathway

Environmental signals such as serum, temperature, nutrients, oxygen, and iron deprivation activate the cyclic AMP (cAMP)-PKA pathway, which positively regulates hyphal gene expression through Efg1p [124]. Gpr1p/Gpa2p, a G-protein coupled receptor complex, is involved in nutrient sensing through the PKA pathway. Signaling through the Gpr1p/Gpa2p complex led to a rise in cAMP levels and hyphal induction [149]. Activation of Ras1p in response to these signals also caused a spike in cAMP levels [124, 150]. Low signaling through the cAMP/PKA pathway was associated with growth on non-fermentable sugars in *S. cerevisiae*, consistent with activation of this pathway by glucose sensing [151, 152]. The PKA isoforms Tpk1/2p are involved in activation of Efg1p in response to changes in the cAMP concentration. Over-expression of Efg1p compensated for hyphal defects in a *tpk2*$^{-}$ mutant [109]. Tpk2p, or another unidentified PKA isoform, may be responsible for the observed phosphorylation of Efg1p [150]. Activation of PKA activity also lowered *EFG1* transcription, indicative of negative auto-regulation by Efg1p itself [124]. While a direct connection between Efg1p and Tkp2 is still speculative, Efg1p is activated in a PKA-cAMP dependent manner.

Efg1p has a putative PKA phosphorylation site in the bHLH domain (T206) and the phosphorylated form is detectable under hyphal-inducing conditions by a mobility shift on an SDS-PAGE gel [150]. Efh1p lacks a similar putative phosphorylation site [111]. When this threonine was mutated to alanine, *C. albicans* was defective in forming hyphae, corresponding to an 'inactive' protein which mimics the deletion of the gene. If a glutamic acid residue was substituted, there was an increase in hyphal formation, and the protein mimicked the phosphorylated form of Efg1p [150]. In addition, the APSES

domain and C-terminal sequences are required for phosphorylation of Efg1p [132]. Despite this data, the locations and number of phosphorylation sites in Efg1p have not yet been directly demonstrated. In summary, Efg1p activity during hyphal induction is regulated at both the transcriptional and post-translational levels.

Phenotypic variation

C. albicans is capable of producing clonal populations which are phenotypically diverse through the mechanisms of hyphal growth and white-opaque switching [153, 154]. White and opaque cells are morphologically distinct: normal yeast cells are considered white phase cells, while opaque phase cells are elongated [155]. White-opaque switching is related to the mating capabilities of *C. albicans* because only opaque cells are competent for mating. Mating competent strains have been isolated from patients, suggesting that white-opaque switching is relevant in a human host [156].

The major regulator of white-opaque switching is the transcription factor Wor1p, which is only expressed in opaque cells [157]. In opaque cells, Wor1p undergoes positive auto-regulation, resulting in bistability of expression within the population [157, 158]. Several transcriptional feedback loops also regulate this process and promote epigenetic stability of these phenotypes [106, 158].

In addition to its role as a regulator of morphogenesis and gene expression, Efg1p is a regulator of white-opaque switching. Only white phase cells express *EFG1* [159], and Efg1p is regulated by a complex feedback network during white-opaque switching. In opaque cells, Wor1p promotes its own expression by inhibiting Efg1p activity through

the secondary regulators Czf1p and Wor2p. This regulation in turn then inhibits the switch from opaque cells to white cells [106].

White-opaque switching affects the physiology of *C. albicans* in multiple ways. White phase cells have increased virulence relative to opaque cells, while opaque cells are better at colonization of specific niches, such as the skin. These differences are related to phase-specific gene expression [160, 161]. Metabolic genes are also co-regulated with white-opaque cell switching, suggesting that cells in either the white or opaque phase may have altered growth properties [162]. For example, white cells express genes associated with fermentation, while opaque cells have a transcriptional profile consistent with oxidative metabolism. It has been suggested that phenotypic plasticity exemplified by white-opaque switching and hyphal growth promote host colonization and pathogenesis [163]. We have proposed that phenotypic variation within a *C. albicans* population may promote either colonization or pathogenesis depending on host immune status. Efg1p is an important regulator of switching between yeast and hyphal states, as well as white and opaque phases in *C. albicans*. Therefore, Efgp1 may contribute to phenotypic variation in colonizing populations, which can lead to altered interactions between *C. albicans* and its host.

Role of Efg1p in gastrointestinal colonization

How *C. albicans* is able to alter its lifestyle to promote colonization in a healthy host and cause disease in an immunocompromised host is a major unresolved question in the field of candidiasis. As systemic infections are thought to arise from endogenous colonizing populations of *C. albicans* in the GI tract [113, 164], understanding how *C.*

albicans interacts with its host in this niche may provide insight as to how *C. albicans* can switch between these alternate lifestyles. Previous work identified several genes important for colonization of the GI tract, such as the putative transcription factor *EFH1*, the adhesin *INT1*, and the lipase *PLB1* [42, 82, 100]. As a homologue of Efh1p [111] and a major regulator of both virulence and morphology in *C. albicans*, we hypothesized that Efg1p may also play a role in colonization of the GI tract.

In order to understand the function of Efg1p during gastrointestinal colonization, we colonized both a healthy host and an immunocompromised host with WT *C. albicans* and a strain lacking both copies of *EFG1* [165]. We observed that in the absence of *EFG1*, cells were capable of initially hyper-colonizing the GI tract, but were defective over time compared to WT *C. albicans*. Hyper-colonization was observed in both a healthy and IC host. However, the defect in colonization of the *efg1*$^{-}$ mutant over time was due to activation of the host immune response and required a T cell dependent response. We showed that the activity and expression of Efg1p differed between colonizing populations depending on the status of the host immune response. Additionally, we observed that Efg1p exhibits phenotypic variation within a population of cells. Based on these data, we proposed a model for how colonizing *C. albicans* cells are capable of sensing changes in the immune status of its host through the activity of Efg1p to promote either colonization or pathogenesis [166]. In a healthy host, Efg1p activity in a colonizing population increases and promotes colonization, while in an IC host, Efg1p activity is low and produces a population poised to initiate disease. Based on these data, Efg1p is a major regulator of colonization and phenotypic heterogeneity in its expression promotes host-dependent selection in a colonizing population.

In order to understand the mechanism controlling the phenotype of *efg1*$^{-}$ cells during colonization, microarrays were used to identify the genes regulated by Efg1p in colonizing cells. Previous work has showed that Efg1p is required for large changes in gene expression during laboratory growth in response to a number of environmental conditions [111]. The results presented here show that Efg1p is also required in order for *C. albicans* to adapt to the GI tract environment through altered gene expression. As previously noted, some genes regulated by Efg1p during laboratory growth did not require Efg1p for their expression in the GI tract, while others were regulated by Efg1p in both conditions [42]. In the absence of Efg1p, increased expression of genes involved in the utilization of non-fermentable carbon sources and carnitine metabolism was noted in colonizing cells which would then promote the observed hyper-colonization of the GI tract by the *efg1*$^{-}$ mutant. The *efg1*$^{-}$ mutant was also able to grow to higher levels during post-exponential phase in laboratory culture, and this growth was enhanced on non-fermentable carbon sources. Growth of the *efg1*$^{-}$ was observed in nutrient poor media in which the WT strain was unable grow unless excess carnitine was added to the media. Therefore, Efg1p is a major regulator of metabolism in both laboratory growth and during colonization of the GI tract.

Efg1p was also required for the expression of a number of genes involved in host interactions, such as genes encoding adhesins (*ALS3*), secreted aspartyl proteases (*SAPs*), and hemoglobin uptake proteins (*RBT5*). In particular, *SOD5*, encoding a superoxide dismutase, was highly up-regulated during colonization in WT cells but not in *efg1*$^{-}$ cells during colonization. *SOD5* is required for the detoxification of ROS produced by phagocytic cells in response to *C. albicans* infections [143]. An *efg1*$^{-}$ mutant which over-

expressed *SOD5* was able to outcompete an *efg1*$^{-}$ mutant alone in the GI tract. Therefore, Efg1p activity protects cells against activation of the host immune response by promoting the expression of a number of genes, such as *SOD5*, during colonization of the GI tract.

Through the phenotypic variation of Efg1p, cells with altered metabolism and differences in their susceptibility to the host immune response are produced in a normal population. Selection by the host immune system can then alter the composition of a colonizing population. Therefore, in a healthy host, growth of the colonizing population is limited and is comprised of cells with high Efg1p activity and increased resistance to the immune response. In an IC host, however, cells with low Efg1p activity are not eliminated and can proliferate to higher levels. Increased fungal burden is a risk factor for developing candidiasis. The characterization of changes in endogenous colonizing populations between healthy hosts and IC hosts, such as Efg1p activity, may allow for improved identification of patients at risk for further disease progression and lead to novel treatment methods for candidiasis.

Chapter 2:

Variation in *Candida albicans EFG1* expression enables host-dependent changes in colonizing fungal populations

The majority of work in this chapter was first published in the journal mBio [165].

Introduction

Candida albicans, one of the many microbes that colonize the human gastrointestinal (GI) tract, is both a commensal and an opportunistic fungal pathogen [1]. Infants can acquire *C. albicans* within the first month of life [167] and colonization persists in the GI tract throughout adulthood. *C. albicans* can be detected in the intestine of the majority of the human population worldwide, yet in most healthy adults, it exists in a balance with its host and does not cause serious disease [1]. When a host becomes immunocompromised, *C. albicans* can become an invasive pathogen, capable of penetrating the GI tract epithelium and reaching the bloodstream to cause a systemic infection [1, 3, 7, 113, 117, 164]. Escape from the GI tract is thought to be the main route of dissemination of endogenous *C. albicans*, and can lead to life-threatening systemic candidiasis in a compromised host. *Candida* infections are one of the most common hospital acquired diseases in the U.S. [168], and systemic infections of *C. albicans* are fatal in up to ~ 50% of cases [169-171].

One major determinant of *C. albicans* behavior is the status of the host immune system [34, 35, 113, 172, 173]. Risk factors for development of candidemia include use of broad spectrum antibiotics and immunosuppression or immunodeficiency [1, 168, 172]. T cells play an important role in the immune response to *C. albicans* and oral candidiasis is a major secondary infection among AIDS patients. Neutrophils and macrophages are involved in the phagocytosis of invading organisms and the production of reactive oxygen species [35, 173, 174]. Neutropenia and chemotherapy are major risk

factors for disseminated candidiasis [172, 175, 176]. In a murine model, neutropenia and mucosal disruption promote dissemination of *C. albicans* from the GI tract [34].

Fungal activities, such as morphogenesis and differential gene expression, also play a role in determining the behavior of *C. albicans* in the host [42, 82, 177-179]. For example, laboratory-grown hyphae (highly elongated cells that remain attached after cell division and lack constrictions at the septa) differ in their interactions with epithelial cells in comparison with laboratory-grown yeast cells, resulting in altered host responses (reviewed in [21]). Several *C. albicans* regulatory factors that control morphogenesis in the laboratory affect GI colonization [42, 177] despite the fact that, in a previous study, fungal cells colonizing the GI tract were found to exhibit yeast cell morphology [42]. For example, the *EFH1* gene, encoding a transcription factor, is highly up-regulated during colonization of the gastrointestinal tract, but not in an animal model of oral candidiasis. Efh1p regulates population size during commensal colonization, but is not required for lethal infection following injection into the bloodstream [42], suggesting that some *C. albicans* genes regulate commensal colonization and other genes regulate pathogenesis.

While these findings provide some insight into the interplay between host and colonizer, the fundamental question of how a change in host immune status influences colonizer behavior remains unanswered. We previously proposed that phenotypic heterogeneity in colonizing cells produces a population that can be shaped by host selective pressure, allowing the population to sense and respond to host immune status [166]. To provide support for this model, we examined the role of the transcription factor Efg1p, a major regulator of *C. albicans* physiology [107, 122, 146], during colonization of the murine GI tract. *EFG1*, a homolog of *EFH1*, is a major regulator of gene

expression and morphology, as well as a virulence factor in systemic infections [111, 125, 135, 137, 139]. Here we show that Efg1p is an important regulator of colonization dynamics in the host and, like certain other proteins [180-184], exhibits cell-to-cell variation in expression and activity. We also demonstrate that the action of the host on a heterogeneous *C. albicans* population changes the composition of the population. Studies of gene expression showed that after colonization was established, *EFG1* expression was higher in *C. albicans* cells that were colonizing healthy mice in comparison to cells colonizing immunodeficient mice. Through this mechanism, the host is able to influence the physiology of its colonizer. Noise in the expression and activity of *EFG1* underlies the ability of a *C. albicans* population to respond to changes in host status to favor either benign colonization or the conversion to pathogenic growth, depending on host immune status.

Results

Cell-to-cell variation in the levels of *EFG1* expression and activity

To test for heterogeneity in a *C. albicans* population, we analyzed the expression and activity of Efg1p. The promoter region of *EFG1* was cloned upstream of the gene encoding yeast enhanced Green Fluorescent Protein (yEGFP) [185, 186]. As a control, the promoter of *DRG1,* a constitutive gene expressed at a similar level to *EFG1* [187], was similarly cloned. Expression of yEGFP was measured following growth to post-exponential phase in rich media at 37°C. Cells carrying either fusion showed relatively low levels of fluorescence that were higher than the fluorescence of an untagged WT strain (Fig. 2-1A). Plots of fluorescence as a function of cell area showed that both

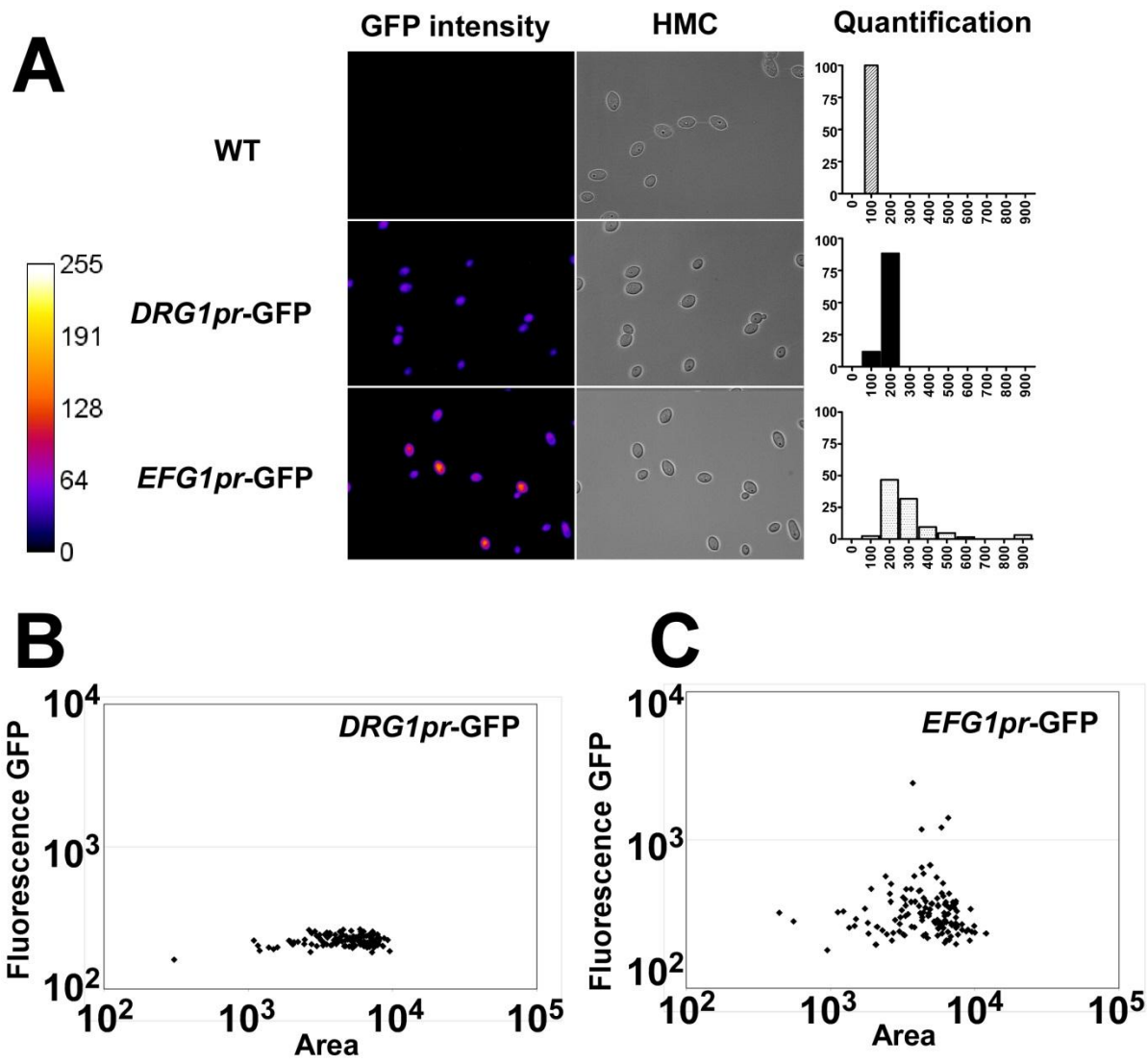


Figure 2-1. *EFG1* is expressed at variable levels within a population of cells.

WT strains carrying *DRG1pr-GFP* or *EFG1pr-GFP* fusions were grown for 24 hours at 37°C in YPD. Cells were washed, stained with Calcofluor white and mounted as described in Materials and Methods. Fluorescence and Hoffman modulation contrast images were collected with a Nikon TE-2000 inverted U microscope. (A) Left panels, heat map of GFP fluorescence intensity. Thresholds were 200 minimum and 820 maximum; legend is shown at left. Right panels, Hoffman modulation contrast (HMC). Graphs at right show % of total cells (y axis) as a function of mean fluorescence per cell in arbitrary units (x axis). The experiment was repeated 3 times and a representative experiment is shown. (B and C) Plot of mean GFP fluorescence per cell as a function of cell area for strains carrying *DRG1pr-GFP* (B) or *EFG1pr-GFP* (C).

strains exhibited a similar range of cell size and that the fluorescence of cells carrying the *EFG1pr-GFP* fusion varied substantially (Fig. 2-1C), while cells carrying the *DRG1pr-GFP* fusion were more uniform in their fluorescence (Fig. 2-1B). The coefficient of variance (COV) for cells expressing *DRG1pr-GFP* was 8.74%, indicating a sharp peak, while cells carrying the *EFG1pr-GFP* fusion showed a broader distribution of fluorescence (COV=70.64%) (Fig. 2-1A). Therefore, expression from the *EFG1* promoter generated greater cell-to-cell variation, while expression from the *DRG1* promoter was uniform within the population.

To test the hypothesis that Efg1p was differentially active in individual cells, expression of an Efg1p target gene was studied. To find target genes that were regulated by Efg1p during both laboratory growth and GI colonization, we tested candidate genes predicted by previous studies [111, 137], because some genes that are regulated by Efg1p during laboratory growth are expressed in an Efg1p-independent manner during colonization [42]. We identified *FDH1* (encoding a putative formate dehydrogenase) as a gene that was negatively regulated by Efg1p in the GI tract of mice and during laboratory growth (Fig. 2-2A and B). To examine the activity of Efg1p as a negative regulator of *FDH1* in individual cells, the *FDH1* promoter was cloned upstream of ubiquitin tagged yEGFP (uGFP; Fig. 2-3). In a previous study, addition of the ubiquitin tag decreased the stability of the GFP protein, providing a more accurate measurement of promoter activity [188]. While fluorescence from fusions of the *DRG1pr* and *EFG1pr* to uGFP was difficult to distinguish from background fluorescence, fluorescence from the *FDH1*pr-*uGFP* fusion was detectable (Fig. 2-4).

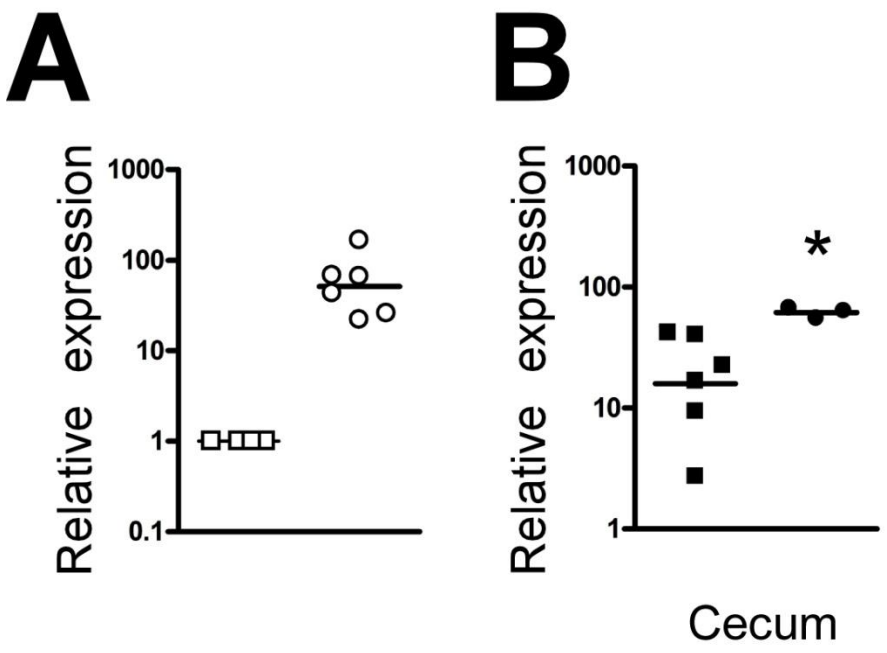


Figure 2-2. Negative regulation of *FDH1* by Efg1p

Cells of WT strain CKY101 (squares) or *efg1*$^{-}$ strain CKY136 (circles) were grown in laboratory culture in rich media or recovered from organs of BALB/c mice at day 18 post-inoculation. *FDH1* expression was measured by qRT-PCR, normalized to *ACT1* levels and expressed relative to expression in laboratory-grown exponential phase cells. Each symbol represents a sample pooled from 2 (cecum contents); for laboratory-grown cells, each symbol represents an independently grown culture. Bars represent the geometric mean; * indicates $p<0.05$; Mann Whitney test. (A) *FDH1* expression in laboratory-grown exponential-phase cells in rich media at 30°C. (B) *FDH1* expression in *C. albicans* cells recovered from the cecum.

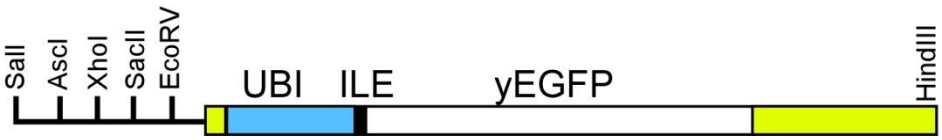


Figure 2-3. Diagram of uGFP.

Polylinker is shown at left. Yellow boxes indicate sequences from the *ACT1* locus. At left, 10 bp from *ACT1* 5' UTR precede the AUG codon of *UBI4*; at right, 396 bp *ACT1* 3' UTR. Blue box indicates *C. albicans* ubiquitin gene from the *UBI4* locus (orf19.6771). Black box indicates isoleucine codon plus 42 bp linker sequence. White box indicates yEGFP (not drawn to scale). This reporter gene is carried in a pNEB193-derived plasmid with *C. albicans URA3*.

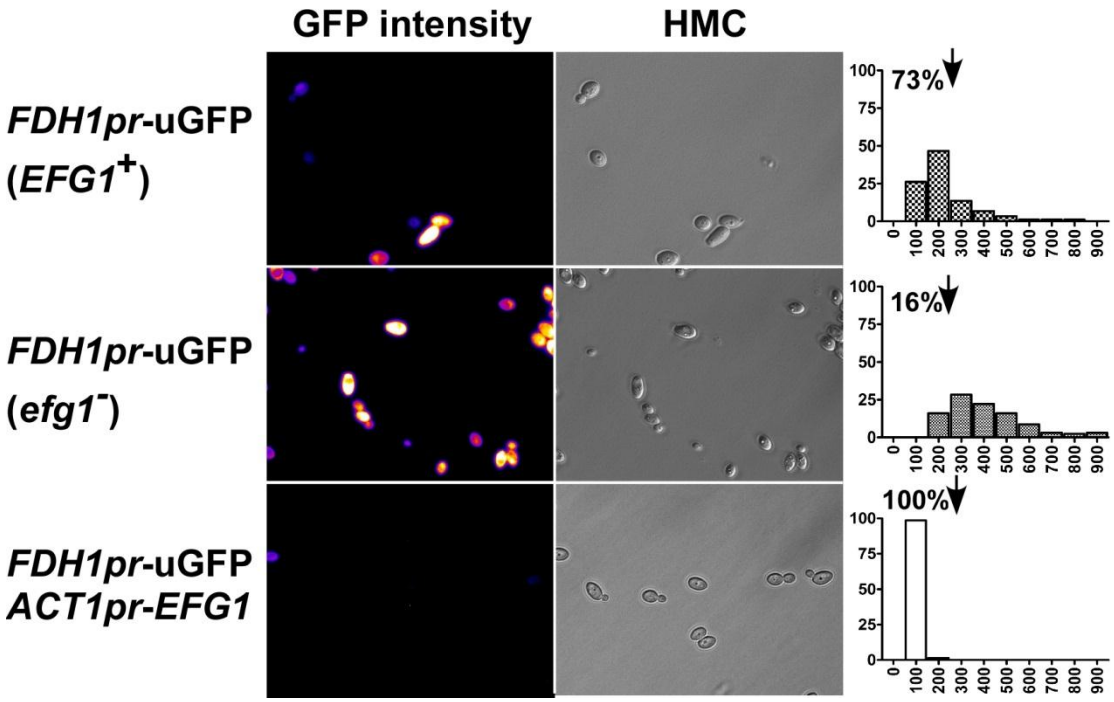


Figure 2-4. High expression of *FDH1* in a subpopulation of cells with low *EFG1* expression.

WT, *efg1*$^{-}$ or *ACT1pr-EFG1* strains carrying *FDH1pr-uGFP* were grown for 24 hours at 37°C in YPD. Cells were stained with Calcofluor white and visualized as in Fig. 1. WT cells carrying either no fusion or both *FDH1pr-uGFP* were grown as above and visualized without Calcofluor white staining. Fluorescence and Hoffman modulation contrast images were collected with a Nikon TE-2000 inverted U microscope. Experiments were repeated at least 2 times and a representative experiment is shown. Left panel shows heat map of GFP fluorescence (same scale as in Fig. 1); right panel shows HMC image. Graphs at right show quantification of mean fluorescence per cell; the arrow demarcates 300 fluorescence units and the number indicates the % of cells with fluorescence lower than 300 units.

A majority of cells carrying the *FDH1pr-uGFP* fusion showed low fluorescence, while some of the cells showed higher fluorescence (Fig. 2-4). Using $\geq$ 300 units (AU) of fluorescence as an arbitrarily chosen threshold to define high fluorescence, 27% of the cells in a population of WT cells exhibited high fluorescence (Fig. 2-4). In the absence of *EFG1*, a majority of the cells carrying the *FDH1pr-uGFP* fusion exhibited high fluorescence (84%) (Fig. 2-4). Additionally, when *EFG1* was expressed from the strong promoter of the actin gene (*ACT1pr-EFG1*), expression of *FDH1pr-uGFP* was very low in most cells (Fig. 2-4). Thus, Efg1p negatively regulated uGFP expression from the *FDH1* promoter and other factor(s) contributed to the variable positive regulation of *FDH1*.

To measure *EFG1* and *FDH1* expression within the same cell, a fusion between the *EFG1* promoter and the gene encoding mCherry protein [189] was transformed into a WT strain carrying the *FDH1pr-uGFP* fusion (Fig. 2-5A). Quantification of the fluorescence of both reporters showed that ~5% of the total population was comprised of cells with high levels of *FDH1* expression and relatively low expression of *EFG1* (Fig. 2-5B). Autofluorescence was lower than the level of fluorescence detected in the presence of these fusions (Fig. 2-5B). Therefore, within a population of WT cells there is variability in Efg1p activity and some WT cells expressed higher levels of *FDH1*, consistent with low Efg1p activity.

Cells lacking Efg1p showed increased colonization of the gastrointestinal tract and hyper-susceptibility to the host response

To determine whether variable levels of Efg1p activity could affect GI

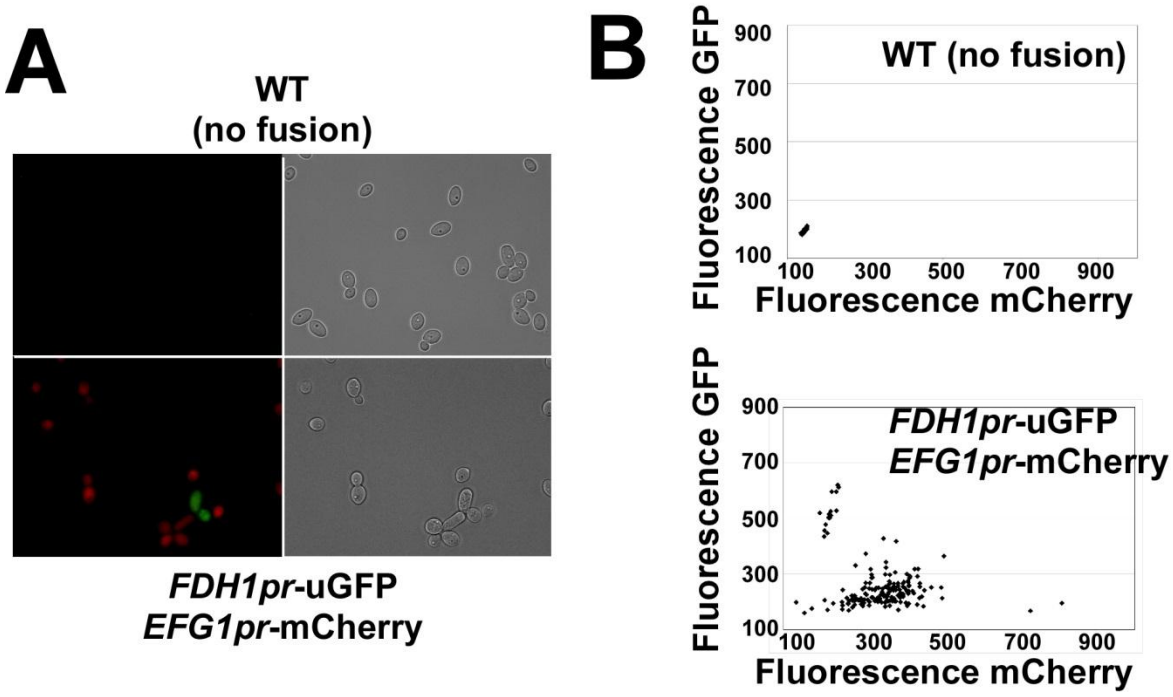


Figure 2-5. Cells with high *FDH1* expression have low levels of *EFG1* transcription.

WT cells carrying either no fusion or both *FDH1pr-uGFP* and *EFG1pr-mCherry* were grown as above and visualized without Calcofluor white staining. (A) Left panel shows overlay of green and red fluorescence for indicated strains. Right panel shows HMC image. (B) Intensity of green fluorescence (*FDH1* expression) as a function of intensity of red fluorescence (*EFG1* expression) is plotted. Each symbol indicates an individual cell. Strains are indicated above each graph.

colonization, a competition experiment was conducted in which BALB/c and Swiss Webster mice were orally inoculated with 1:1 mixtures of a nourseothricin-resistant (nou^R) WT strain and an unmarked strain (either WT, an *efg1*$^-$ null mutant, or the complemented mutant). The WT population contains both high and low *EFG1* expressing cells; mixing with *efg1*$^-$ null mutant cells increases the proportion of low *EFG1* expressing cells. At various times post-inoculation, fecal pellets were collected from mice, homogenized, and plated to determine the number of colony forming units per gram of sample (CFU/g) and the competitive index (CI: the ratio of the unmarked strain over the nou^R strain, divided by their ratio in the inoculum; see Materials and Methods). The *efg1*$^-$ null strain out-competed WT *C. albicans* (CI>1) at early time points (e.g. day 1, Fig. 2-6A, p=0.0001; Mann Whitney test). In comparison, the CI for competition between marked and unmarked WT strains was close to one, and the CI for the complemented strain versus the marked WT strain was intermediate. When colonized mice were sacrificed on day six post-inoculation, the *efg1*$^-$ mutant was found to out-compete WT *C. albicans* in each of the gastrointestinal organs (Fig. 2-6B). At later times post-inoculation, WT *C. albicans* cells began to out-compete the *efg1*$^-$ mutant in fecal pellets. For example, at day 18, the average CI for mutant/WT dropped to below one (Fig. 2-6A; p=0.0003, Mann Whitney test). In GI tract organ homogenates of mice sacrificed at day 18, the CIs were consistent with the CIs measured in fecal pellets (Fig. 2-6B). Thus, at day 18, WT cells were more fit than the mutant. The dynamics of colonization over time are also shown (Fig. 2-7). In summary, when both strains share the same host, the *efg1*$^-$ mutant had an initial colonization advantage over WT *C. albicans*, but was preferentially eliminated at later times during colonization. We

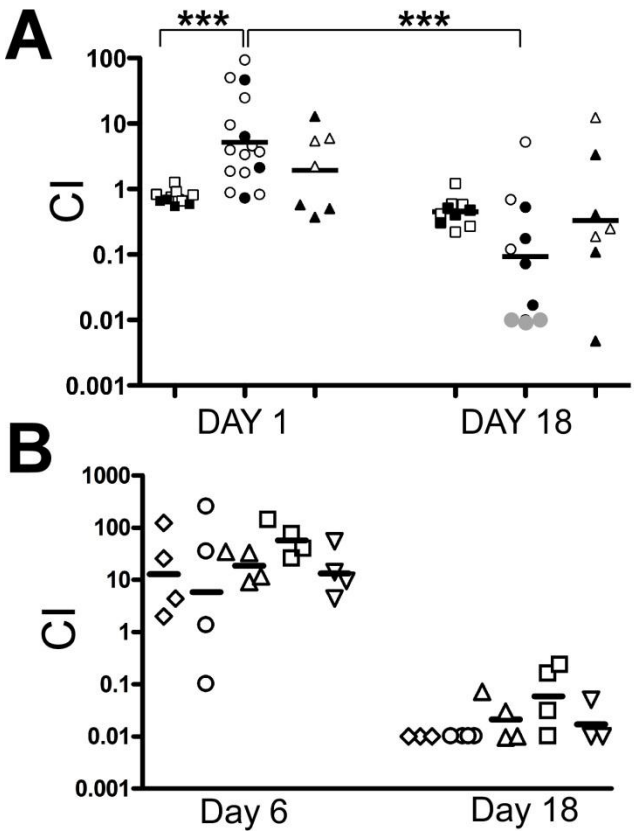


Figure 2-6. *efg1*$^{-}$ cells out-compete wild type *C. albicans* but are preferentially lost over time.

(A) CI measured in fecal pellets at days 1 and 18 post-inoculation. Cells of strain JPY105 (nourseothricin (nou)-resistant WT) were mixed 1:1 with either CKY101 (WT), CKY136 (*efg1*$^{-}$ null) or HLC74 (*EFG1/efg1*$^{-}$ complemented mutant) and inoculated by oral gavage into BALB/c (filled symbols) or Swiss Webster mice (open symbols). Competition data is shown as the competitive index (CI, the ratio of CFU for the unmarked mutant strain and the marked WT strain divided by input ratio). Each symbol represents one mouse; bars are the geometric mean. Squares, nou^{S} WT/nou^{R} WT; circles, *efg1*$^{-}$ strain/nou^{R} WT; triangles, complemented *efg1*$^{-}$ strain/nou^{R} WT. Grey circles shown at CI=0.01 indicate samples with levels of the *efg1*$^{-}$ strain below the limit of detection. *** $p<0.001$ Mann Whitney test. (B) Competition of *efg1*$^{-}$ strain/nou^{R} WT strains in each of the GI tract organs at day 6 and day 18 post-inoculation in Swiss Webster mice. Stomach contents, diamonds; stomach wall, circles; ileum, triangles; cecum contents, squares; cecum wall, inverted triangle.

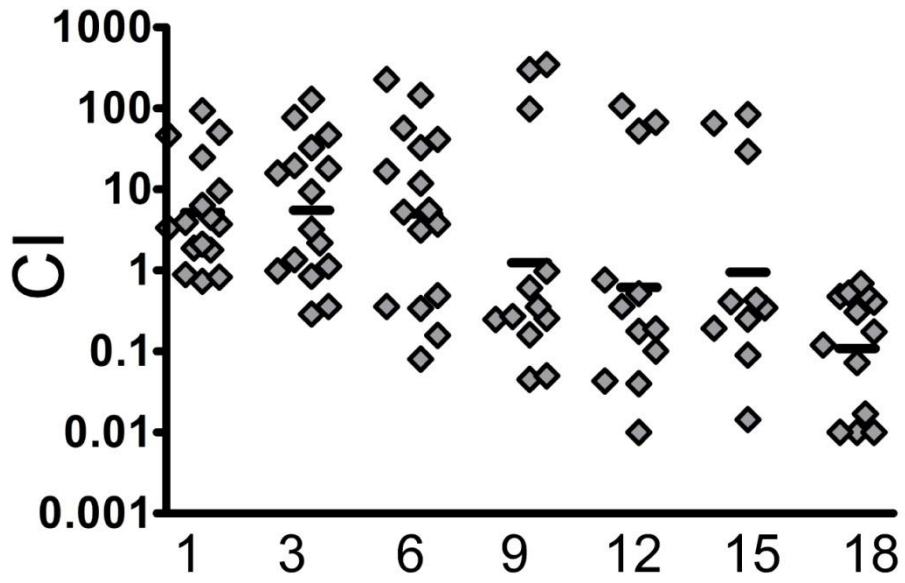


Figure 2-7. Dynamics of competition between *efg1*$^{-}$ cells and wild type *C. albicans.*

CI measured in fecal pellets over time; days post-inoculation are shown on the x-axis. Cells of strain JPY105 (nourseothricin (nou)-resistant WT) were mixed 1:1 with CKY136 (*efg1*$^{-}$ null) and inoculated by oral gavage into BALB/c and Swiss Webster mice. Competition data is shown as the competitive index (CI, the ratio of CFU for the unmarked mutant strain and the marked WT strain divided by input ratio). Each symbol represents one mouse; bars are the geometric mean.

hypothesize that the *efg1*$^{-}$ strain shows increased susceptibility to the host response that develops over time.

Similar results were obtained from single strain colonization of Swiss Webster mice, an outbred mouse model. Following oral gavage, *efg1*$^{-}$ null mutant cells initially hyper-colonized ($p=0.001$, day 1) and continued to colonize at high levels until day 6, but were defective for colonization at day 21 post-inoculation ($p=0.0047$) (Fig. 2-8A). Colonization in the organs at day 21 by the *efg1*$^{-}$ mutant was also defective in the stomach wall ($p=0.00043$) and cecum contents ($p=0.0082$); other organs showed a similar trend (Fig. 2-9). As a control, fecal pellets were also taken at 6 hours post inoculation and mice were sacrificed at this time point to observe the colonization levels in the GI tract organs (Fig. 2-8B). At 6 hours post-inoculation, no difference in colonization was observed between WT and *efg1*$^{-}$ strains in any GI tract organ. This shows that both strains were inoculated at similar levels and differences in colonization were not because of restricted growth in any organ due to pH or other environmental conditions. No defect in growth by the *efg1*$^{-}$ mutant at acidic pH other than a defect in filamentation has been reported [128].

Colonizing WT *C. albicans* cells in the lumen of the GI tract are predominantly in the yeast form ([42] and Table 2-1). Nonetheless, to ensure that CFU measurements accurately reflected cell numbers and were not biased by different levels of filamentation in these strains, *C. albicans* genomes were measured during colonization by quantitative real time PCR (qPCR). A single hypha may contain multiple nuclei [190, 191], yet only produce a single colony when plated. If differences in hyphal formation affected the number of CFUs per sample, a non-linear correlation between CFUs and number of

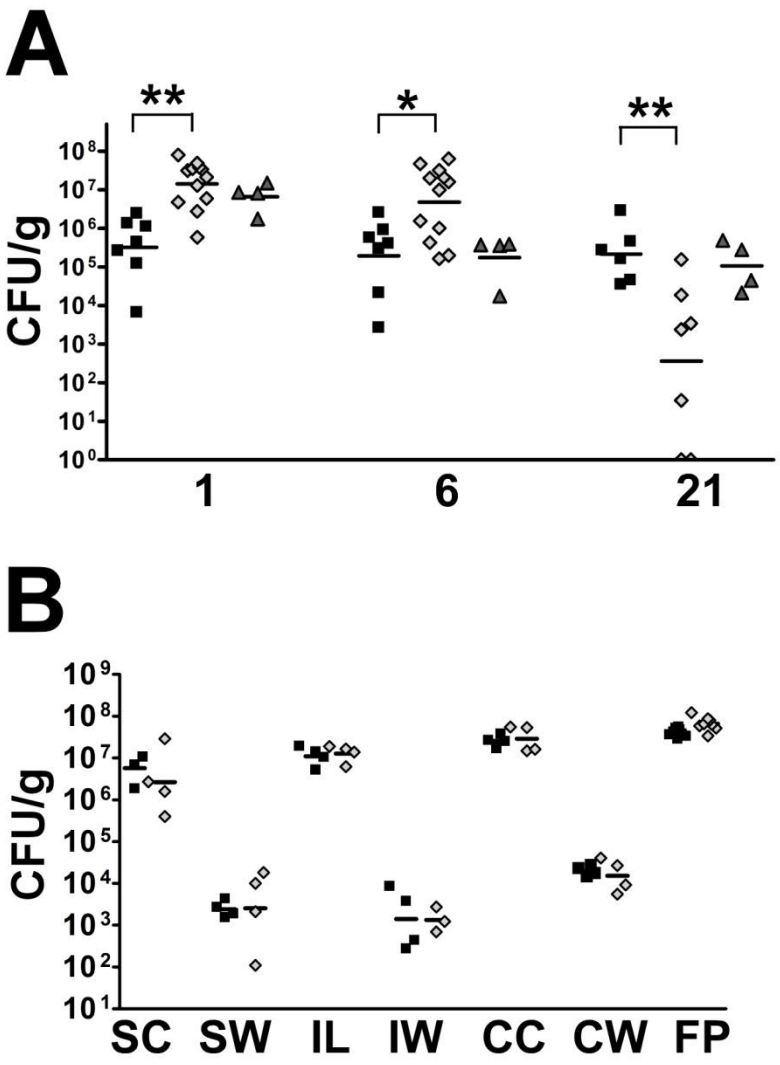


Figure 2-8. Analysis of single strain colonization by *C. albicans* WT and *efg1*$^{-}$ strains.

(A) Colonization over time in Swiss Webster mice by WT (squares), *efg1*$^{-}$ (diamonds) and a complemented p*EFG1*/ *efg1*$^{-}$ mutant (triangles). Days 1, 6, and 21 post-inoculation are shown. (B) Colonization of GI tract organs and fecal pellets measured at 6 hrs. post-inoculation as colony forming units/gram tissue (CFU/g). WT (squares) and *efg1*$^{-}$ (diamonds) strains were inoculated into Swiss Webster mice by oral gavage and colonization measured in the SC (stomach contents), SW (stomach wall), IL (ileum contents), IW (ileum wall), CC (cecum contents), CW (cecum wall), and FP (fecal pellets). Each symbol represents an individual mouse; bars are the geometric mean. *$p<0.05$, **$p<0.01$ Mann whitney test.

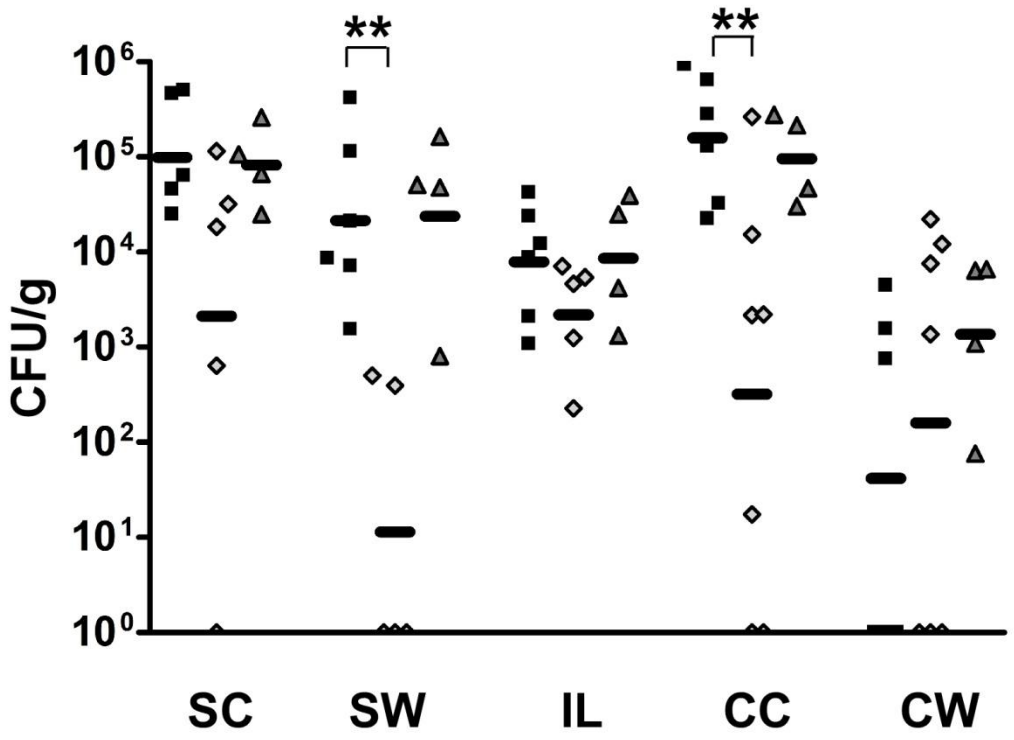


Figure 2-9. Organ colonization by *C. albicans* WT and *efg1*$^-$ strains at day 21.

Colonization over time in Swiss Webster mice by WT (squares), *efg1*$^-$ (diamonds) and a complemented p*EFG1*/ *efg1*$^-$ mutant (triangles) at day 21 post-inoculation. Strains were inoculated into Swiss Webster mice by oral gavage and colonization measured in the SC (stomach contents), SW (stomach wall), IL (ileum), CC (cecum contents), and CW (cecum wall). Each symbol represents an individual mouse; bars are the geometric mean. **p<0.01 Mann whitney test.

Host	D.p.i.	% yeast	% hyphae	Total number of cells observed
BALB/c	3	86.7	13.3	30
BALB/c *nu/nu*	3	78.0	22.0	82
BALB/c	18	78.9	21.9	90
BALB/c *nu/nu*	18	98.2	1.8	57

Table 2-1. Prevalence of yeast and hyphae during GI tract colonization.

BALB/c and BALB/c *nu/nu* mice were orally gavaged with WT *C. albicans* cells expressing GFP promoter fusions. Cells from the ileum and stomach were observed by microscopy to determine the numbers of yeast and hyphae present in each sample. D.p.i., days post-inoculation.

genomes present would be detected. Cecum contents were either plated for CFU/g or used for the isolation of genomic DNA and qPCR. The number of genomes detected correlated well with the CFU (R^2=0.72) (Fig. 2-10). WT samples had lower levels of both CFUs and genomes detected, while *efg1*$^-$ samples had higher levels of both, confirming the conclusion of hyper-colonization by the *efg1*$^-$ mutant.

Results of single strain oral gavage inoculations of BALB/c mice, an inbred mouse strain, with WT, *efg1*$^-$, or the complemented *EFG1*/*efg1*$^-$ strains were consistent with the competition results. No sign of disseminated disease was noted during either competition or single strains colonization experiments: no *C. albicans* were recovered from the tongue, esophagus, liver, or kidney (data not shown). At early times post-inoculation, the *efg1*$^-$ strain exhibited increased colonization levels in fecal pellets compared to WT *C. albicans* (e.g. day 1, Fig. 2-11; $p<0.0001$, Mann Whitney test). In fecal pellets collected at various times post-inoculation, colonization by the *efg1*$^-$ strain remained higher than WT for 6-9 days but then declined. By day 18 post-inoculation, the colonization of the *efg1*$^-$ mutant was no longer above WT levels (Fig. 2-11). Cecum homogenates from mice sacrificed on day 3 or 18 showed results that were consistent with results observed from fecal pellets (Fig. 2-12A and B). Colonization by the complemented strain was intermediate. Intermediate colonization by this strain may be due to effects of having one copy of the gene present instead of two (haplo-insufficiency) or altered expression patterns. The overall trends in colonization were consistent; however, the level of variability in colonization increased over the course of our experiments. This effect may be due to differences between individual mice, such as the composition of the microbiota or the timing of activation of the immune response, as well

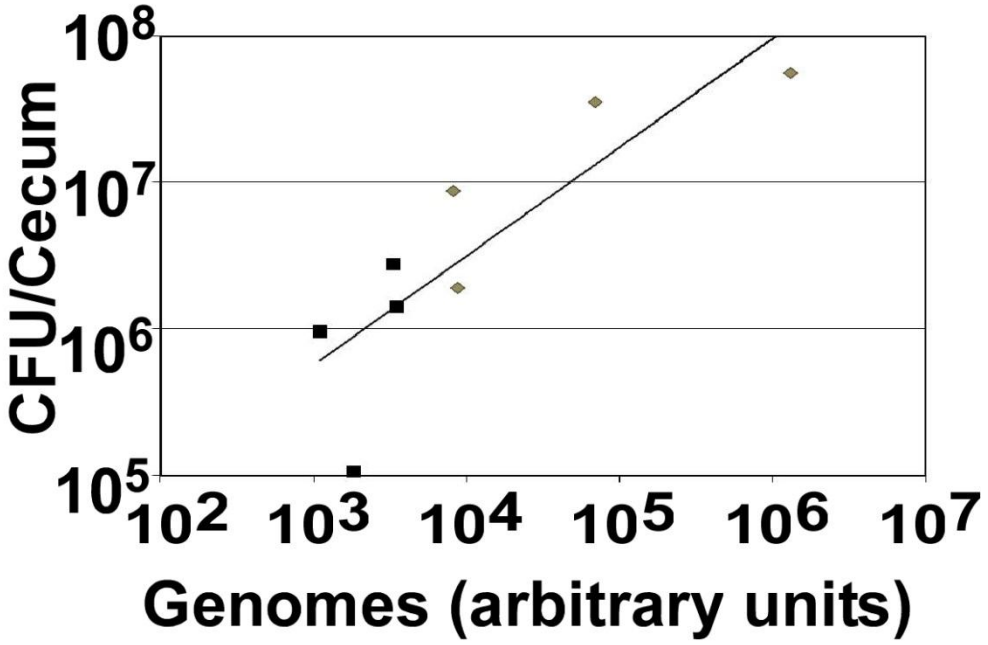


Figure 2-10. Quantification of colonization measured by qPCR.

Cecum contents from BALB/c mice gavaged with WT or *efg1*$^{-}$ null mutant cells were collected at day 3 post-inoculation. One aliquot of cecum contents was plated and another was used for extraction of DNA. The number of CFU is plotted as a function of fungal genomes (in arbitrary units), measured using qPCR.

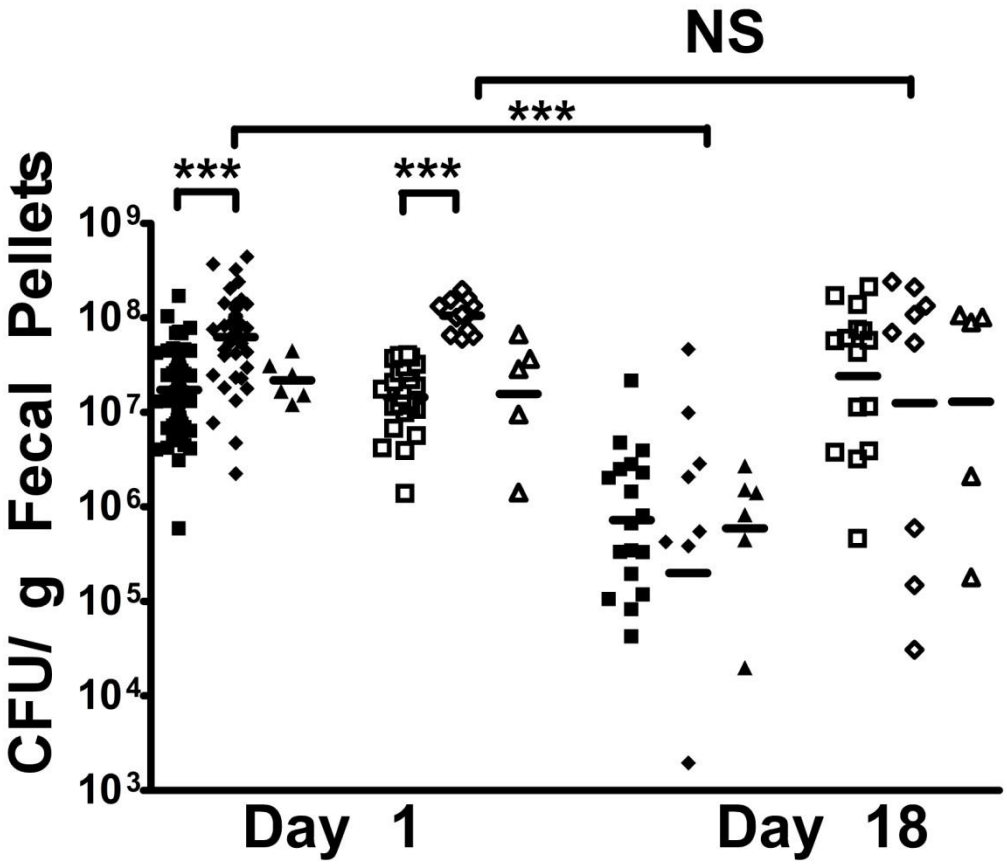


Figure 2-11. ***EFG1*-dependent colonization of healthy and immunocompromised hosts.**

Colonization measured in fecal pellets at days 1 and 18 post-inoculation. Single strains listed above were inoculated by oral gavage into BALB/c mice (closed symbols) or BALB/c *nu/nu* mice (open symbols). Squares, WT *C. albicans*; diamonds, *efg1*$^{-}$ strain; triangles, complemented *efg1*$^{-}$ strain. Each symbol represents an individual mouse and bars are the geometric means. Samples were statistically significant by Kruskal-Wallis test ($p<0.0001$) and Dunn's multiple comparison post-test. *$p<0.05$, ***$p<0.001$ Mann Whitney test for pairwise analysis.

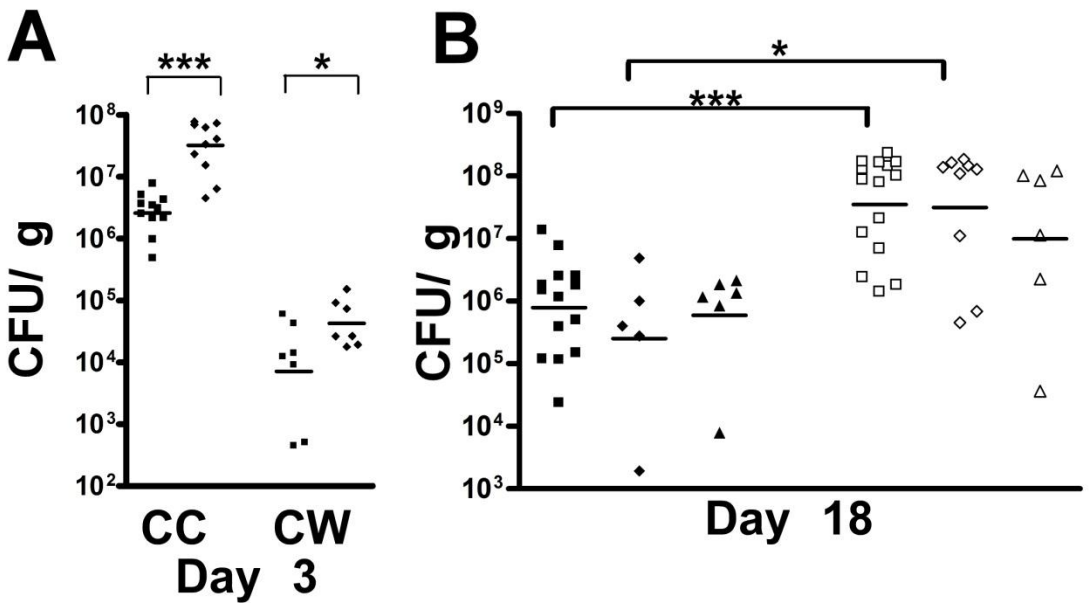


Figure 2-12. Organ colonization by the *efg1*$^{-}$ mutant and WT *C. albicans* in healthy and immunocompromised hosts.

Colonization measured in the organs at days 3 and 18 post-inoculation. Single strains inoculated by oral gavage into BALB/c mice (closed symbols) or BALB/c *nu/nu* mice (open symbols). Squares, WT *C. albicans*; diamonds, *efg1*$^{-}$ strain; triangles, complemented *efg1*$^{-}$ strain. (A) Colonization in GI tract organs of BALB/c mice at day 3 post-inoculation. CC, cecum contents, CW, cecum wall. (B) Colonization of cecum contents in BALB/c and BALB/c *nu/nu* mice at day 18 post-inoculation. *$p<0.05$, ***$p<0.001$ Mann Whitney test for pairwise analysis.

as potentially different levels of *EFG1* activity in each individual [192, 193].

Histological analysis of tissue was used to assess inflammation and tissue invasion during colonization by both WT and *efg1*$^{-}$ strains of *C. albicans* (Fig. 2-13A and B, Table 2-2). No evidence of adhesion or inflammation was detected in tissue samples from the ileum, cecum, tongue, esophagus, kidney or liver by either strain. The only region in which there were detectable levels of inflammation and adhesion was the limiting ridge of the stomach wall, as previously observed [7, 175]. Inflammation and adhesion occurred in multifocal regions, while other areas of the tissue appeared normal. Inflammation, as well as hyperplasia, of varying levels was observed in the lamina propria and submucosa, especially in the area of the limiting ridge. The submucosa also showed evidence of edema in the affected regions. *C. albicans* cells, when observed, were detected in association with the limiting ridge and with exudated inflammatory cells, comprised mainly of neutrophils and, less frequently, lymphocytes.

Very little evidence of inflammation was observed in response to WT *C. albicans* at 3 days post-inoculation and no cells were observed in association with host tissue (Fig. 2-13A and Table 2-2). In the absence of *EFG1*, cells were observed adhering to the stomach wall and produced mild levels of inflammation. At day 6 post-inoculation, inflammation in response of WT *C. albicans* peaked, especially at the limiting ridge (Fig. 2-13A). Large pockets of both yeast and hyphae were observed in the keratin layer in association with neutrophils (Fig. 2-13B). Increased inflammation was also observed in response to the *efg1*$^{-}$ cells, although less prominently at the limiting ridge, and very few adherent cells were observed compared to WT levels (Fig. 2-13A and B). In colonized Swiss Webster mice at day 6 post-inoculation, *efg1*$^{-}$ cells were observed adhering to

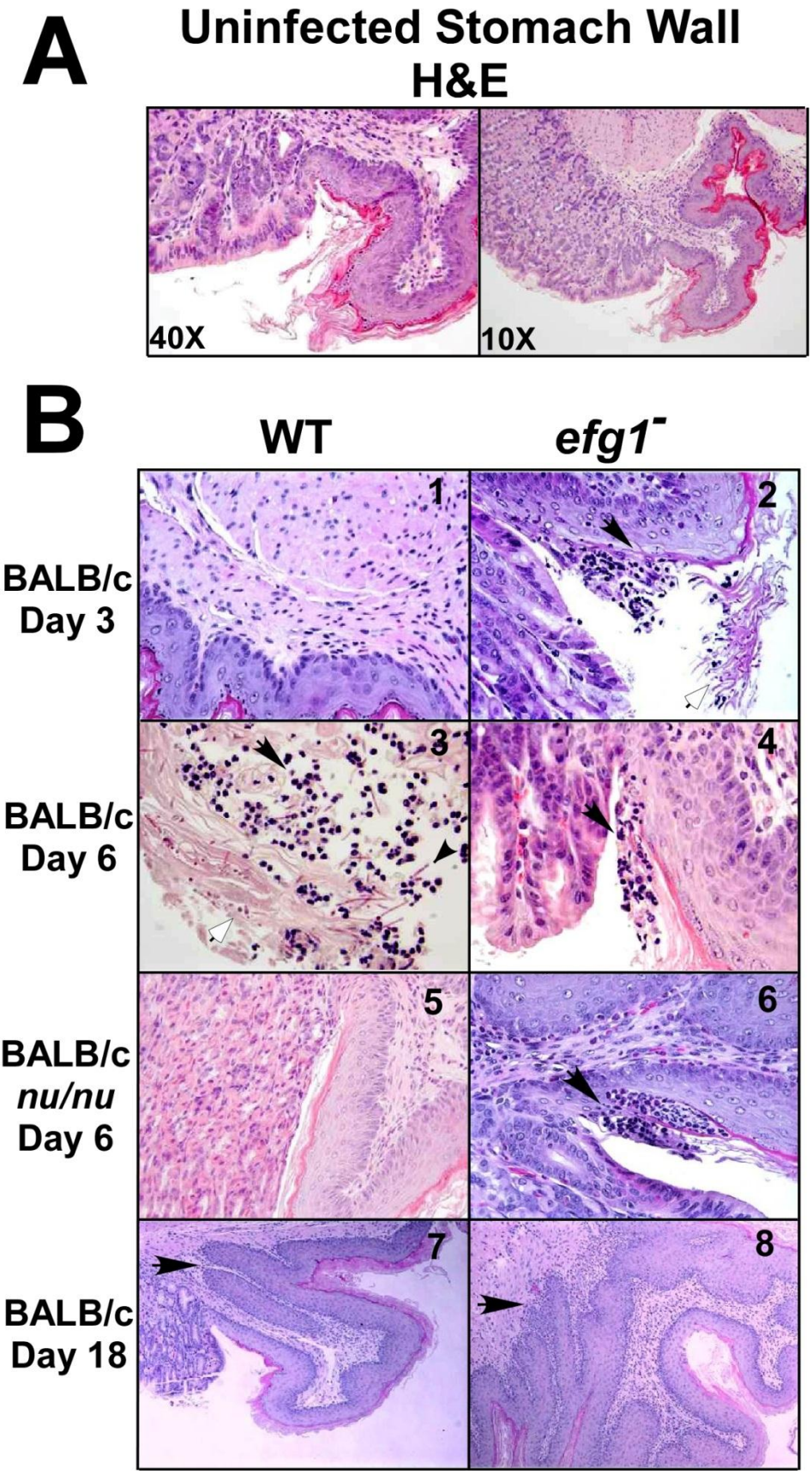
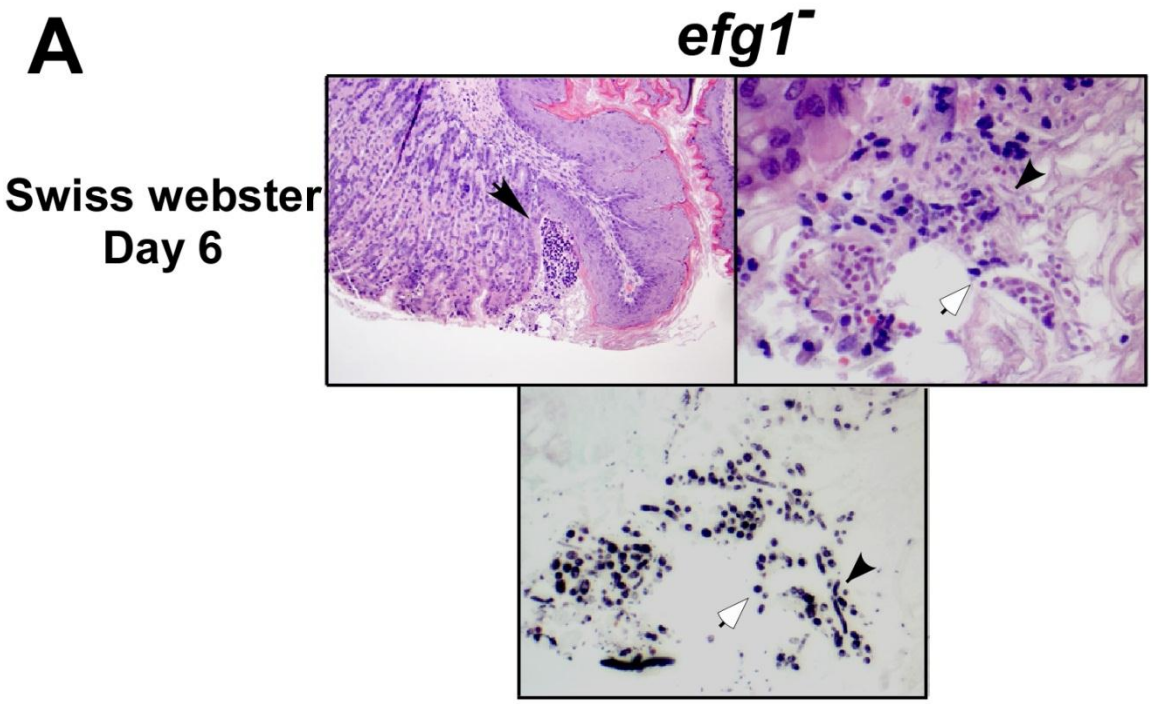

A
Uninfected Stomach Wall
H&E
40X
10X
B
WT
efg1-
BALB/c
Day 3
BALB/c
Day 6
BALB/c
nu/nu
Day 6
BALB/c
Day 18
1
2
3
4
5
6
7
8

Figure 2-13A. Inflammation during GI tract colonization by *C. albicans*.

Stomach wall tissue was taken from BALB/c and BALB/c *nu/nu* mice, fixed, and paraffin sections near the limiting ridge stained with H&E to detect inflammation. (A) Sections were taken from uninfected BALB/c mice. (B) Stomach tissue from mice colonized with either WT or *efg1*$^{-}$ strains. Panels 1, 2: BALB/c day 3, 40X. Panels 3, 4: BALB/c day 6, 40X. Panels 5, 6: BALB/c *nu/nu* day 6, 40X. Panels 7, 8: BALB/c day 18, 10X. Black arrows indicate focal inflammation; white arrows indicate yeast cells; black arrowheads indicate hyphal or pseudohyphal cells.


A
efg1-
Swiss webster
Day 6

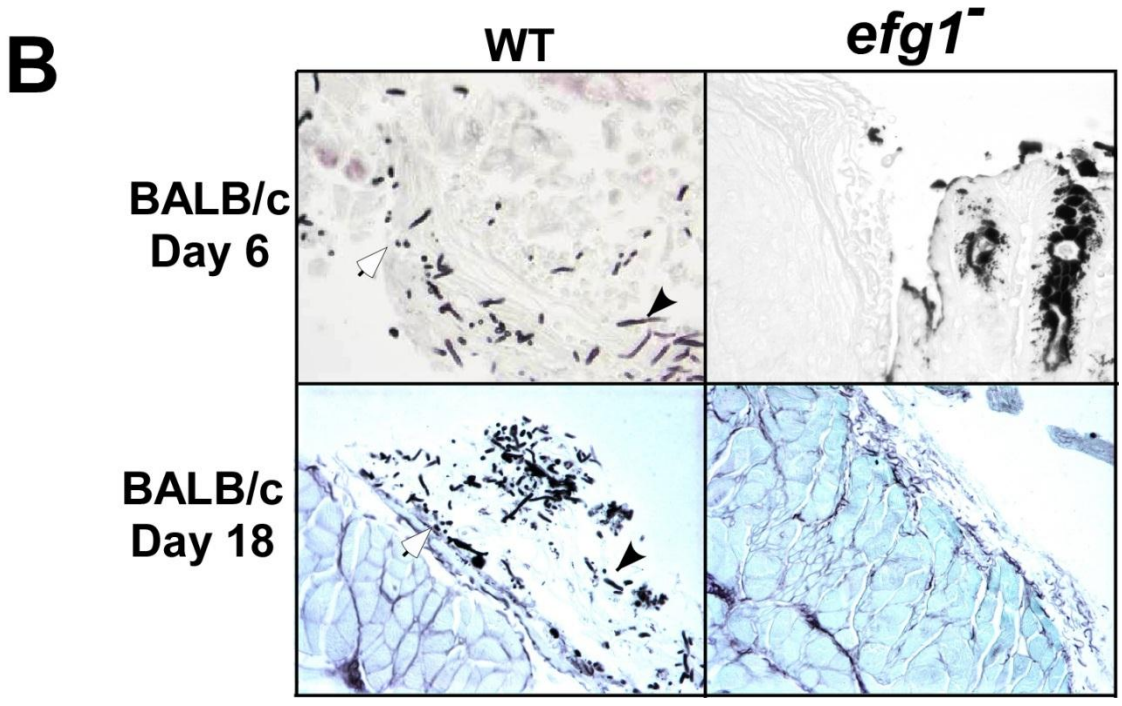

B
WT
efg1-
BALB/c
Day 6
BALB/c
Day 18

Figure 2-13B. Presence of adherent fungi during colonization of the GI tract.

Stomach wall tissue was taken from Swiss Webster and BALB/c mice, fixed, and paraffin sections near the limiting ridge stained with either H&E to detect inflammation or GMS to detect fungi. (A) Tissue from Swiss Webster mice colonized with an *efg1*$^{-}$ strain at day 6 is shown with H&E staining at 10X and 60X, and a 60X GMS image of adherent cells is also shown. (B) BALB/c mice colonized with WT or *efg1*$^{-}$ strains. Upper row: GMS staining at day 6 post-inoculation, 40X. Lower row: GMS staining at day 18, 10X. Black arrows indicate focal inflammation; white arrows indicate yeast cells; black arrowheads indicate hyphal or pseudohyphal cells.

Strain	D.p.i.	Host	N	Fungal burden	Presence of fungi	Forestomach			Limiting Ridge	Glandular stomach	
						Neutrophils	Mono-nuclear cells	Epidermal hyperplasia			
WT	3	BALB/c	3	2.5×10^4	None	1	0	0	0	1	1
efg1$^-$	3	BALB/c	4	3.2×10^4	Yeast, pseudo-hyphae	2	1	2	1	1	0
WT	6	BALB/c	3	4.1×10^4	Yeast, hyphae	3	4	4	4	1	0
WT	6	BALB/c *nu/nu*	4	ND	None	1	1	2	0	1	0
efg1$^-$	6	BALB/c	3	6.4×10^4	Yeast (rare)	3	2	3	1	3	2
efg1$^-$	6	BALB/c *nu/nu*	3	ND	Yeast, pseudo-hyphae	1	0	2	1	1	1
WT	18	BALB/c	4	5.2×10^4	Yeast, hyphae	4	2	4	0	2	2
WT	18	BALB/c *nu/nu*	ND	7.9×10^4	None	ND	ND	ND	2	ND	ND
efg1$^-$	18	BALB/c	4	3.7×10^4	None	1	1	3	0	1	1
efg1$^-$	18	BALB/c *nu/nu*	ND	1.5×10^4	None	ND	ND	ND	1	NA	ND

Table 2-2. Inflammation during GI tract colonization by WT and *efg1*$^-$ *C. albicans* strains.

Histology slides were scored for inflammation and the presence of *C. albicans* associated with the stomach wall using both WT and *efg1*$^-$ strains. Samples were taken at 3, 6, 18 days post-inoculation (D.p.i.) and the number of samples analyzed is shown (N). Fungal burden in the stomach wall at the indicated time points is shown as well. The presence of associated fungi is indicated as either none, yeast, pseudohyphae, or true hyphae. Inflammation was scored in the forestomach, limiting ridge, and glandular stomach (specific information on neutrophils, mononuclear cells, and hyperplasia is noted in specific regions; ND indicates not determined). Inflammation was multifocal, and the most severe region was analyzed. Inflammation score: 0, none; 1, slight; 2, mild; 3, moderate; 4, severe.

stomach tissue in association with inflammatory exudate (Fig. 2-13B). More adhesion by the *efg1*$^{-}$ mutant was evident at day 6 in Swiss Webster mice compared to BALB/c mice, although this may be due to the focal nature of the lesions. The level of inflammation between the Swiss Webster and BALB/c mice was similar in response to the *efg1*$^{-}$ mutant. Inflammation decreased over time at day 18 post-inoculation in BALB/c mice, but high levels of neutrophils remained in the forestomach of mice colonized with WT *C. albicans* (Fig. 2-13A). In mice colonized with either WT or *efg1*$^{-}$ strains, there was evidence of severe epidermal hyperplasia at this time (Fig. 2-13A).

These results show that colonization by *C. albicans* produced an inflammatory response in which neutrophils and mononuclear cells were present at sites of adhesion by *C. albicans*. Inflammatory cells were extruded into the lumen of the stomach after 6 days post-inoculation. The *efg1*$^{-}$ mutant produced an earlier inflammatory response and *efg1*$^{-}$ cells were observed to be adherent earlier than WT *C. albicans*. Over time, evidence of the immune response remained, although it appeared less acute. These data support our hypothesis that the decline in *efg1*$^{-}$ mutant levels of colonization is due to activation of the immune response during colonization.

To test the role of the host immune response, colonization was studied in BALB/c *nu/nu* mice, which are athymic and T cell deficient. In these mice, colonization by both WT *C. albicans* and *efg1*$^{-}$ null mutants remained high at later times post-inoculation, consistent with previous results [7]. The *efg1*$^{-}$ strain hyper-colonized both BALB/c and BALB/c *nu/nu* mice at one day post-inoculation (Fig. 2-11) indicating this phenotype is independent of the immune response. At day 18 post-inoculation, colonization levels of the *efg1*$^{-}$ null mutant in the fecal pellets (Fig. 2-11; $p=0.0002$, Mann Whitney test) and

cecum (Fig. 2-12B) of *nu/nu* mice were significantly higher than in BALB/c mice. Therefore, the hyper-susceptibility of the *efg1*$^{-}$ strain to the immune response was not observed in this immunodeficient mouse.

In the absence of T cells, a much lower level of inflammation was observed after 6 days in response to both WT and *efg1*$^{-}$ C. albicans (Fig. 2-13A). After 18 days, there was a moderate increase in inflammation in response to both WT and *efg1*$^{-}$ strains (Table 2-2). *C. albicans* did not disseminate or cause systemic disease in either BALB/c or BALB/c *nu/nu* mice (data not shown). T cells are thus required for the observed mucosal inflammatory response during colonization by *C. albicans*.

To test the importance of possible differences in microbiota, in two experiments, BALB/c and BALB/c *nu/nu* mice were co-housed for two weeks prior to inoculation with *C. albicans*. This treatment results in sharing of the microbiota and can alter the susceptibility of a mouse to colonization by some microbes [194, 195]. Co-housing did not alter the colonization dynamics of WT *C. albicans* (Fig. 2-14) suggesting that differences in colonization between BALB/c and *nu/nu* mice are not dependent on historical differences in their microbiota.

In summary, in the absence of Efg1p, *C. albicans* hyper-colonized the GI tract at early time points post-inoculation. In a healthy mouse but not an immunodeficient mouse, this phenotype was unsustainable and the mutant was less fit than WT *C. albicans* at later times post-inoculation. These results suggest that, depending on the strength of the host response, selection during colonization will affect the overall Efg1p activity in the colonizing population. In this model, the differential sensitivity of individual cells in a heterogeneous population with cell-to-cell variation in Efg1p activity allows the

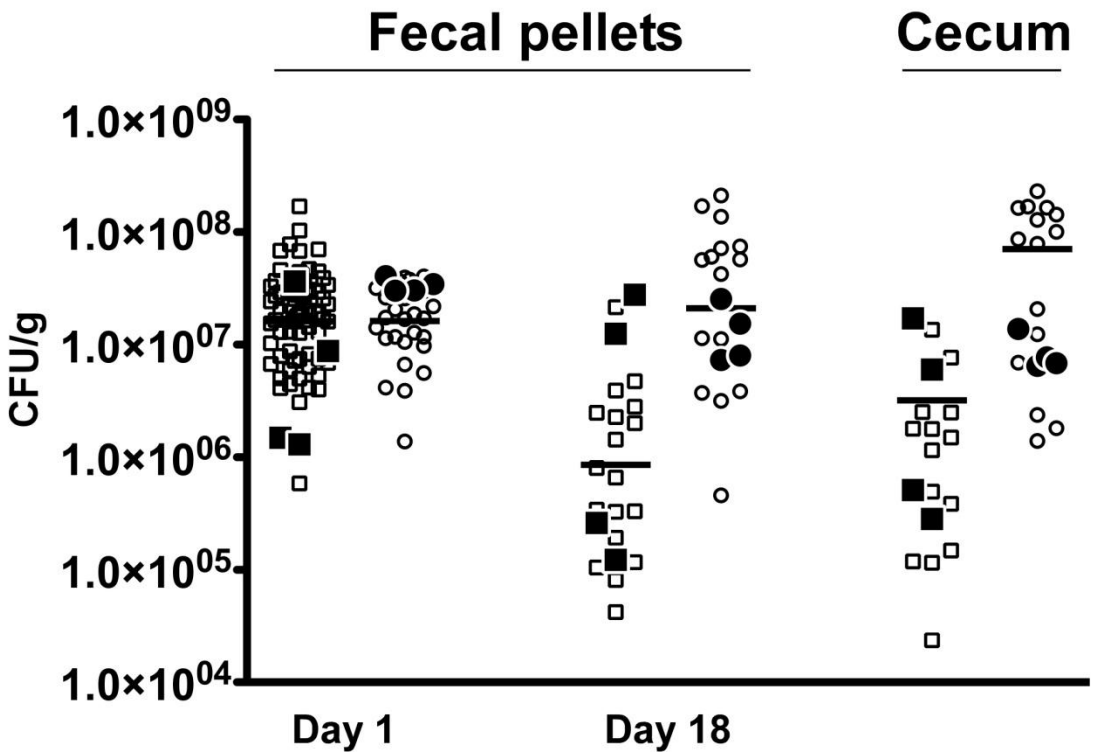


Figure 2-14. Colonization after cohousing BALB/c and BALB/c *nu/nu* mice.

BALB/c (closed squares) and BALB/c *nu/nu* (closed circles) mice were co-housed and then inoculated with wild type *C. albicans* by oral gavage. Results obtained with mice that were not co-housed (BALB/c, open squares and BALB/c *nu/nu*, open circles) are shown for comparison. Fecal pellets were collected and plated at days 1 and 18 post-inoculation; colonization in cecum contents at day 18 is also shown. Each symbol represents an individual mouse and the bars are the geometric mean.

population to respond to host status.

Differential *EFG1* expression during GI colonization of different hosts

The model predicts that, during colonization of different hosts, the average expression of *EFG1* would differ because the host acts differentially on individual *C. albicans* cells with either high or low *EFG1* expression. To test this prediction, we measured the levels of *EFG1* transcript expressed by *C. albicans* cells during GI colonization. WT *C. albicans* cells were isolated from the lumen of the cecum or ileum of WT BALB/c mice. For comparison, *C. albicans* cells were grown in laboratory media under the conditions used to prepare the inoculum that was given to the mice so that changes due to host environmental signals, including hypoxia and pH, could be detected. Quantitative real time RT-PCR (qRT-PCR) was used to measure the level of *EFG1* transcript normalized to the transcription of the actin gene, *ACT1*. In the cecum at three days post-inoculation, expression of *EFG1* was lower than in laboratory grown cells (Fig. 2-15A; p=0.0087, Mann Whitney test). However, in the cecum at eighteen days post-inoculation, *EFG1* expression was similar to expression in laboratory-grown cells (Fig. 2-15A) Expression of *EFG1* in the ileum was consistent with this trend (Fig. 2-15B). Therefore, *EFG1* transcript levels were low during the initial adaptation of *C. albicans* to the GI tract and increased over time.

To determine whether the expression of *EFG1* was different in an immunocompromised host, *EFG1* transcript levels were measured during colonization of T-cell deficient BALB/c *nu/nu* mice. *EFG1* transcript levels were again found to be low at three days post-inoculation relative to levels in laboratory-grown cells (Fig. 2-15A, B;

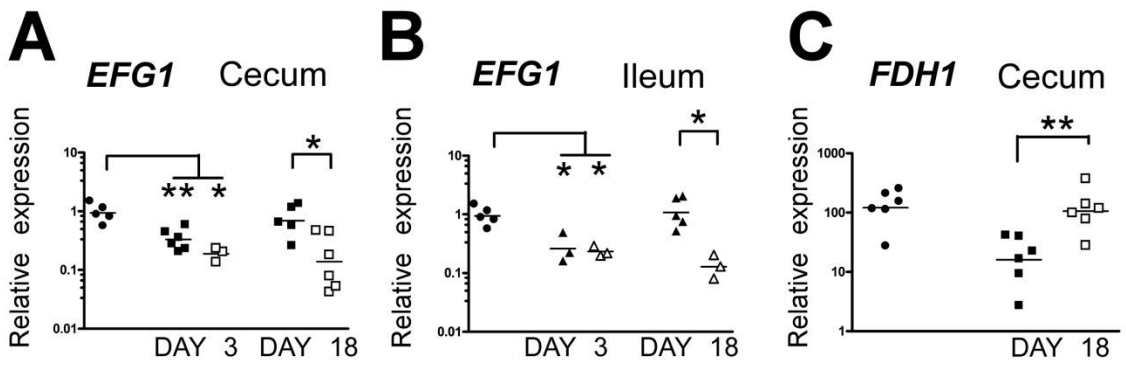


Figure 2-15. Transcription and activity of *EFG1* differ during colonization of healthy and immunocompromised hosts.

Cells of WT strain CKY101 was grown in laboratory culture in rich media or recovered from organs of BALB/c (closed symbols) or BALB/c *nu/nu* mice (open symbols) on day 3 or 18 post-inoculation. Expression of *EFG1* (A, B) or *FDH1* (C) was measured by qRT-PCR, normalized to *ACT1* levels and expressed relative to expression in laboratory-grown exponential phase cells. Each symbol represents a sample pooled from 2 (cecum contents) or 4 mice (ileum contents). For laboratory-grown cells, each symbol represents an independently grown culture. Closed circles, laboratory-grown post-exponential phase cells; triangles, ileum samples; squares, cecum samples. Bars represent the geometric mean; * indicates $p<0.05$; ** $p<0.01$; Mann Whitney test.

p=0.0357 for both organs, Mann Whitney test), showing that the initial down-regulation of *EFG1* during colonization does not require the presence of T cells. However, in BALB/c *nu/nu* organs at eighteen days post-inoculation, *EFG1* transcript levels remained low. At this time point, *EFG1* transcript levels in fungi colonizing *nu/nu* mice were significantly lower than levels in fungi colonizing the ceca (p=0.0173; Mann Whitney test) and ileum (p=0.0357; Mann Whitney test) of BALB/c mice. Thus, colonizing populations of *C. albicans* in immunocompetent and immunocompromised hosts differed in expression of *EFG1*. Since Efg1p is a transcriptional regulator of a large number of genes [111, 135, 137, 139, 144], many differences in gene expression are expected in response to changes in *EFG1* expression.

To test the hypothesis that Efg1p was differentially active in cells colonizing different hosts, transcription of *FDH1* was studied. In the cecum at day 18 post-inoculation, *FDH1* expression was higher in *nu/nu* mice than in BALB/c mice (Fig. 2-15C; p=0.0087, Mann Whitney test). Additionally, in the absence of *EFG1*, *FDH1* levels increased in BALB/c mice at day 18 (Fig. 2-2B). Thus, *EFG1* transcription and activity as a negative regulator differed between *C. albicans* cells colonizing either healthy or T cell deficient hosts, consistent with the proposed model.

Preventing low *EFG1* expression reduces colonization

To test the hypothesis that cells with low expression of *EFG1* are important for establishing colonization, we constructed a strain in which the promoter of the actin gene (*ACT1pr*) was placed upstream of the *EFG1* ORF. This construct was integrated at an exogenous locus in the genome of wild type *C. albicans*, resulting in a strain with the two

endogenous copies of *EFG1* under the control of the native promoter, as well as a third copy driven by the *ACT1* promoter. Expression of *EFG1* from the *ACT1* promoter produced cells which were mainly in the yeast form during growth in rich media. At day 3 post-inoculation of BALB/c mice, expression of *EFG1* was approximately 10-fold higher in the strain carrying the *ACT1pr-EFG1* construct than in WT *C. albicans* ($p<0.0001$) (Fig. 2-16A). Colonization by this strain was reduced at three days post-inoculation in both the fecal pellets and cecum contents of BALB/c mice ($p<0.0001$, fecal pellets; $p=0.0011$, cecum contents) (Fig. 2-16B). Colonization remained lower than wild type over time as well and showed a similar trend the stomach wall ($p=0.0344$), cecum wall ($p=0.0159$), and cecum contents ($p=0.0393$) (Fig. 2-16C). These results show that low Efg1p activity cells support higher levels of initial colonization, and that blocking the formation of these cells results in sub-optimal levels of colonization.

Recapitulation of *C. albicans* colonization dynamics with a mixture of *efg1*$^-$ null cells and *EFG1*-constitutive cells

The differences in *EFG1* expression between *C. albicans* cells colonizing WT and immunodeficient mice showed that colonizing *C. albicans* populations differ under various circumstances. These differences could arise through regulated changes in *EFG1* transcription. In addition, the effects of host selective pressure exerted on colonizing cells with low Efg1p activity could provide a mechanism for producing differences in colonizing populations. To test whether the host was capable of changing the composition of a colonizing population, we created a heterogeneous population by mixing two strains that were unable to regulate their expression of *EFG1*. The *ACT1pr-*

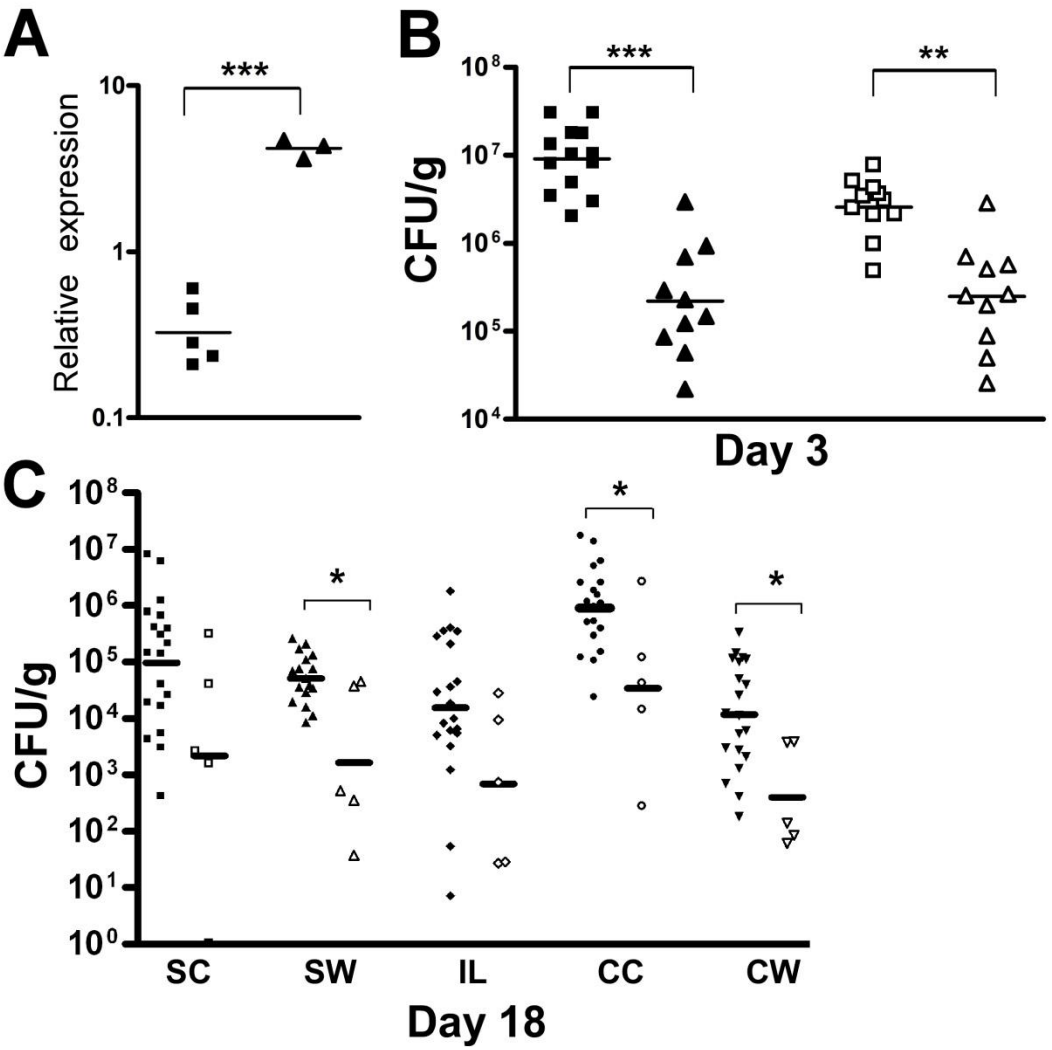


Figure 2-16. Over-expression of *EFG1* causes a decrease in colonization.

(A) BALB/c mice were inoculated by oral gavage with strains CKY101 (WT) or JPY104 (*ACT1pr-EFG1*). Expression of *EFG1* in cells recovered from the cecum at day 3 post-inoculation measured by qRT-PCR, normalized to *ACT1* expression and shown relative to expression in laboratory-grown exponential phase cells. Each symbol represents a sample pooled from 2 mice. Squares, WT *C. albicans*; triangles, *ACT1pr-EFG1* strain. ***$p<0.001$ Student's t test. (B) Colonization of BALB/c mice with either WT (squares) or *ACT1pr-EFG1* strains (triangles) in the fecal pellets (filled in symbols) or cecum contents (open symbols) at day 3 post-inoculation. Symbols represent an individual mouse; bars are the geometric mean. (C) Day 18 organ colonization. WT, closed symbols, *ACT1pr-EFG1*, open symbols. SC, stomach contents; SW, stomach wall; IL, ileum; CC, cecum wall; CW, cecum wall.

EFG1 fusion strain was mixed 1:1 with a drug resistant mutant lacking *EFG1* and the artificial heterogeneous population was orally gavaged into BALB/c mice.

Initially, the *efg1*$^{-}$ strain out-competed the constitutively-expressing *EFG1* strain (Fig. 2-17A). However, over time, the *ACT1pr-EFG1* strain eventually out-competed the *efg1*$^{-}$ mutant (Fig. 2-17A), even though the *ACT1pr-EFG1* strain colonized worse than WT cells when inoculated singly into BALB/c mice (Fig. 2-16B). At day 18 post-inoculation, the *ACT1pr-EFG1* strain out-competed the *efg1*$^{-}$ strain throughout the GI tract (Fig. 2-17B). These results show clearly that changes in the composition of the colonizing population can occur in the absence of changes in *EFG1* expression in individual cells. Thus, the effects of the host response on the makeup of the population promote the ability of a colonizing *C. albicans* population to change its pattern of gene expression in response to host immune status.

Discussion

The results presented in this communication demonstrate that over time, *C. albicans* populations colonizing healthy or compromised hosts became different from each other in terms of gene expression. The differences in colonizing cells occurred despite the fact that tissue invasion and fungal dissemination were not observed in T cell deficient mice under the conditions of these experiments. Alterations in the expression of *EFG1* and the increased colonization levels seen in T cell deficient mice may represent a first step towards the initiation of disease, which could occur if the host were to become further compromised. Other models which permit invasion and disseminated disease

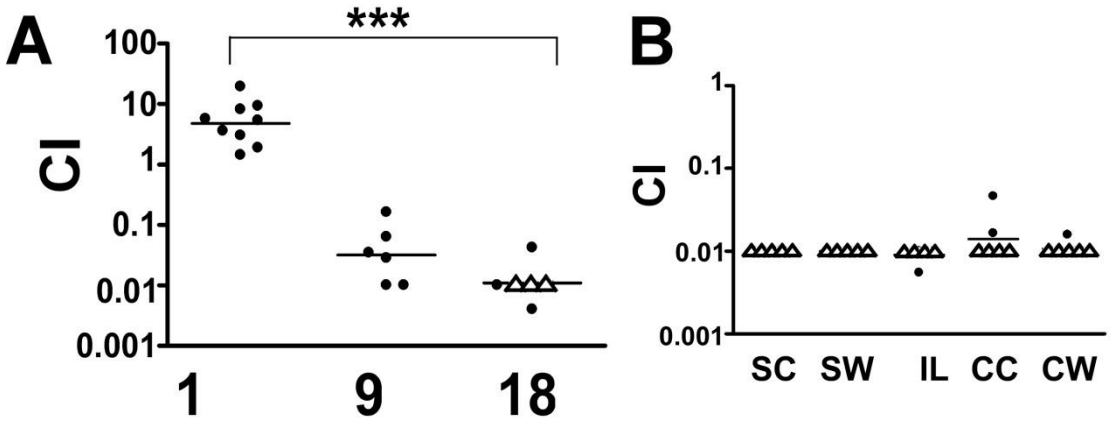


Figure 2-17. Cells with forced high expression of *EFG1* out-compete *efg1*$^{-}$ cells over time.

(A) CI determined in the fecal pellets at day 1, 9, and 18 post-inoculation. JPY106 (*efg1*$^{-}$ nou^{R}) and JPY104 (*ACT1pr-EFG1* cells) were co-inoculated into BALB/c mice at a 1:1 ratio by oral gavage. Competitive index (CI, ratio of the *efg1*$^{-}$ strain / *ACT1pr-EFG1* strain divided by the input ratio) is shown. Each symbol represents one mouse; bars are the geometric mean. Open triangles shown at CI=0.01 indicate samples with levels of the *efg1*$^{-}$ strain below the limit of detection. (B) CI in organ homogenates at day 18 post-inoculation. JPY106 (*efg1*$^{-}$ nou^{R}) and JPY104 (*ACT1pr-EFG1* cells) were co-inoculated into BALB/c mice at a 1:1 ratio by oral gavage. Competitive index (CI, ratio of the *efg1*$^{-}$ drug resistant strain / *ACT1pr-EFG1* sensitive strain divided by the input ratio) is shown. Each symbol represents one mouse; bars are the geometric mean. Open triangles shown at CI=0.01 indicate samples with levels of the *efg1*$^{-}$ strain that were below the limit of detection. SC, stomach contents; SW, stomach wall; IL, ileum; CC, cecum contents; CW, cecum wall. *$p<0.05$, **$p<0.01$ Mann whitney test.

from the GI tract would allow further study of the progression from colonization to pathogenesis.

Results also showed that Efg1p is an important regulator of *C. albicans* colonization. The homologue of *EFG1*, *EFH1*, was previously shown to be a pro-colonization factor as it is up-regulated in the GI tract, but not during oropharyngeal candidiasis, and is required for persistence in the GI tract [42]. In contrast, *EFG1* is required for both colonization and pathogenesis [107]. This suggests that *C. albicans* has adapted its gene expression patterns to reflect these alternate lifestyles. While some genes may be specific colonization factors or virulence factors, others may confer the ability to switch between commensalism and pathogenesis. Since Efg1p is a transcriptional regulator of a large number of genes [111, 137], many differences in gene expression are expected in response to changes in *EFG1* expression.

Previous observations showed that fungal cells colonizing the GI tract as commensals exhibited yeast cell morphology and expressed adhesins and other virulence factors [42, 177]. Expression of many of these factors was Efg1p-independent in the GI tract [42], in contrast to their Efg1p-dependent expression under laboratory conditions [111, 137]. Therefore, in the GI tract, WT cells of both morphologies and cells with low Efg1p activity are expected to express factors that allow them to interact with host epithelial cells.

We propose that WT cells with low Efg1p activity, arising due to natural variation in Efg1p activity from cell to cell, provide a mechanism that allows the colonizing population to respond to host immune status (Fig. 2-18). Upon entry into a naïve or compromised host, low Efg1p activity cells promote growth to higher levels. Over time

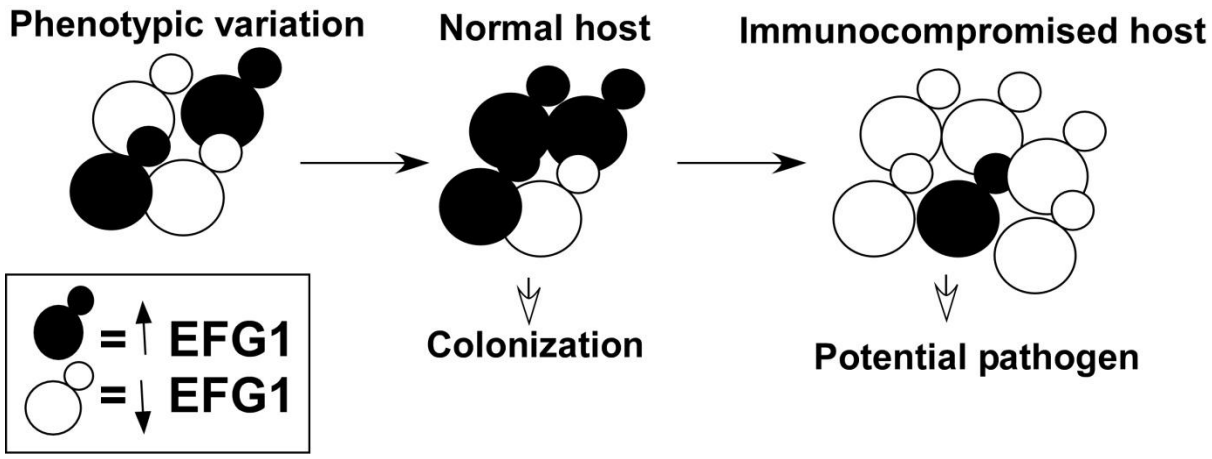


Figure 2-18. Model of *C. albicans* population dynamics.

Model of colonization dynamics based on variation in Efg1p activity in healthy and immunocompromised hosts. Normal growth in the absence of selective pressure produces a population composed of cells with both high and low Efg1p activity. In a healthy host, cells with low Efg1p activity are eliminated by the immune response, allowing cells with high Efg1p activity to predominate and produce a population adapted for colonization. When a host becomes immunocompromised, low Efg1p activity cells are able to grow to a higher population density and may produce a more pathogenic population.

in a healthy host, selective pressure leads to preferential loss of low Efg1p activity cells and an increased proportion of cells with higher Efg1p activity. Cells with high levels of *EFG1*, as in the *ACT1pr-EFG1* fusion strain, colonize poorly but may have altered capacities for invasion or virulence.

Populations differing in levels of Efg1p activity that arise in different hosts may differ in their propensity to become pathogenic. Some evidence suggests that cells lacking Efg1p are more likely to initiate invasion of host tissue and to escape from the GI tract than WT cells. The *efg1*$^{-}$ mutant was shown to exhibit enhanced escape from the GI tract and colonization of mesenteric lymph nodes in mice stressed by hypoxia [114]. Additionally, cells lacking Efg1p adhere to tissues in the murine stomach [33, 35] and, in our studies, adhered earlier than WT *C. albicans.* Therefore, accumulation of low Efg1p activity cells in the GI tract may establish a population which is poised to produce invasive lesions and disseminate in response to further stress upon the host.

In our model, we propose that under all conditions, the colonizing population is heterogeneous. Thus, if a change in host status occurs, diminishing the selective pressure on the population, a higher representation of low Efg1p activity cells would result, poising the population to initiate pathogenic interactions. We use the term 'immunosensing' to describe the ability of colonizing *C. albicans* populations to change in response to host status [166].

In humans, *C. albicans* colonization is typically established in infancy [167, 196]. By analogy with the results presented above, it is likely that colonizing populations in human infants exhibit low Efg1p activity due to an immature immune system and microbiota. This fact may contribute to the incidence of *Candida* disease (oral thrush,

diaper rash) in infants. In human adults, Efg1p activity in colonizing *C. albicans* cells is likely to be high, unless the person becomes immunocompromised. If the host's ability to eliminate fungal cells with low Efg1p activity becomes deficient, the composition of the colonizing population will change, potentially setting the stage for the development of candidiasis. The accumulation of low Efg1p activity cells may thus represent an early warning sign that a particular person is at high risk for development of disease. In conclusion, the findings presented in this communication show that changes in Efg1p activity underlie one of the most important capabilities of *C. albicans* as a commensal and opportunistic pathogen: the ability to adjust its physiology depending on the status of its host.

Materials and Methods

Strains: *C. albicans* strains used in this study are listed in Table 2-3. A CAI-4 strain carrying two copies of chromosome 1 was used. Strains CAI-4 and HLC67 were kindly provided by Drs. W. Fonzi and G. Fink respectively. *E. coli* strains XL1-Blue or DH5alpha were used for propagating plasmids. The promoter regions of the *EFG1* and *DRG1* genes were amplified and cloned upstream of the genes encoding either yEGFP [185] or mCherry [189], while the *FDH1* promoter was cloned upstream of a ubiquitin-Green fluorescent protein gene fusion. For expression of *EFG1* from the *ACT1* promoter, the *EFG1* ORF was cloned onto plasmid Clp10 ACT1p-gLUC59 [197, 198].

Plasmids: GFP constructs were constructed by amplifying the promoter regions of the *EFG1*, *FDH1*, and *DRG1* genes with primers described in Table 2-4. *EFG1* and *DRG1* promoter fragments were digested with AscI and SacII and cloned into vector pLIB2, a pLIB1 [115] derivative with a larger polylinker. The *FDH1* promoter fragment was digested with AscI and SacII and cloned into vector pLIB3, a pLIB2 derivative that contains a fusion of *C. albicans* ubiquitin at the N-terminus of yEGFP (diagrammed in Figure 2). The fusion is based on the construct previously described [188]. Each plasmid was linearized by digestion with a restriction enzyme that cuts uniquely within the cloned promoter region: SapI for *DRG1pr*-GFP, Tth111I for *EFG1pr*-GFP, and PacI for *FDH1pr*-uGFP. Constructs were transformed into either CAI-4 or HLC67 using *URA3* as the selectable marker and proper integration was confirmed by PCR.

For double labeled strains, the *EFG1* promoter fragment was cloned upstream of the gene encoding mCherry [189] in vector pCherS. pCherS carries the mCherry ORF flanked upstream by a polylinker and 10 bp from the *ACT1* 5' UTR and downstream by

the *ADH1* (orf19.3997) 3' UTR. This fragment is cloned in the SAT placer [199], conferring nourseothricin resistance.

To construct a strain in which *EFG1* expression showed altered regulation, the Clp10 ACT1p-gLUC59 plasmid [197] was digested with HindIII and NheI and the g*LUC-PGA59* fusion was replaced with the *EFG1* ORF downstream of the actin promoter (*ACT1*pr). The plasmid was linearized with StuI and transformed into strain CAI-4 using *URA3* as the selectable marker. Proper integration at the *RPS1* locus was confirmed by PCR.

Drug resistant wild type or *efg1*$^{-}$ null strains were constructed by digesting the plasmid iSAT [42] with BsgI and transforming into CAI-4 or HLC67 using *URA3* as the selectable marker. The resulting strains (JPY105 and JPY106) are resistant to nourseothricin on plates containing sucrose as the carbon source.

Media and Growth conditions: YPD (1% yeast extract, 2% peptone, 2% glucose) and YPS (1% yeast extract, 2% peptone, and 2% sucrose) were used as rich growth media. Minimal CM media lacking uracil was made as previously described [198]. Cells from the GI tract were plated on YPD agar plus streptomycin (100ug/ml) and ampicillin (50ug/ml) (YPD-SA). YPD-SA plates were replica plated onto YP-sucrose plates with nourseothricin (200ug/ml) for competition experiments. Log phase cells were grown to an OD=1 in YPD at 30°C; post-exponential phase cells were grown from a single colony for 24 hrs. in YPD at 37°C.

Animal models: All experiments were done in compliance with regulatory guidelines defined by Tufts University IACUC committee. Female 18-20g Swiss Webster mice (Charles Rivers Laboratories), or 5-7week old BALB/c and BALB/c *nu/nu*

mice (NCI) were treated with antibiotics (tetracycline 1mg/ml, streptomycin 2mg/ml, gentamycin 0.1mg/ml) as described [42]. Mice were tested for fungal contamination prior to each experiment. Mice were inoculated with 0.1ml *C. albicans* cells at $5x10^8$ cells/mL by oral gavage. Colonization was tested over time by collecting fresh fecal pellets and plating homogenates on YPD agar plus streptomycin (100ug/ml) and ampicillin (50ug/ml) (YPD-SA). Mice were sacrificed at various time points post-inoculation to determine levels of colonization in cecum contents, cecum wall and ileum homogenates. Combined results from at least 2 experiments are shown for all data. Homogenates of kidney, liver, and tongue were plated on YPD-SA. No colonization of these organs was detected in any experiment. Mann Whitney, Kruskal-Wallis, and Student's t tests were done using Graphpad Prism software with $p<0.05$ as the cutoff for significance.

For competition experiments, colonies on YPD-SA plates were replica plated onto YP-sucrose-nourseothricin and the ratio of drug sensitive to drug resistant cells was measured. The competitive index= CFU *efg1*$^-$/CFU WT or *ACT1pr-EFG1* at time x, divided by CFU *efg1*$^-$/CFU WT or *ACT1pr-EFG1* in the input. For *efg1*$^-$/WT competitions, the WT strain was nou^R. For *efg1*$^-$/ *ACT1pr-EFG1* competitions, the *efg1*$^-$ strain was nou^R.

Tissues samples for histology were preserved in 10% formalin before being embedded in paraffin for sectioning by the Tufts Division of Laboratory Animal Medicine. Samples were stained by hematoxylin and eosin (H&E) and Gomori's Methenamine silver stain (GMS) to observe both inflammation and fungi. Inflammation

scoring and photography was performed in part by Lauren Richey, who was blinded during the scoring.

Microscopy: To determine fluorescence, strains were grown to post-exponential phase at 37°C in YPD for 24hrs. Cells were resuspended in a 1mg/ml solution of Calcofluor white for 5 min., and then washed twice in PBS. To disrupt clumps, cells were diluted and shaken for 5 min. at maximum speed using a Tissuelyser II (Qiagen). Cells were visualized using a Nikon Eclipse TE2000-U inverted microscope and Roper Scientific CCD black and white camera using NIS-elements-BR software. GFP images were taken with a 460-490nm filter and Calcofluor white staining was detected with a UE-2E/C filter. mCherry was visualized with a G-2E/C filter. Photographs were taken at 90X magnification. Image analysis was performed using the Image J program (NIH) to quantify GFP fluorescence per cell. 80-200 cells were imaged per strain and repeated in triplicate. Coefficients of variance (COV) were used to measure the broadness of the distribution and were based on the image quantification (COV=average fluorescence/standard deviation*100).

RNA and cDNA preparation: Laboratory grown cells or contents of cecum or ileum samples taken at 3 or 18 days post-inoculation were mixed with RNA-Later (Ambion) and frozen at -80°C. RNA was extracted from laboratory grown samples using mechanical disruption with glass-zirconium beads and an RNeasy mini kit (Qiagen) with on column DNaseI digestion. For samples of mouse GI tract contents, samples were first filtered through 250um polypropylene mesh (Small Parts, Inc), then pelleted and resuspended in Trizol. RNA was isolated using the Purelink kit Trizol extraction

procedure (Invitrogen) with DNaseI digestion. Concentration of RNA was measured using a Nanodrop spectrophotometer and samples were stored at -80°C.

cDNA was generated from 1-10ug RNA with Superscript II or III Reverse transcriptase (Invitrogen) and an oligo-dT primer. Samples were heated to 70°C for 10 min. and the enzyme was added. The sample was then incubated at 42°C or 50°C, respectively, for 1.5 hrs. Following addition of 2.5M NaOH, the reaction was incubated at 37°C for 15 min. 2M Hepes free acid was then added to neutralize the pH and cDNA was purified using Qiaquick columns (Qiagen). Concentrations were determined using a Nanodrop spectrophotometer and samples were stored at -20°C.

qRT-PCR: Primers used to detect expression of genes are listed in Table 2-4. qRT-PCR reactions were performed with SYBR green reaction mix (Qiagen) with reference dye ROX using an MX3000p or MX4000 instrument (Stratagene). Each sample was tested in triplicate and melting curve analysis in addition to agarose gel electrophoresis was used to confirm primer specificity. Samples tested with no cDNA or cDNA from an *efg1*$^{-}$ strain using *EFG1* primers showed no detectable signal. Standard curves were generated and all results normalized to the level of actin expression in each respective sample. All samples are presented normalized to the level of expression for each respective gene in wild type cells grown in YPD at 30°C to an OD=1.

qPCR: Cecum contents, including fungal cells, from BALB/c mice collected at day 3 post-inoculation were diluted with PBS. One aliquot was plated on YPD-SA as described above, and another was pelleted and weighed. DNA was extracted from 70mg wet weight of pelleted cecum contents by bead beating in phenol-chloroform, followed by chloroform extraction, and ethanol precipitation [42]. The DNA was further purified

using a Qiagen DNeasy kit and RNase digestion. The amount of C. *albicans* DNA present in each sample was determined using qPCR as described [42].

Name	Genotype	Source
CAI-4	SC5314 Δ*ura3::imm434/*Δ*ura3::imm434*	[200]
CKY101	CAI-4 (pDB152)	[61]
HLC67	CAI-4 *efg1*::*hisG*/*efg1*::*hisG*	[107]
CKY136	CAI-4 *efg1::hisG/efg1::hisG* (pDB152)	[62]
HLC74	CAI-4 *efg1::hisG/efg1::hisG* (p*EFG1*)	[107]
JPY100	CAI-4 *FDH1*::*FDH1pr*-uGFP	This study
JPY101	HLC67 *FDH1*::*FDH1pr*-uGFP	This study
JPY102	CAI-4 *EFG1*::*EFG1pr*-GFP	This study
JPY103	CAI-4 *DRG1*::*DRG1pr*-GFP	This study
JPY 104	CAI-4 *rps1*::*ACT1pr-EFG1*	This study
JPY 105	CAI-4 *ade2*::pISAT	This study
JPY 106	HLC67 *ade2*::pISAT	This study
JPY 107	CAI-4 *rps1::ACT1pr-EFG1 FDH1::FDH1pr-uGFP*	This study
JPY 108	CAI-4 *FDH1*::*FDH1*pr-uGFP *EFG1*::*EFG1*pr-mCherry	This study

Table 2-3. *C. albicans* strains used in these studies.

QPCR PRIMERS		
GENE	PRIMER NAME	SEQUENCE
ACT1	ABAF3	5’-TATGAAAGTTAAGATTATTGCTCCACCAGAAA -3’
ACT1	ABAR3	5’-GGAAAGTAGACAATGAAGCCAAGATAGAAC-3’
FDH1	FDH1 F1	5’-CCAGTTCAACCAGCTCCAAAAGACAT-3’
FDH1	FDH1 R1	5’-GTGGCATAGTCACCATCAATAACAATAACATC-3’
EFG1	EFG1 F1	5’-CAGTATGGTCAGTATAATGCTGGTAAGAA-3’
EFG1	EFG1 R1	5’-TTGTTGTTGCTGTTGGTATGGATATGATGATG-3’
rDNA	RDNAF1	5’-GCCCAGCCTGCCGCCAGAGGTCTAAA-3’
rDNA	RDNAR1	5’-CGCCTTACCACTACCGTCTTTCA-3’
CLONING PRIMERS		
DRG1	DRG1F1	5’-ttggcgcgccCCTGCAATAATTGTTGACGACGAC-3’
DRG1	DRG1R1	5’-tcctccgcggTTTTTATTTTAATTGGGGAATGAA-3’
EFG1	EFGpF	5’-ttggcgcgccATGGGCTTATTATGCATTCAGTCA-3’
EFG1	EFGpR	5’-tcctccgcggTATGGGTTATATTCTTGGTAGTCA-3’
FDH1	REP1F	5’-ttggcgcgccAATACTTGGGTCCAGCAGTCTTGATG-3’
FDH1	REP1R	5’-tcctccgcggTTTTGTTTTTCGTTAAGCTTTTTGATTTAGT-3’

Table 2-4. Primers used in these studies.

Chapter 3:

EFG1-dependent gene expression during GI tract colonization

Introduction

Candida albicans is a medically relevant opportunistic pathogen which commonly colonizes the human GI tract, skin, and genitourinary tracts [1, 2, 5, 167]. Normally, *C. albicans* benignly interacts with its host, but can cause serious disease when a host becomes immunocompromised [172, 176, 196, 201, 202]. *C. albicans* is capable of causing several different types of diseases, including gastrointestinal candidiasis, yeast infections, thrush, skin infections, and systemic infections [22, 117, 203]. Systemic infections are thought to be caused by endogenous *C. albicans* escaping the GI tract and circulating via the bloodstream to infect deep tissues such as the lungs, kidney, and liver [7, 113, 114, 164].

As a colonizing organism of the GI tract, *C. albicans* grows mainly in the yeast form [42], but as a pathogen, the virulence of *C. albicans* is often associated with its ability to form hyphal cells [3, 7, 83]. Hyphal morphology is induced by an array of environmental signals which converge through a network of transcription factors, allowing *C. albicans* to fine tune its responses to changes in the environment [44, 46, 61, 123, 125, 126].

The transcription factor Efg1p was identified as major regulator of hyphal formation under most laboratory conditions [111]. An *efg1*$^{-}$ null mutant was attenuated for virulence in a systemic model of candidiasis and was more susceptible to phagocytosis [107]. Studies have shown that Efg1p regulates widespread changes in gene expression in response to serum, temperature, hypoxia, low iron availability, as well as in kidney lesions [8, 9, 44, 111, 137, 146]. These data show that Efg1p is responsible for the expression of a large number of cell surface proteins, stress response genes, and

metabolic genes in response to various environmental cues. We have shown that Efg1p is a major regulator of colonization in the GI tract [165]. In the absence of Efg1p, cells hyper-colonized the GI tract, but were more susceptible to the host immune response. Therefore, Efg1p is a major regulator of gene expression and is involved in the regulation of GI tract colonization, pathogenesis and hyphal growth.

Several other transcription factors which regulate morphogenesis have also been shown to regulate GI tract colonization (Fig. 1-2). The transcription factor Efh1p, a homologue of Efg1p, was up-regulated during GI tract colonization and regulated persistence in the GI tract [42, 111]. The transcription factor Cph2p [130] was required for normal levels of GI tract colonization by *C. albicans* [177]. Cph2p and Tec1p, another regulator of morphogenesis, both regulated gene expression during colonization [131, 177]. The transcription factor Cph1p also regulated the yeast to hyphal transition through an alternate signaling pathway [126, 127]. Cph1p is partially redundant with Efg1p, and previous studies showed that deletion of both *CPH1* and *EFG1* caused additive defects in morphogenesis and virulence of *C. albicans* in the bloodstream [107, 126].

Previous data showed that *C. albicans* cells colonizing the GI tract expressed genes that are characteristic of laboratory-grown log phase and post-exponential phase cells [177]. Additionally, colonizing cells expressed cell surface proteins associated with hyphal gene expression, despite the fact that a majority of cells in the population were in the yeast form [42]. Several cell surface proteins regulated by Efg1p during hyphal growth, such as *ECE1*, were up-regulated during GI tract colonization and expressed

independently of Efg1p [42]. This finding suggests that during gastrointestinal colonization the Efg1p regulon differs from laboratory growth.

To understand how Efg1p regulates GI tract colonization, we used microarray analysis to globally identify genes which are regulated by Efg1p during GI tract colonization in the cecum and ileum. These data show that Efg1p regulates the expression of genes involved in metabolism and host interactions in colonizing cells. Genes which contribute to the hyper-colonization of the *efg1*$^{-}$ null mutant and its increased sensitivity to the host immune response during GI tract colonization were identified.

Results

A general program of *C. albicans* gene expression within the host

Previous studies examined the effects of the cecum environment on gene expression by WT *C. albicans* [42]. Here, we have extended these studies to examine changes in gene expression by WT *C. albicans* between cells colonizing the cecum and the ileum. There are considerable differences between the ileum and cecum environment. The ileum has a more alkaline pH and increased levels of oxygen relative to the cecum [47, 204]. After passage through the ileum, many nutrients have been absorbed during digestion, and undigestable polysaccharides are then metabolized by bacteria in the cecum [68]. Bacteria in both the ileum and cecum ferment polysaccharides to produce short chain fatty acids, but there are greater numbers of commensal bacteria in the cecum compared to the ileum [59, 205].

To understand differences in fungal cells colonizing the cecum and the ileum, BALB/c mice were inoculated by oral gavage with WT *C. albicans*, as described in Material and Methods (Fig. 3-1, schematic of microarray analysis). At days 1 and 3 post-inoculation, fecal pellets were homogenized and plated to measure colonization levels (Fig. 3-2, squares). Cecum and ileum contents were harvested for gene expression analysis at day 3. RNA was extracted from the GI tract contents, amplified, and labeled for hybridization to microarrays (Material and Methods). For each microarray, gene expression was measured relative to a reference sample of WT *C. albicans* cells grown in the laboratory to log phase in rich medium at 37°C. Hybridization, scanning and analysis of microarray results using GeneSpring was performed (Material and Methods). Colonization experiments, sample collection, and RNA isolation were performed by J. Pierce in the Kumamoto lab; sample amplification, dye coupling, microarray hybridization and scanning were done by D. Dignard in the Whiteway lab. Genes which were 1.5 fold up- or down-regulated with a p-value of <0.05 were considered to be significantly differentially expressed. Gene expression ratios between microarrays (WT cecum or ileum sample/WT reference) were then used to define genes differentially expressed between cells colonizing the cecum or the ileum. Venn diagrams and GO (Gene ontology) term analyses were used to identify key changes in gene expression during colonization of the GI tract [206].

First, we analyzed gene expression of WT *C. albicans* cells in the cecum or ileum relative to the reference sample (Fig. 3-3A and Table 3-1). WT *C. albicans* differentially expressed 607 genes in the cecum, and 778 genes in the ileum relative to the laboratory grown cells. Roughly half of these genes (348) were differentially regulated by *C.*

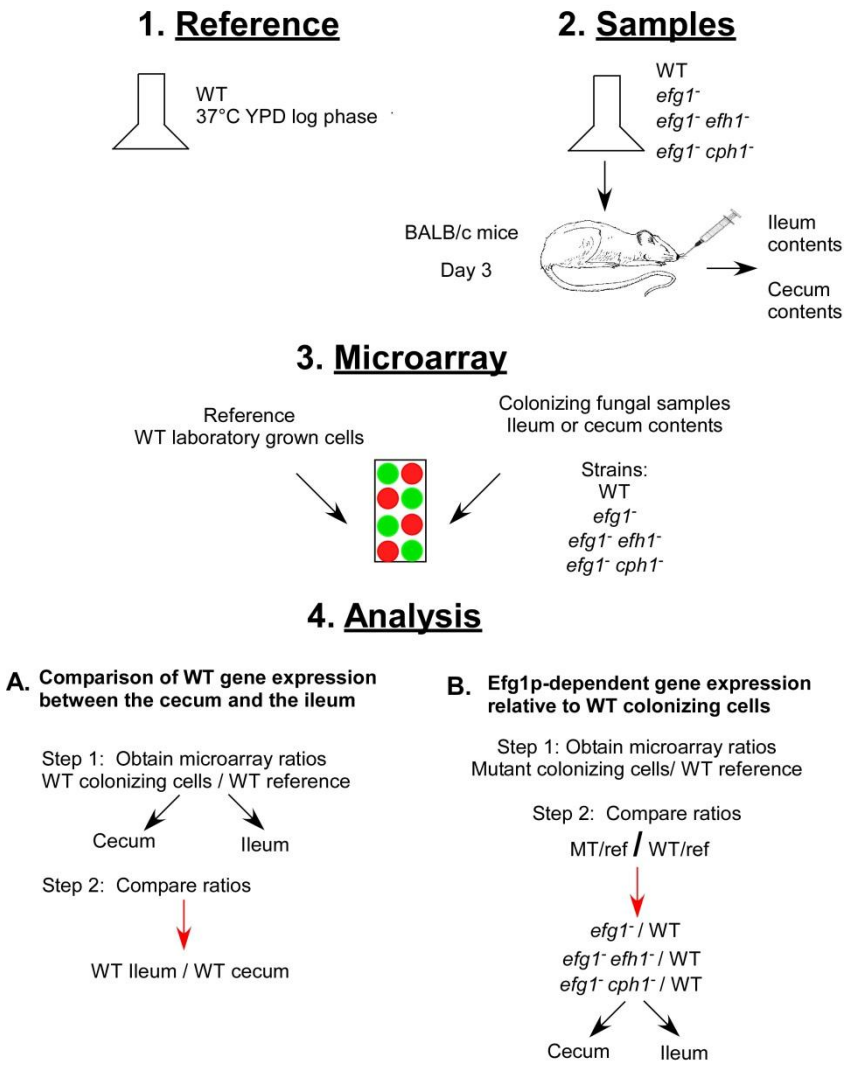


Figure 3-1. Schematic of microarray analysis.

Laboratory cells were grown in rich medium at 37°C as a reference sample. Samples from colonizing fungal cells were harvested from the cecum and the ileum at day 3 in BALB/c mice. Microarrays were done comparing each condition to the reference sample using several *C. albicans* strains. Analysis compared WT gene expression in the ileum and the cecum relative to a reference sample, as well as mutant strains relative to WT gene expression in each organ.

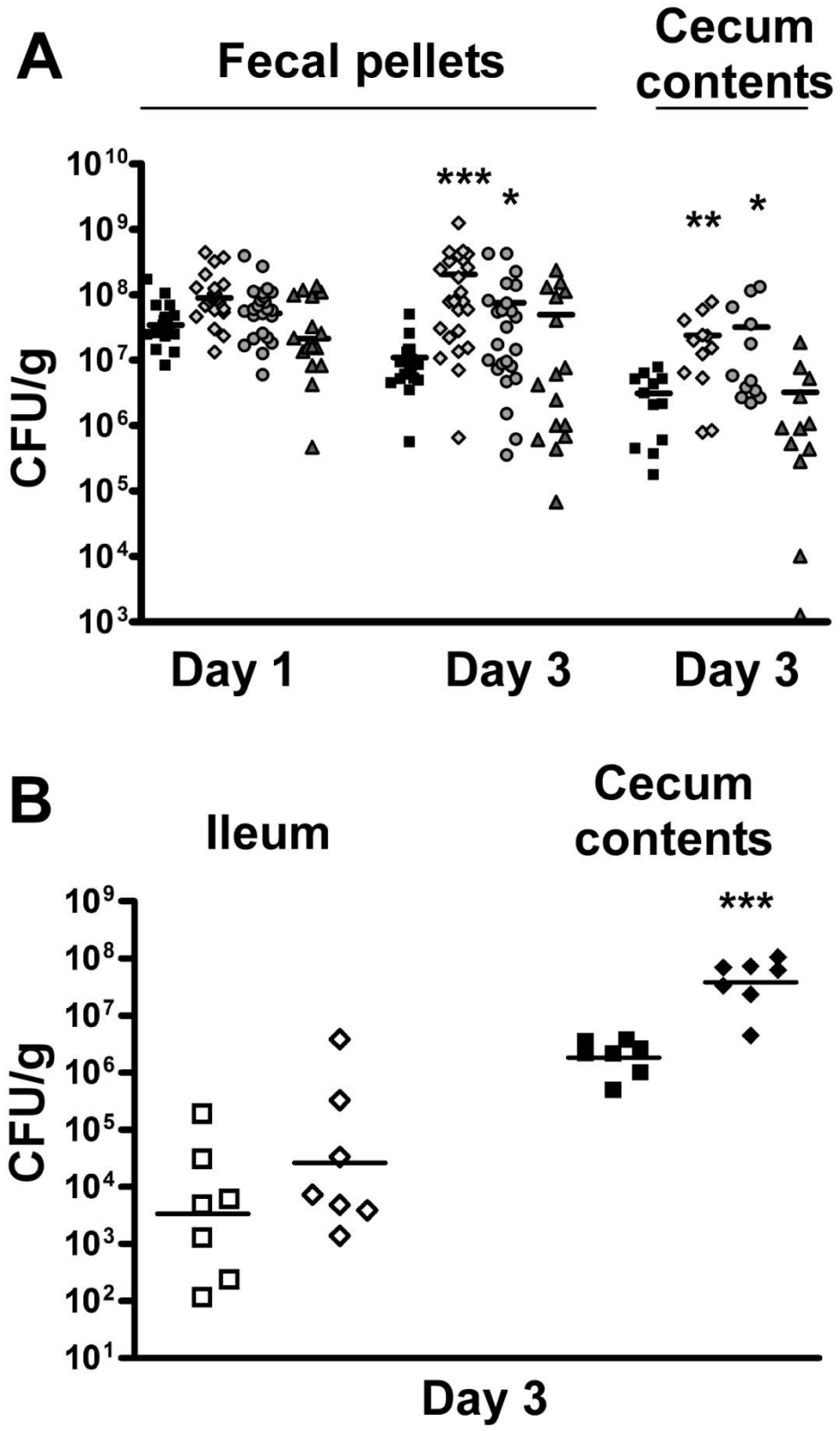

A
Fecal pellets
Cecum contents
CFU/g
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
***
*
**
*
Day 1
Day 3
Day 3
B
Ileum
Cecum contents
CFU/g
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
***
Day 3

Figure 3-2. Gastrointestinal colonization of *efg1*$^{-}$ null mutants.

(A) Colonization of the fecal pellets and cecum contents by *C. albicans* is shown at days 1 and 3 post-inoculation by oral gavage for WT and *efg1*$^{-}$ null mutant strains in BALB/c mice. WT, squares; *efg1*$^{-}$, diamonds, *efg1*$^{-}$ *efh1*$^{-}$, circles, *efg1*$^{-}$ *cph1*$^{-}$, triangles. (B) Colonization of whole ileum (open symbols) and cecum contents (closed symbols) by WT (squares) and *efg1*$^{-}$ null mutant **(**diamonds) strains at day 3 post-inoculation of BALB/c mice. Each symbol represents an individual mouse; bars are the geometric mean. ***$p<0.0001$, **$p<0.01$, *$p<0.05$ Mann whitney test.

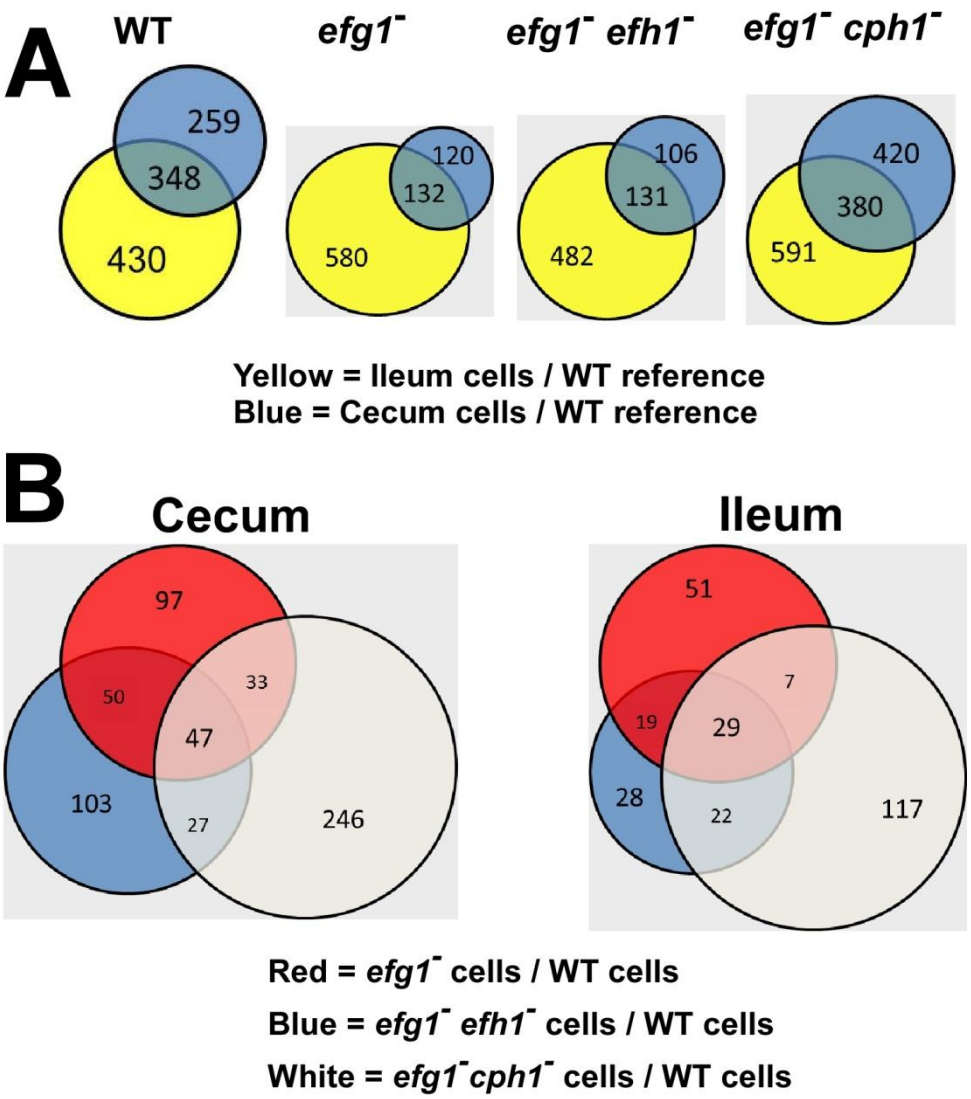


Figure 3-3. Overview of gene expression changes during colonization by *C. albicans*.

Venn diagrams depicting overlapping gene expression patterns during GI tract colonization by *C. albicans* WT, *efg1*$^{-}$, *efg1*$^{-}$ *efh1*$^{-}$, and *efg1*$^{-}$ *cph1*$^{-}$ strains. (A) Gene expression for each strain was compared to the laboratory reference sample as determined by microarray analysis. (B) Microarray data for the *efg1*$^{-}$ null mutant strains was first compared to the reference sample on each slide, and then ratios were generated with WT *C. albicans* microarray data in either the cecum (left) or the ileum (right). Percent of genes uniquely expressed by each strain (cecum and ileum): *efg1*$^{-}$, 43% and 48%; *efg1*$^{-}$ *efh1*$^{-}$, 45% and 33%; *efg1*$^{-}$*cph1*$^{-}$ 70% and 63%.

Condition	Cecum	Ileum
WT / reference	607	778
efg1^{-} / reference	252	712
efg1^{-} *efh1*$^{-}$ / reference	237	613
efg1^{-} *cph1*$^{-}$ / reference	800	971
efg1^{-} / WT	227	106
efg1^{-} *efh1*$^{-}$ / WT	227	86
efg1^{-} *cph1*$^{-}$ / WT	353	187

Table 3-1. Total genes differentially expressed during colonization of the GI tract.

Genes were defined as differentially expressed if they were more than 1.5 fold up- or down-regulated in each microarray, and had a p-value<0.05 in at least one comparison. Initial comparisons were done for each strain relative to a laboratory reference; genes specifically affected by *EFG1* were identified by generating a gene expression ratio (mutant/reference) / (WT/reference). Analysis was done on cells from both the cecum and the ileum.

albicans cells in both organs (Fig. 3-3A). Relative to laboratory grown cells, more genes were down-regulated (415/607 in the cecum, 465/778 in the ileum) during colonization than up-regulated (192/607 in the cecum, 313/778 in the ileum). GO term analysis identified significantly over-represented categories of genes which were either up- or down- regulated within our data sets relative to laboratory grown cells (Tables 3-2A, 3-2B, 3-3).

One major group of genes up-regulated by WT *C. albicans* during colonization of both the cecum and ileum were genes involved in carbon metabolism (Table 3-4). Included among these were genes involved in galactose metabolism (*GAL1* and *GAL10*). Additionally, genes involved in alcohol metabolism, such as *ALD5* (aldehyde dehydrogenase) and *CTN1* (carnitine acetyl transferase) were up-regulated in both the cecum and ileum (Table 3-4). The increased expression of *CTN1* by cells colonizing the cecum relative to laboratory grown cells was confirmed by qRT-PCR (Fig. 3-4). Increased expression of genes required for gluconeogenesis, such as *FBP1* and *PCK1*, was also noted in fungal cells colonizing the cecum and ileum (Table 3-2A). Expression of these genes increases in the laboratory in the absence of glucose, and up-regulation of these pathways in colonizing cells is consistent with the low availability of glucose in the cecum [207-210].

In these studies, we observed that cells in the cecum and the ileum were capable of fermentation of carbons, such as galactose, and alcohol metabolism. However, colonizing fungal cells were also exposed to nutrient limiting conditions in both environments. Increased expression of genes involved in gluconeogenesis in the cecum and ileum was similar to the patterns of gene expression observed during phagocytosis

Gene Ontology	Corrected P-value	Genes
Up-regulated		
Host interaction	<0.0001	*ALS10, CAT1, CDC42, DAC1,HSX11, HWP1, HXK1, HYR1, PGA10, PHR1, RBT1, RBT4, RBT5, SAP4, SAP5, SAP6, SOD5, TEC1, TPS2, UME6, VPS21, WOR1*
Response to heat	0.005	*HSP60, HSP70, HSP78, HSP104*
Carbohydrate metabolism	0.007	*AMS1, ARD, FBP1, GAL1, GAL10, PCK1, SKN1*
Catabolic processes	0.025	*AUT7, FDH1, PUT1, PUT2, RPN10, RPT1, SEC4*
Hyphal growth	0.027	*CYC3, DDR48*
Small molecule metabolism	0.03	*AAT21, ALD5, ARG1, CTN1, CYS3, FBP1, IDP2, MAE1, PRO3, STF2, SNZ1*
Catalytic activity	<0.0001	*ARG3,CDR11, CRP1, ENA2, POT1,SPC3, ECM4, PEX4, YHB5*
GTP binding	0.017	*RAC1*
Phosphate transport	0.02	*GIT2, PHO89, PHO84*
Cell surface	<0.0001	*ALS3, EXG2, FRP3, HGT1, HSP30, OPT4, PGA7, PGA54, PGA34*
Cellular localization	<0.0001	*CIP1, FTH1, GAR1, KIP4, PGA7, SBA1, SEC13, SPC3, TAR1*

Table 3-2A. Genes up-regulated by WT *C. albicans* in the cecum.

GO terms listed were significantly enriched ($p<0.05$) when comparing gene expression of WT *C. albicans* in the cecum relative to a laboratory grown reference sample. Duplicate genes are only listed under one category and overlapping categories were combined. P-values were corrected for false discovery rate.

Gene Ontology	Corrected P-value	Genes
Down-regulated		
Cellular metabolism	<0.0001	*ACB1, ARF2, ATP14, BIG1, BIO2, BMS1, ENP1, FCA1, GCR3, GCS1, GLO1, GPI1, HEM4, HEM14, LIP7, MEU1, MLP1, MNN4, MRT4, MSI3, MSK1, NAN1, NEP1, NHP6A, NP1, OPI3, PRE3, PRE8, PRE9, PRP8, PSF1, PT4, PTC8, PUP3, PUS7, PYC1, QRC9, RAD59, RAV2, RFC5, RHD1, RPG1A, RPN6, RPT4, RNR21, RTF1, SET3, SMD2, SNP3, TOP1, UTP4, VAS1, VPS23, VRG4*
RNA polymerase activity		*RPA12, RPA190, RPB1, RPB8, RPB11, RPC10, RPC21, RPC31, RPC40*
Amino acid metabolism		*ILV6, LYS9, MET3, MET8, MET14, MET18, THR1*
Sulfur metabolism	0.009	*BIO2, GCS1, GLO1, MEU1*
Cellular processes	<0.0001	*APL5, CLB4, CSI2, ECM3, ECM25, GZF3, HAC1, HGT10, MDS3, MXR1, MYO1, NIP17, PHO87, RGD1, SKO1, SLA1, SLA2, SPC98, TIF5, TOM22, TOM71, YMC1*
Transferase activity	0.005	*AKR1, ANP1, APT1, ARP4, DIE2, EFG20, FUR1, GUK1, HOC1, IPL1, MCD4, MNT2, OPI1, URA2, URA5*
Small congujating protein-specific protease activity	0.03	*DOA4, DOT4, ULP1, ULP2*
Catalytic activity	<0.0001	*FRE10, PHO112, WRS1*
Nucleus	0.004	*ASG1, CLB4, HRT2, STP3, WAR1*
Non-membrane bounded organelle	0.004	*MRPL6, MRPL10, MRP90, RPS30*
Intracellular component	<0.0001	*ACO2, ASG1, FMP27, MRP20, WH11*

Table 3-2B. Genes down-regulated by WT *C. albicans* in the cecum.

GO terms listed were significantly enriched ($p<0.05$) when comparing gene expression of WT *C. albicans* in the cecum relative to a laboratory grown reference sample. Duplicate genes are only listed under one category and overlapping categories were combined. P-values were corrected for false discovery rate.

Gene Ontology	Corrected P-value	Genes
Up-regulated		
Pathogenesis	<0.0001	*ALS10, CAT1, CDC11, CDC24, CNH1, DAC1, HOG1, HWP1, HXK1,NAG3, NAG6, NDT80, NIK1, PHR1, RBT1, RBT4, RBT5, RIM101, RSV16, SAP4, SAP5, SAP6, SOD5, TEC1, TPS2, UME6, VPS21,* ORF19.3070.1
Acetyl-CoA metabolism	0.009	*ACS1, CTN3, CIT1, CTN1, ICL1, MDH1-1, MVD, FUM12*
Multi-organism process	0.016	*CST5; HSP70, SSA2,* ORF19.93
Small molecule catabolism	0.031	*ARO10, CAR1, DTD2, FDH1, GAL1, GAL10, PUT1, PUT2, PXP2,* ORF19.317, ORF19.5159, ORF19.7108
Alcohol metabolic process	0.040	*FBP1, INO1, PCK1, STD1, UPC2,* ORF19.5611, ORF19.6423, ORF19.7108
Down-regulated		
Glycosylation	0.002	*ALG2, ALG11, ANP1, CWH8, DIE2, MNN2, MNT2, MNT3, OST1, PMT5, VRG4,* ORF19.1092, ORF19.1761, ORF19.7426, ORF19.5065
Phospholipid biosynthesis	0.019	*CHO2, EFG20, GPI1, MCD4, OPI3,* ORF19.2045, ORF19.3447, ORF19.3695, ORF19.4558, ORF19.4581, ORF19.60, ORF19.6154
Transferase activity	0.002	*ALG5, ANP1, APT1, GLC3, HOC1, URA5*

Table 3-3. Genes differentially expressed by WT *C. albicans* in the ileum.

GO terms listed were significantly enriched ($p<0.05$) in groups of genes either up- or down-regulated when comparing gene expression of WT *C. albicans* in the ileum relative to a laboratory grown reference sample. Duplicate genes are only listed under one category and overlapping categories were combined. P-values were corrected for false discovery rate.

Function	Gene	WT Cecum	WT Ileum	Rosenbach et al.	Walker et al. (kidney)	Thewes et al. (liver)
Galactose metabolism	*GAL1*	Up	Up	Up	-	Down
	GAL10	Up	Up	Up	-	-
Fermentation	*ALD5*	Up	Up	Up	-	-
Gluconeogenesis	*FBP1*	Up	Up	Up	-	-
	PCK1	Up	Up	Up	-	Up
Glyoxylate cycle	*ICL1*	Up RT-PCR	Up	Up	-	-
	MLS1	S. Up	Up	Up	Up	-
TCA cycle	*CIT1*	-	Up	-	-	-
	MDH1-1	-	Up	-	-	-
Inositol biosynthesis	*INO1*	Up RT-PCR	Up	Up	-	Up
Phosphate transport	*PHO84*	Up	Up	Up	Up	Up
	PHO89	Up	Up	Up	Up	-
pH response	*PHR1*	Up	Up	Up	Down	Up
Iron uptake	*HMX1*	Up	-	Up	-	Down
	PGA10	Up	-	-	Down	-
	RBT5	Up	Up	Up	Down	-
Acetyl-CoA metabolism	*ACS1*	S. Up	Up	Up	Up	-
	CTN1	Up RT-PCR	Up	Up	-	-
	CTN3	S. Up	Up	Up	Up	-
Secreted proteases	*SAP4*	Up	Up	-	-	-
	SAP5	Up	Up	Up	-	Up
	SAP6	Up	Up	-	-	-
Cell surface proteins	*ALS3*	Up	Up	Up	-	-
	ALS10	Up	Up	Up	-	Up
	HYR1	Up	S. Up	Up	-	-
	HWP1	Up	Up	Up	-	-
Transcription factors	*FLO8*	S. Up	S. Up	-	-	-
	RIM101	-	Up	Up	-	-
	TEC1	Up	Up	Up	-	-
	WOR1	Up	S. Up	-	-	-
Hyphal signaling	*DCK1*	S. Up	S. Up	Up	-	-
	DFI1	S. Up	Up	Up	-	Down
	NGT1	S. Up	-	-	-	-
	RAC1	Up	S. Up	-	-	-
Oxidative stress response	*CAT1*	Up	Up	Up	Up	Down
	CIP1	Up	Up	-	-	-
	SOD5	Up	Up	Up	Down	Up
Stress response	*DDR48*	Up	Up	-	-	Up
Heat shock response	*HSP30*	Up	Up	Up	-	-
	HSP31	Up	Up	Up	-	-
	HSP60	Up	Up	-	-	-
	HSP70	Up	Up	Up	-	-
	HSP78	Up	Up	Up	-	-
	HSP104	Up	Up	Up	-	-
Glycosylation	*MNT2*	Down	Down	-	-	-
	MNT3	-	Down	-	-	-
	PMT5	S. Down	Down	-	-	-
White phase specific	*WH11*	Down	Down	Up	-	-

Table 3-4. Select gene expression by WT *C. albicans* during colonization and invasive disease.

Expression of select genes by WT *C. albicans* is shown in both the cecum and the ileum. These data are compared to previous gene expression studies from the cecum, as well as from fungal cells within kidney and liver lesions. Genes are noted as up- or slightly up- (S. Up) regulated in yellow; down- or slightly down- (S. down) regulated genes are shown in blue. Conditions in which a gene was not differentially regulated are shown in grey (-).

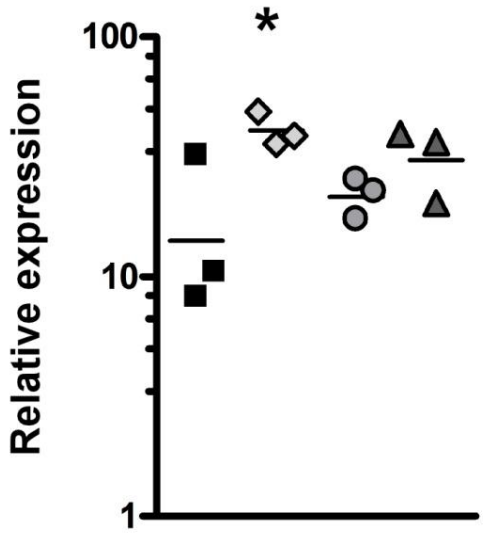


Figure 3-4. *CTN1* is induced during colonization by WT *C. albicans* and repressed by Efg1p.

Expression of *CTN1* shown by RT-PCR. Expression is normalized to *ACT1* for each sample and expressed relative to levels present during laboratory growth. Samples were taken at 3 days post-inoculation from BALB/c mice. Each symbol represents 2 ceca, bars are the geometric mean. WT, squares; *efg1*$^-$, diamonds; *efg1*$^-$ *efh1*$^-$, circles, *efg1*$^-$ *cph1*$^-$, triangles. *$p<0.05$ ANOVA.

[63]. Up-regulation of both fermentation and gluconeogenesis may indicate that some colonizing fungal cells undergo fermentation, while others utilize gluconeogenesis as observed previously [177, 211]. These results may indicate that some colonizing cells have access to nutrients for fermentation, while others are in a nutrient-poor local environment.

Gene expression consistent with increased uptake and utilization of several essential nutrients was observed by cells colonizing the cecum and ileum, in agreement with previous results obtained from the cecum [177]. In the cecum and the ileum, up-regulation of genes involved in phosphate transport (*PHO84* and *PHO89*) was observed, and up-regulation of *PHO89* (Fig. 3-5B) relative to laboratory grown cells was confirmed by qRT-PCR. Expression of *PHO84* by invasive *C. albicans* cells in both the liver and kidney has been previously observed [8, 9], indicating a general need for phosphate transport activity during growth within a host.

Increased expression of hemoglobin utilization genes (*RBT5*, *PGA10*, and *HMX1*) compared to the reference sample was also observed by microarray (Tables 3-2A, 3-4). Up-regulation of *RBT5* (Fig. 3-5A) in colonizing cells relative to laboratory grown cells was confirmed by qRT-PCR. The GI tract has been described as an iron-replete environment [104]. However, up-regulation of hemoglobin uptake and utilization genes during colonization suggests a role for this activity during colonization (Fig. 3-5A). Other genes involved in iron uptake and utilization were also up-regulated during systemic infections [8, 9]. This suggests that iron availability is an important signal for both colonizing and invasive cells.

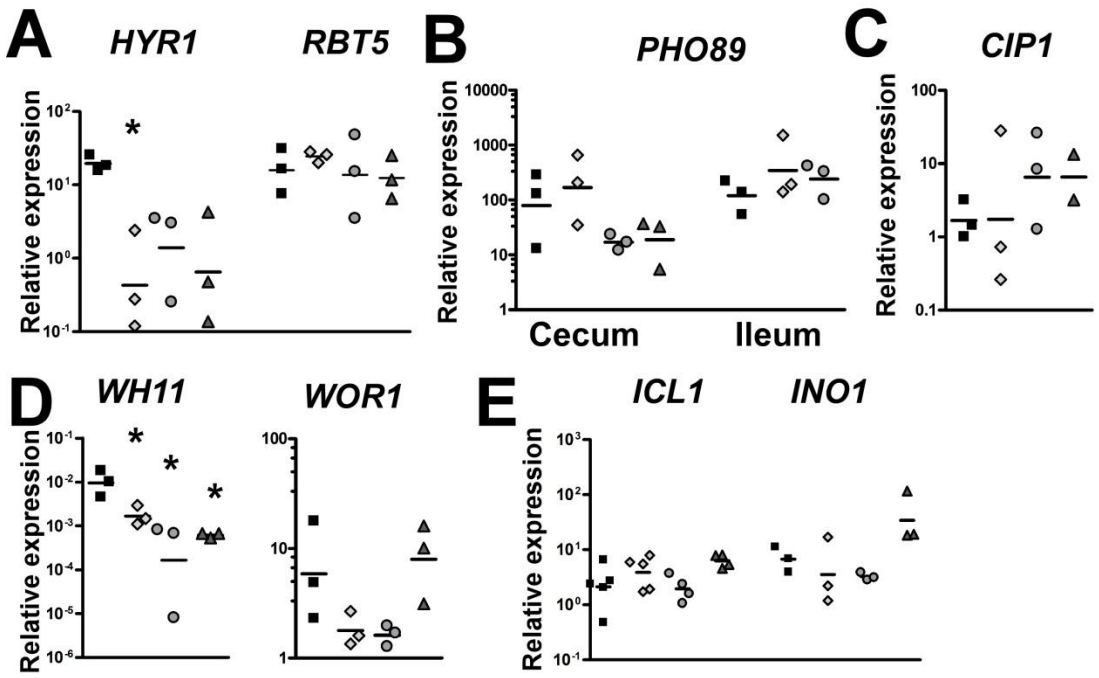


Figure 3-5. RT-PCR analysis of gene expression patterns during GI tract colonization.

RT-PCR was used to validate microarray results in either the cecum or ileum by *C. albicans*. Expression is normalized to *ACT1* for each sample and expressed relative to levels present during laboratory growth. Samples were taken at 3 days post-inoculation. Each symbol represents two ceca or 4 ilea; bars are the geometric mean. WT, squares; *efg1*$^{-}$, diamonds; *efg1*$^{-}$ *efh1*$^{-}$, circles, *efg1*$^{-}$ *cph1*$^{-}$, triangles. (A) Expression of genes involved in host interactions: *HYR1* and *RBT5* in the cecum. (B) Expression of *PHO89* in the cecum and ileum. (C) Increased *CIP1* expression in the cecum. (D) Down-regulation of white phase specific gene *WH11* in the cecum of day 3 and increased expression of *WOR1*. (E) Expression of *ICL1* and *INO1* in the cecum at day 3 post-inoculation. * $p<0.05$, ANOVA comparing mutant to WT gene expression.

INO1, a gene involved in inositol-1-phosphate biosynthesis, a membrane component and signaling molecule, was also up-regulated by microarray (Table 3-4) in fungal cells colonizing the cecum and ileum. Increased expression of *INO1* in the cecum relative to laboratory grown cells was confirmed by qRT-PCR (Fig. 3-5E). Increased expression of *INO1* was previously noted in the cecum as well as invasive kidney lesions, suggesting this is also part of a general transcriptional response to the host environment.

A large number of genes involved in host interactions were up-regulated in both the cecum and the ileum by WT *C. albicans* relative to laboratory grown cells. These included genes annotated as being important for both pathogenesis and symbiosis. *HYR1*, a hyphal-induced cell wall protein, was up-regulated by WT *C. albicans* during colonization ($p<0.05$) (Fig. 3-5A) [212, 213]. *SAPs 4, 5,* and *6* (secreted aspartyl proteases), *ALS3* (an adhesin) and *HWP1* (a hyphal wall protein) were also among the group of genes up-regulated by WT *C. albicans* whose expression increased in both the cecum and the ileum compared to the reference sample (Table 3-2A, 3-3) [116, 125, 140, 144, 214, 215]. *SAP*s 4-6 were up-regulated in an oral model of thrush, suggesting that these genes are important for both colonization and pathogenesis. These genes have all been described as up-regulated during hyphal growth [137, 140]. Therefore, consistent with previous data, cells colonizing both the cecum and the ileum have gene expression patterns that are similar to hyphal cells even though most colonizing cells are yeast [42].

Based on the expression of hyphal genes (*ALS10, HWP1*) in both the cecum and the ileum, we analyzed the expression patterns of genes involved in the signal transduction pathways which regulate morphogenesis. Clustering by arrays showed that the expression of genes which regulate hyphal growth were similar in the cecum and

ileum (Fig. 3-6). The genes involved in the pathways which respond to matrix embedding (*DCK1*, *RAC1*, *DFI1*) and N-acetylglucosamine (*NGT1*, *RIM101*, *FLO8*) were all up-regulated in cells colonizing the cecum and ileum relative to the reference sample [190] (Fig. 3-6 and Table 3-4). N-acetylglucosamine (GlcNAc) is a component of both bacterial and fungal cell walls, and may be a relevant signaling molecule in the GI tract environment. *C. albicans* has been shown to filament in response to GlcNAc present in serum, indicating that this signal may also play an important role in the bloodstream [49]. Luminal contents and mucus layers present in the gut may stimulate hyphal gene expression in a similar manner to cells in embedded conditions in the laboratory through contact sensing and pressure differences in the cell wall. GlcNAc and cell contact therefore may represent important signals which *C. albicans* responds to via the hyphal signaling network in the gut to alter gene expression.

Several transcription factors were identified as up-regulated in colonizing cells, including *TEC1* and *WOR1* (Tables 3-2A, 3-3). Tec1p was previously shown to alter gene expression during colonization, but did not affect the ability of *C. albicans* to colonize the GI tract [177]. *WOR1* is the major transcription factor responsible for white–opaque switching, and increased expression of *WOR1* is associated with the mating-competent opaque phase [157]. *WOR1* was up-regulated during colonization in the cecum (Fig. 3-5D). Down-regulation of white phase specific genes, such as *WH11* and *EFG1*, was also observed by RT-PCR (Fig. 3-5D and Table 3-2B) [165]. This observation suggests that the GI tract environment is permissive for white-opaque switching [155].

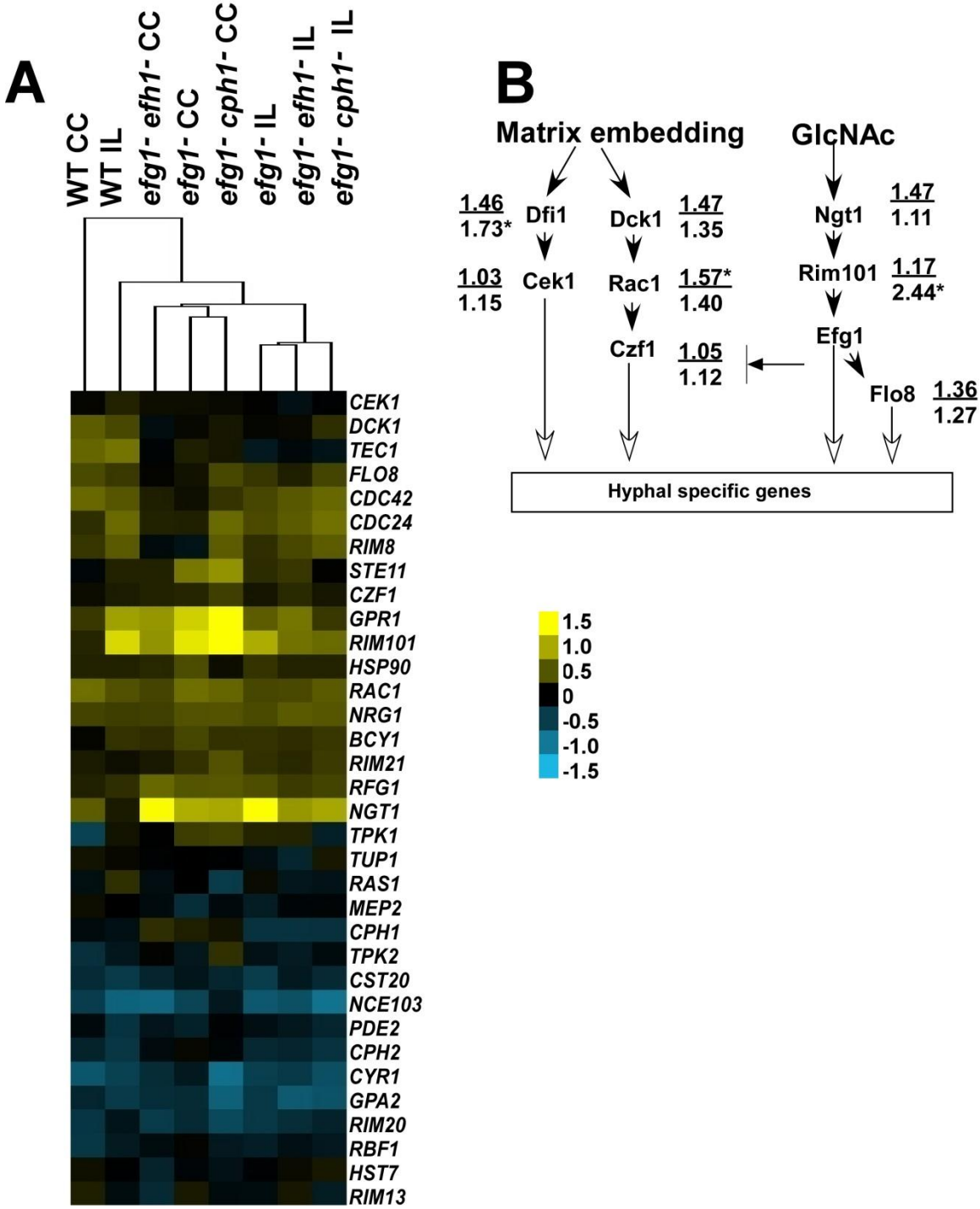

A
WT CC
WT IL
efg1- efh1- CC
efg1- CC
efg1- cph1- CC
efg1- IL
efg1- efh1- IL
efg1- cph1- IL
CEK1
DCK1
TEC1
FLO8
CDC42
CDC24
RIM8
STE11
CZF1
GPR1
RIM101
HSP90
RAC1
NRG1
BCY1
RIM21
RFG1
NGT1
TPK1
TUP1
RAS1
MEP2
CPH1
TPK2
CST20
NCE103
PDE2
CPH2
CYR1
GPA2
RIM20
RBF1
HST7
RIM13
B
Matrix embedding
GlcNAc
1.46
1.73*
Dfi1
Dck1
1.47
1.35
Ngt1
1.47
1.11
1.03
1.15
Cek1
Rac1
1.57*
1.40
Rim101
1.17
2.44*
Czf1
1.05
1.12
Efg1
Flo8
1.36
1.27
Hyphal specific genes
1.5
1.0
0.5
0
-0.5
-1.0
-1.5

Figure 3-6. Differential expression of genes regulating hyphal formation during GI tract colonization.

(A) Heat map showing the expression patterns of genes involved in the regulation of hyphal growth based on microarray data of cells colonizing the cecum and ileum at 3 days post-inoculation. Expression levels are relative to a laboratory reference sample. Yellow indicates increased expression during colonization; blue indicates decreased expression. (B) Differential expression of genes involved in sensing matrix embedding and N-acetylglucosamine (GlcNAc). Ratio of expression relative to a laboratory reference is shown next to each gene (cecum, top; ileum, bottom). * indicates p-value <0.05 for microarray data comparing WT gene expression in the cecum or ileum to the reference sample.

A significant stress response was detected in colonizing WT cells relative to laboratory grown cells in both the cecum and the ileum. Increased expression of several stress response genes, such as *DDR48*, was detected (Tables 3-2A, 3-3). The expression of heat shock genes, including *HSP60*, *HSP78*, and *HSP104*, also increased in colonizing cells, despite the fact that the reference sample was grown at physiological temperature (Tables 3-2A, 3-3 and 3-4). Genes involved in the oxidative stress response were also highly induced. *SOD5*, a superoxide dismutase, was the most highly up-regulated gene in our study (Tables 3-2A, 3-3, and Fig. 3-7) [142, 143]. *SOD5* was up-regulated in the ileum as well as the cecum. *CAT1*, the major catalase in the cell, and *CIP1*, a second putative oxidoreductase, were also up-regulated during colonization relative to laboratory grown cells (Tables 3-2A, 3-3, and Fig. 3-5C) [216, 217]. Oxidative stress was not observed in cells invading liver tissue in a systemic infection model, suggesting that this response is more important in colonizing cells than in invasive cells. Based on these results, *C. albicans* undergoes various types of stress during colonization, particularly oxidative stress, and expresses a number of genes to counteract these effects.

In summary, there was significant overlap in gene expression between cells colonizing the cecum and ileum. Genes involved in host interactions, metabolism, and hyphal growth were expressed at higher levels in both organs relative to laboratory grown cells. In addition, induction of the oxidative stress response and phosphate uptake was detected in cells colonizing both the ileum and the cecum. Cells in the ileum therefore exhibited similar stress responses as cells colonizing the cecum, suggesting that this is a general response by colonizing cells to the host environment.

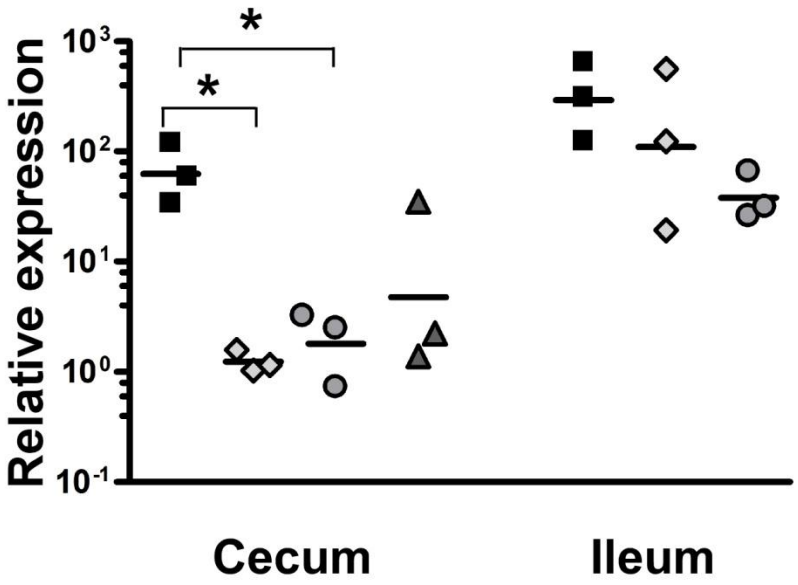


Figure 3-7. *SOD5* is expressed in colonizing cells in an Efg1p-dependent manner.

Expression of *SOD5* determined by RT-PCR. Expression is normalized to *ACT1* for each sample and expressed relative to levels present during laboratory growth. Samples were taken at 3 days post-inoculation from BALB/c mice. Each symbol represents 2 ceca or 4 ilea; bars are the geometric mean. WT, squares; *efg1*$^-$, diamonds; *efg1*$^-$ *efh1*$^-$, circles, *efg1*$^-$ *cph1*$^-$, triangles. *p<0.05 ANOVA.

Niche specific gene expression by WT *C. albicans* in the ileum

While many features of gene expression were similar between cells colonizing the two organs, there were also significant differences in gene expression patterns. Over half the genes that were differentially expressed between colonizing cells in the ileum and laboratory grown cells were not similarly regulated in the cecum (Fig. 3-3A). In the ileum, fewer GO terms were identified as being significantly over-represented during colonization by WT *C. albicans* than the cecum (Table 3-3).

Metabolic gene expression was altered between cells colonizing the cecum and the ileum. Expression of genes involved in acetyl-CoA metabolism by WT *C. albicans* was higher in the ileum than the cecum (Table 3-3), relative to the reference sample. This included the expression of the carnitine acetyl transferase *CTN3*, as well as the putative acetyl-CoA synthase, *ACS1*. *CTN1* was similarly induced in both organs relative to the reference sample. The TCA cycle genes, *CIT1* and *MDH1-1* were also expressed at higher levels in the ileum than the cecum (Tables 3-3, 3-4). The two main enzymes of the glyoxylate pathway were up-regulated in the ileum as well: increased expression of *ICL1* during colonization (Fig. 3-5E) was shown by qRT-PCR, and *MLS1* was slightly up-regulated by microarray (Table 3-4). This suggests that the glyoxylate pathway is important for colonization as well as for systemic virulence [110]. Cells undergoing hypoxia have decreased levels of respiration, consistent with the observed decrease in expression of the TCA cycle in the cecum relative to the ileum by colonizing fungal cells. These data show that acetyl-CoA metabolism and the TCA cycle are more important for metabolism in the ileum than the cecum. Increased expression of metabolic genes in the

ileum involved in acetyl-CoA metabolism may indicate increased utilization of non-fermentable carbon sources during colonization.

Several genes were up-regulated in response to pH during colonization by WT *C. albicans*. *PHO89* and *PHR1* (glycosidase) are both induced in alkaline pH and were up-regulated during colonization (Tables 3-2A, 3-3, 3-4) [103, 218]. Expression of *PHO89* and *PHR1* was higher in the ileum than the cecum in WT *C. albicans* cells by microarray analysis relative to the reference sample (Table 3-3). Consistent with these observations, the pH of the digestive tract is alkaline in the ileum and closer to neutral in the cecum.

PHO89 and *PHR1* are both activated by the *RIM101* pathway under alkaline conditions [219, 220]. Therefore, we used qRT-PCR to measure the expression of *RIM101*. We observed that *RIM101* was up-regulated in the cecum by RT-PCR (Fig. 3-8), and in the ileum as well by microarray (Table 3-4). However, a *rim101*$^{-}$ mutant was able to colonize at WT levels as measured in fecal pellets and GI tract organs (Fig. 3-9A and B). Rim101p was required for systemic virulence; therefore there may be differential requirements for this gene between colonization and systemic infections [43]. Although *RIM101* is not necessary for WT levels of colonization, Rim101p may contribute to gene expression in colonizing cells, similar to the transcription factor Tec1p [177].

One major group of genes down-regulated by WT *C. albicans* in the ileum was the glycosylation pathway. This category included the down-regulation of genes responsible for N-linked glycosylation, hexosylation, and mannosylation (*MNT2*, *MNT3*, *and PMT5*) (Tables 3-3, 3-4). Decreased or altered patterns of glycosylation may allow colonizing cells to avoid the immune response. A decreased inflammatory response was observed in response to *C. albicans* mutants defective in glycosylation, although these

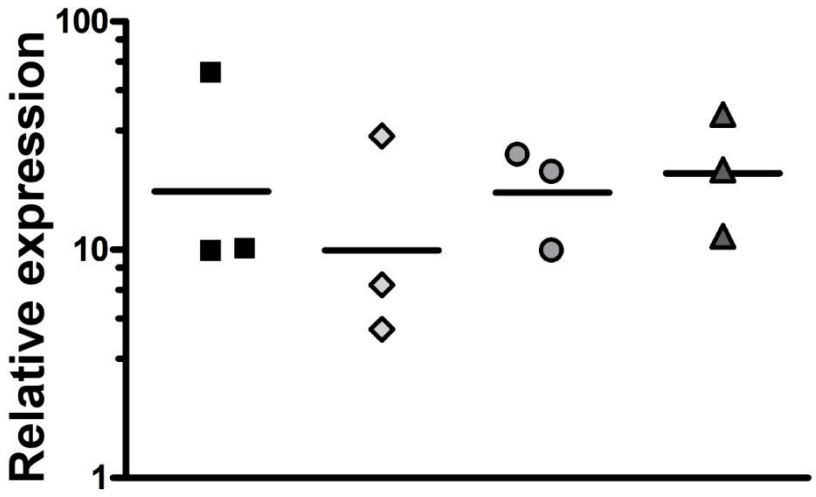


Figure 3-8. *RIM101* expression is increased during colonization by *C. albicans.*

Expression of *RIM101* in the cecum at day 3 post-inoculation. Expression is normalized to *ACT1* for each sample and expressed relative to levels present during laboratory growth. Samples were taken at 3 days post-inoculation from BALB/c mice. WT, squares; *efg1*$^{-}$, diamonds; *efg1*$^{-}$ *efh1*$^{-}$, circles, *efg1*$^{-}$ *cph1*$^{-}$, triangles. Each symbol represents 1 sample from 2 ceca; bars are the geometric mean.

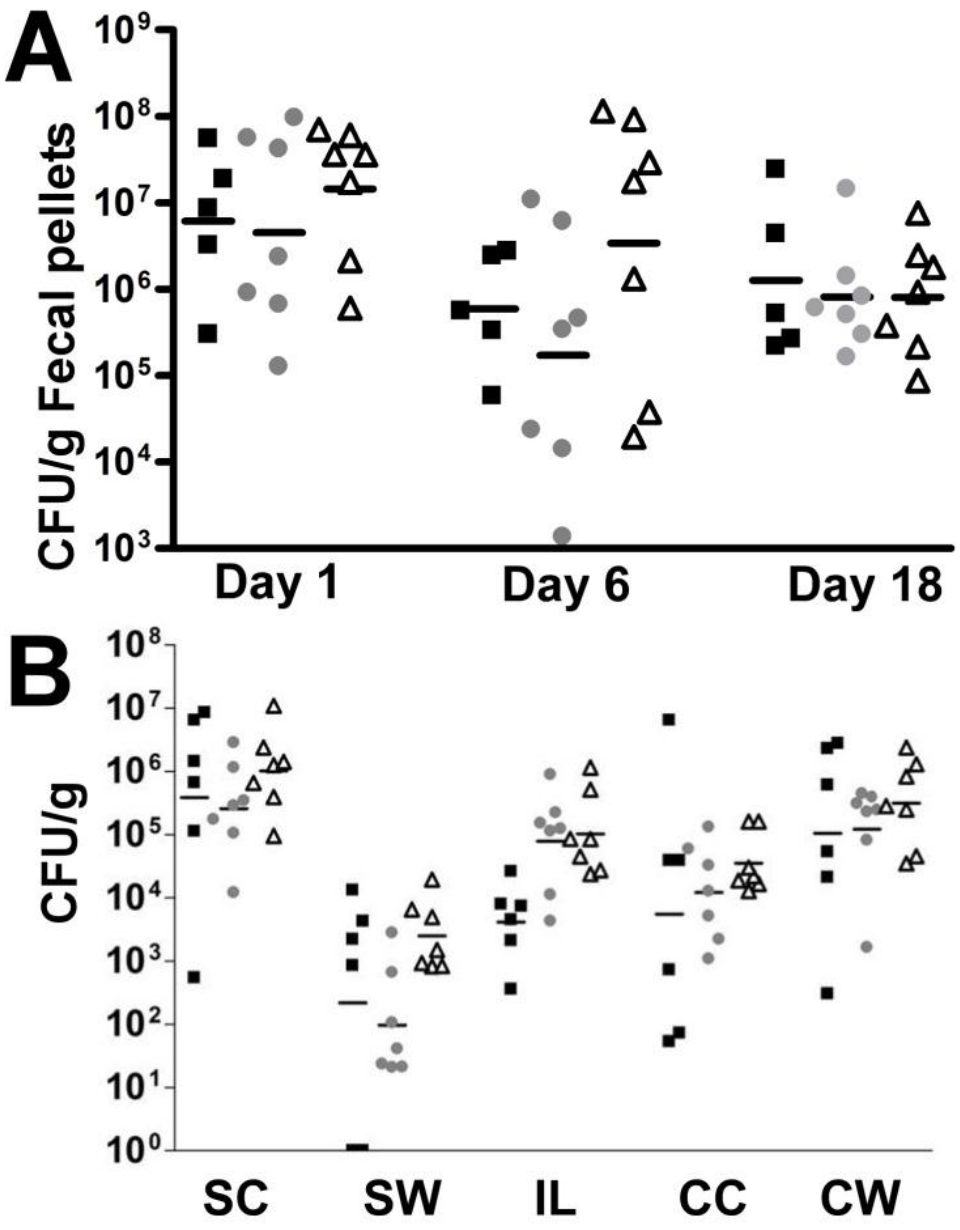


Figure 3-9. *RIM101* is not required for WT levels of GI tract colonization.

BALB/c mice were colonized with WT (squares), *rim101*$^-$ (circles), and p*RIM101*/*rim101*$^-$ (triangles) strains. (A) Colonization of the fecal pellets at days 1, 6, and 18 post-inoculation. (B) Colonization of the GI tract organs at day 18 post-inoculation. SC, stomach contents; SW, stomach wall; IL, ileum; CC, cecum contents; CW, cecum wall. Each symbol represents one mouse; bars are the geometric mean.

mutants have pleotropic effects [221]. In addition, cells with deficiencies in mannosylation were more resistant to phagocytosis by neutrophils [222]. The mannosyltransferases *MNT1* and *MNT2* were required for virulence in systemic infections, while *PMT5* (protein mannosyl transferase) was not [223-225]. Therefore, there may be differential requirements for glycosylation during colonization and systemic infections. A decrease in glycosylation may allow colonizing cells to prevent excessive inflammation and phagocytosis in this environment, in contrast to invasive cells which require glycosylation to cause disease.

In summary, overall patterns of gene expression were similar when *C. albicans* was colonizing the ileum or the cecum, but several differences in gene expression between the two organs were also identified. Cells in the ileum showed increased expression of genes involved in acetyl-CoA metabolism and the TCA cycle. Cells in the ileum also had increased expression of genes regulated by alkaline pH. Down-regulation of genes involved in glycosylation was specific to cells colonizing the ileum. Differences in extracellular signals such as pH, nutrient availability, and the immune response between the cecum and the ileum produce altered gene expression between cells colonizing these two organs. Our data shows that some genes are expressed by colonizing cells in both the cecum and ileum, while others are specific to each particular environment.

Efg1-dependent gene expression during colonization

Under laboratory growth conditions, *EFG1* has been shown to have large effects on transcription in response to various environmental signals [111, 146]. Previous data

showed that a few genes regulated by *EFG1* under laboratory growth conditions are regulated independently of *EFG1* during GI tract colonization [42]. To understand the effect of Efg1p on gene expression during colonization, microarray analysis of gene expression in colonizing *efg1*$^-$ null mutant cells was conducted. BALB/c mice were colonized with the *efg1*$^-$ null strain of *C. albicans*, and fecal pellets were plated to determine the level of colonization. Cecum and ileum contents were used to determine gene expression in colonizing cells. As previously observed [165], the *efg1*$^-$ null mutant strain showed increased colonization relative to WT *C. albicans* at day 3 in the fecal pellets and in the cecum contents ($p<0.0001$, $p=0.0024$, respectively, Mann whitney test) (Fig. 3-2).

Samples of *efg1*$^-$ null mutant cells from either the cecum or the ileum were hybridized to microarray slides along with the WT laboratory reference sample. Data from these microarray samples showed that 252 genes were differentially expressed in the cecum and 712 genes were differentially expressed in the ileum (Table 3-1) by the *efg1*$^-$ null mutant colonizing cells compared to the WT laboratory reference sample. The overlap in gene expression by *efg1*$^-$ null mutant cells colonizing the cecum and ileum was only 132 genes, indicating a larger effect of the niche on gene expression patterns in the *efg1*$^-$ null mutant when compared to the WT laboratory reference. In contrast, WT colonizing cells differentially regulated 348 overlapping genes in the cecum and the ileum samples relative to the WT laboratory reference (Fig. 3-3A). Therefore, there is a greater effect of the environment on gene expression patterns in the *efg1*$^-$ null mutant compared to the WT strain.

Microarray data comparing the mutant and WT colonizing samples to the reference sample were then used to generate gene expression ratios between arrays (mutant/reference / WT/reference). This second step of the analysis allowed us to determine which genes were differentially expressed between the *efg1*$^{-}$ null mutant and WT strains in colonizing cells of both the ileum and the cecum. 227 genes were identified as differentially expressed between the *efg1*$^{-}$ null mutant and WT colonizing cells in the cecum, while only 106 genes were differentially expressed between these two strains in the ileum (Table 3-1, Fig. 3-3B). Therefore, Efg1p regulates a larger number of genes in the cecum than the ileum in colonizing cells. The larger number of genes differentially expressed between the *efg1*$^{-}$ null mutant in the ileum and the WT laboratory reference thus represent genes which are regulated in response to the host environment and not Efg1p. Based on these results, Efg1p-dependent gene expression is more active in response to the environmental signals present in the cecum than the ileum.

Efg1p both positively and negatively regulated gene expression in the cecum and the ileum. In the cecum, GO term analysis identified several categories of genes that were expressed at lower levels in the absence of Efg1p compared to WT colonizing cells (Table 3-5). Efg1p was required for the increased expression of genes involved in host interactions, adhesion, and the oxidative stress response during colonization. Efg1p positively regulated the expression of the adhesins Als3p and Als10p, as well as the secreted aspartyl proteases Saps4-6p in both the cecum and the ileum. Reduced expression of genes involved in the oxidative stress response (*SOD5, CIP1, CAT1,* and *DDR48*) was observed in the absence of Efg1p in the cecum (Table 3-4, 3-5, Fig. 3-7). Efg1p was also required for the full expression of *WH11* in cells colonizing the cecum

Gene Ontology	Corrected P-value	Genes
Higher expression in the absence of Efg1p		
Cecum		
None		
Ileum		
Carbohydrate transport activity	<0.0001	*AGP2, CNT, GIT1, HGT2, HGT9, HXT5, JEN1, NGT1*, ORF19.409
Cell surface/cell membrane	<0.0001	*ALS7, ENG1, OP4, PGA26, PGA31, PGA45, PLB4, PLC2, RBE1, RHD3*, ORF19.1172, ORF19.2045, ORF19.6066
Lower expression in the absence of Efg1p		
Cecum		
Host interaction	0.001	*ALS10, CAT1, HSP70, HSX11, HYR1, PGA10, PTP3 RBT4, RCK2, SAP4, SAP5, SAP6, SPD5, TPS2, UME6, WH11*, ORF19.60, ORF19.6589
Response to oxidative stress	0.002	*AHP1, CAT1, CIP1, DDR48, EBP1, PST1, PST2, MET10, SOD5*, ORF19.86
Fungal cell wall	<0.0001	*ALS3*
Purine transport	0.009	*ANT1, YHM1*
Transaminase activity	0.009	*ARO8*, ORF19.1180
Catalytic activity	0.026	*ARO3, CCC2, EMC4, EFG9, GPI3, MET1, MET3, PRP22, SPC3, TRP3*, ORF19.1249, ORF19.3982, ORF19.4567, ORF19.496, ORF19.5617
Ileum		
Adhesion	<0.0001	*ALS10, HYR1, MNT2, SAP4, SAP5, SAP6, TEC1*
Host interaction	0.0001	*SOD5, RBT1, RCK2, UME6*
Fungal cell wall	0.012	*ALS3, GPM2, PST2*

Table 3-5. Genes differentially expressed by the *efg1*$^{-}$ null mutant strain during colonization.

GO terms listed were significantly enriched ($p<0.05$) when comparing gene expression of the *efg1*$^{-}$ null mutant strain relative to WT gene expression. Groups which were either up- or down-regulated in the cecum or the ileum are listed. Duplicate genes are only listed under one category and overlapping categories were combined. P-values were corrected for false discovery rate.

(Fig. 3-5D). These data suggest that Efg1p plays a large role in positively regulating host interactions through cell surface protein expression as well as protection against oxidative stress in colonizing cells.

Fewer categories of genes had increased expression in the absence of Efg1p relative to WT *C. albicans* during colonization. In the ileum, more functional groups of genes were repressed by Efg1p than in the cecum. GO term analysis identified genes involved in carbohydrate transport (*HGT2* and *HGT9*) and cell surface proteins (*ALS7*, *PLB4*, *PLC2*) as increased in expression in the *efg1*$^{-}$ null (Table 3-5) compared to WT colonizing cells in the ileum. Expression of carnitine biosynthesis and utilization genes (*AGP2*, *CAT2*, orf19.6306) increased in the *efg1*$^{-}$ null mutant in the ileum relative to WT gene expression in colonizing cells (Table 3-6). The expression of *CTN1*, a carnitine acetyltransferase, was higher in the absence of Efg1p in the cecum relative to WT *C. albicans* as measured by qRT-PCR (Fig. 3-4). Increased expression of genes involved in the utilization of non-fermentable carbon sources such as β-oxidation of fatty acids and alcohol metabolism was noted in both the cecum and the ileum in the absence of Efg1p. Enhanced carnitine utilization would be expected to increase the ability of the *efg1*$^{-}$ null mutant strain to utilize the non-fermentable carbon sources present in the GI tract. In laboratory growth on glucose-containing medium, a previous study showed that genes involved in glycolysis and oxidative metabolism had increased expression in the *efg1*$^{-}$ null mutant [111]. However, these trends were not observed in colonizing cells. The altered expression of carnitine metabolism genes observed in colonizing *efg1*$^{-}$ null mutant cells was not noted in laboratory studies [111], perhaps because of differences in carbon sources available in the gut and laboratory culture medium. Increased expression of

Gene	Gene name	*efg1*$^{-}$ Cecum	*efg1*$^{-}$ *efh1*$^{-}$ Cecum	*efg1*$^{-}$ *cph1*$^{-}$ Cecum	*efg1*$^{-}$ Ileum	*efg1*$^{-}$ *efh1*$^{-}$ Ileum	*efg1*$^{-}$ *cph1*$^{-}$ Ileum
AGP2	Carnitine transporter activity	1.44	1.17	1.27	1.69*$^{+}$	1.35	1.41
CAT2	Carnitine acetyl transferase	1.30	1.26	1.25	2.11*	1.70*	1.33
CTN1	Predicted carnitine acetyl transferase	0.69	1.17	0.71	1.19	0.83	0.99
CTN3	Predicted carnitine acetyl transferase	0.88	0.72	0.71	1.16	0.90	0.80
Orf 19.4316	Trimethyllysine dioxygenase	1.23	1.35	0.85	1.16	1.02	1.00
Orf 19.6305	Hydroxytrimethyllysine aldolase	0.81	1.09	0.89	1.00	0.98	1.12
Orf 19.6306	Trimethylaminobutyraldehyde dehydrogenase	0.87	0.94	0.96	1.70*	1.26	1.13
Orf 19.7131	Butyrobetaine dioxygenase	0.96	0.97	1.41	1.02	1.00	1.23

Table 3-6. Expression of genes involved in carnitine biosynthesis and utilization during colonization.

Gene expression is shown for each of the *efg1*$^{-}$ null mutant in the cecum and ileum relative to the expression in WT colonizing cells in the cecum using microarray data. *indicates fold-change greater than 1.5 relative to WT colonizing cells based on microarray ratios. + indicates p-value <0.05 between mutant and WT colonizing cell samples.

metabolic genes in the absence of Efg1p may promote the hyper-colonization of the *efg1*$^{-}$ null mutant in the GI tract that we observed previously (Fig. 3-2) [165].

Previous studies showed that a few hyphal-regulated genes are expressed independently of Efg1p during colonization, despite being regulated by Efg1p during laboratory growth [42]. In these studies, we observed that a subset of genes previously identified as Efg1p-dependent also require Efg1p for their expression in colonizing cells. The genes encoding the adhesin Als3p, the hyphal-regulated cell surface protein Hyr1p, the secreted aspartyl proteases Saps4-6p, and the superoxide dismutase Sod5p (Table 3-7; Fig. 3-5A and 3-7) all required Efg1p for their expression during colonization. However, other genes, including the gene encoding hyphal wall protein *HWP1*, the pH-responsive glycosidase *PHR1*, and the hemoglobin utilization gene *RBT5* (Table 3-7 and Fig. 3-5A) were not affected by the absence of Efg1p in colonizing cells [42]. In these studies, we therefore identified additional genes which are regulated independently of Efg1p during colonization. Therefore, genes regulated by hyphal growth in the laboratory are expressed during colonization, but only a subset of these genes requires Efg1p for their expression.

Effect of Efh1p and Cph1p on Efg1p-dependent gene expression patterns

To further understand the role of Efg1p in the regulation of gene expression during colonization, we expanded our study to include the *efg1*$^{-}$ *efh1*$^{-}$ and *efg1*$^{-}$ *cph1*$^{-}$ double null mutant strains of *C. albicans*. These strains were inoculated into BALB/c mice, and at days 1 and 3 post-inoculation, fecal pellets were plated to determine colonization levels. Microarray analysis was used to determine the global gene

Genes regulated by Efg1p during colonization	**WT Cecum**	***efg1*$^{-}$ Cecum**	***efg1*$^{-}$ *efh1*$^{-}$ Cecum**	***efg1*$^{-}$ *cph1*$^{-}$ Cecum**	**WT Ileum**	***efg1*$^{-}$ Ileum**	***efg1*$^{-}$ *efh1*$^{-}$ Ileum**	***efg1*$^{-}$ *cph1*$^{-}$ Ileum**
ALS3	2.3*	1.0+	0.9+	1.5+	2.2*	0.8+	0.9+	1.1+
DDR48	2.1*	1.1+	1.2+	1.6+	1.90*	1.5	1.3+	1.8+
HYR1	1.8*	0.8+	0.8+	1.0+	1.4	0.8+	0.8+	0.8
RBT4	1.9*	0.9+	1.0+	1.4+	2.0*	1.5	1.6	1.8
SAP4	3.7*	1.0+	0.9+	1.0+	5.1*	0.9+	0.9+	0.8+
SAP5	2.7*	1.5+	1.0+	1.0+	3.9*	1.5+	1.1+	1.00+
SAP6	1.9*	0.7+	0.8+	0.6+	2.8*	0.6+	0.8+	0.6+
SOD5	11.9*	1.2+	1.4+	1.2+	4.4*	2.2+	1.9+	1.1+
TPS2	1.6*	1.0+	1.1	1.4	1.5*	1.1+	1.3	1.5
Genes regulated independently of *EFG1* during colonization								
HWP1	1.7	1.4	1.3	1.5	1.8*	1.5	1.7	1.9
PHR1	1.9*	2.2	2.0	3.0+	2.8*	2.2	2.1	2.7
RBT1	2.3*	1.6	1.4+	1.7	2.6*	1.6+	1.5+	1.9
RBT5	2.5*	2.5	1.7	3.3	2.7*	2.2	2.4	3.5

Table 3-7. Comparison of genes regulated by Efg1p in the laboratory or colonization.

Regulation of previously identified targets of Efg1p during GI tract colonization is shown by microarray data. Fold changes are relative the WT laboratory grown reference sample. Genes regulated by Efg1p in both laboratory conditions and colonization are shown; Efg1p-independent gene expression of known targets is also shown. * $p<0.05$ from microarray analysis comparing WT colonizing cells to the laboratory reference; + $p<0.05$ comparing an *efg1*$^{-}$ null mutant array data (single or double) to WT colonizing cells microarray data in either the cecum or the ileum. Ratios of gene expression between colonizing cells and the references sample are color-coded: bright yellow >1.5, light yellow >1.25, light blue <0.75.

expression patterns from cells colonizing the cecum and ileum. The *efg1*$^{-}$ *efh1*$^{-}$ double null strain hyper-colonized at day 3 in the fecal pellets and cecum contents (p=0.0305, p=0.0304, respectively), similar to the *efg1*$^{-}$ null mutant alone (Fig. 3-2). Similar patterns of gene expression between the *efg1*$^{-}$ single null and *efg1*$^{-}$ *efh1*$^{-}$ double null strains were consistent with their colonization phenotype. More genes were differentially regulated by the *efg1*$^{-}$ *efh1*$^{-}$ double null strain compared to WT *C. albicans* colonizing cells in the cecum (227) than the ileum (86) (Table 3-1).

Genes involved in host interactions, oxidative stress response, and cell surface proteins had decreased expression in *efg1*$^{-}$ *efh1*$^{-}$ double null cells compared to WT cells in the GI tract (Table 3-8). Expression of *CAT2,* a carnitine acetyltransferase, was increased in the *efg1*$^{-}$ *efh1*$^{-}$ double null mutant in the ileum compared to WT colonizing cells (Table 3-6). Increased expression of genes involved in carnitine metabolism may contribute to the hyper-colonization of the *efg1*$^{-}$ *efh1*$^{-}$ double null mutant. Overall, the *efg1*$^{-}$ *efh1*$^{-}$ double null mutant had similar patterns of gene expression to the *efg1*$^{-}$ mutant, supporting previous data that these two mutants act similarly and express a common group of genes during laboratory growth [111].

While colonization of the *efg1*$^{-}$ null mutant was not changed by the addition of the *efh1*$^{-}$ deletion, addition of a *cph1*$^{-}$ deletion to an *efg1*$^{-}$ null mutant caused a return to WT levels of colonization (Fig. 3-2). Analysis of gene expression during colonization showed that more genes were differentially expressed by the *efg1*$^{-}$ *cph1*$^{-}$ double null mutant (cecum, 353; ileum, 187) than either the *efg1*$^{-}$ single null or *efg1*$^{-}$ *efh1*$^{-}$ double null mutants relative to WT gene expression in the gut (Table 3-1). This finding mirrors the previous observation that more genes are altered in expression in the *efg1*$^{-}$ *cph1*$^{-}$

Gene Ontology	Corrected P-value	Genes
Higher expression in the absence of Efg1p and Efh1p		
Cecum		
None		
Ileum		
Proteasome Regulation	0.048	*RPT2, RPT4*
Lower expression in the absence of Efg1p and Efh1p		
Cecum		
Host interaction	0.001	*ALS10, CAT1, HSX11, HYR1, PGA10, RBT1, RBT4, SAP4, SAP5, SAP6, SOD5, TEC1, UME6*
Peptidase activity	0.02	*SPC3*, ORF19. 1195
Fungal cell wall	<0.0001	*ALS3, CAT1, DDR48, HYR1, PGA10, PST2, RBT1, RBT4, SAP4, SAP5, SAP6, SOD5*
Ileum		
Adhesion	<0.0001	*AHR1, ALS10, HYR1, MNT2, SAP4, SAP5, SAP6, TDH3, TEC1*
Host interaction	<0.0001	*ECE1, RBT1, RIM101, SOD5, UME6, WH11*
Alpha glucosidase activity	0.013	*GDB1*, ORF19.3982
Fungal cell wall	<0.0001	*ACB1, ALS3, DDR48, PGA23, PST2*

Table 3-8. Genes differentially expressed by the *efg1*$^{-}$ *efh1*$^{-}$ double null mutant strain during colonization.

Up- or down-regulated GO terms listed were significantly enriched ($p<0.05$) when comparing gene expression of the *efg1*$^{-}$ *efh1*$^{-}$ double null mutant strain relative to WT gene expression in colonizing cells of either the cecum or ileum. Duplicate genes are only listed under one category and overlapping categories were combined. P-values were corrected for false discovery rate.

double null mutant than the *efg1*$^{-}$ single null mutant relative to WT cells during laboratory growth [111].

The expression of several cell surface genes was altered in the *efg1*$^{-}$ *cph1*$^{-}$ strain relative to WT colonizing cells, and this effect was more prominent in the ileum than the cecum (Table 3-9). Increased expression of genes involved in carbohydrate catabolism and sugar transport by the *efg1*$^{-}$ *cph1*$^{-}$ double null relative to WT colonizing cells in the ileum was observed (Table 3-9). In the cecum, GO term analysis identified increased expression of genes involved in signal transduction and the glyoxylate cycle by the *efg1*$^{-}$ *cph1*$^{-}$ double null strain compared to WT colonizing cells (Table 3-9). Interestingly, increased expression of genes involved in carnitine metabolism relative to WT colonizing cells was not detected in this mutant (Table 3-6). The lack of higher expression of genes involved in carnitine metabolism may contribute to the lack of hyper-colonization by the *efg1*$^{-}$ *cph1*$^{-}$ double null strain (Fig. 3-2). Thus, for gene expression during colonization, the addition of the *cph1*$^{-}$ deletion either reversed the effects of the *efg1*$^{-}$ null mutant alone, or generated a compensatory effect in contrast to the partial redundancy between Efg1p and Cph1p observed under other conditions [107, 111].

A number of genes were identified as regulated by Efg1p in all three mutants: 47 genes in the cecum and 29 in the ileum (Table 3-10A, B). Of these, 30 genes represent unidentified ORFs (Table 3-10B). This observation suggests that a large number of conserved Efg1p targets represent previously uncategorized genes which may have functions specific to colonization. Genes co-regulated between the three *efg1*$^{-}$ mutants included the adhesins *ALS1* and *ALS10*, as well as the heat shock genes *HSP30*, *21*, and *78*. The family of secreted aspartyl proteases Saps4-6p and the superoxide dismutase

Gene Ontology	Corrected P-value	Genes
Higher expression in the absence of Efg1p and Cph1p		
Cecum		
Signal transduction	0.001	*GPR1, HGT4, MKC1, NIK1, OPY2, PTC1, RAS2, RIM15, RIM101, SAC7, SKO1, SOG2, STE11, TPK2, YVH1*
Glyoxylate cycle	0.012	*ICL1, MLS1,* ORF19.3070.1
Ileum		
Carbohydrate transport	0.012	*GIT2, HGT2, HGT9, HGT12, JEN1, JEN2, NGT1, PHO87, YMC1*
Fungal cell wall	0.02	*CHT2, ENG1, OP4, RBE1, RHD3*
Lower expression in the absence of Efg1p and Cph1p		
Cecum		
Fungal cell wall	0.015	*ALS3, GCA1, HYR1,LIP10, SAP4, SAP5, SAP6, SOD5*
Ileum		
Host interaction	0.002	*ALS10, HYR1, RIM101, SAP4, SAP5, SAP6, SAP9, SOD5, TEC1, VSP11*
Adhesion	0.005	*AHR1*
Carbohydrate catabolism	0.01	*AMS1, DAC1, GAL10, GDB1, SGA1, SUC1,* ORF19.3982

Table 3-9. Genes differentially expressed by the *efg1*$^{-}$ *cph1*$^{-}$ double null mutant strain during colonization.

Up- or down-regulated GO terms listed were significantly enriched ($p<0.05$) when comparing gene expression of the *efg1*$^{-}$ *cph1*$^{-}$ double null mutant strain relative to WT gene expression. Duplicate genes are only listed under one category and overlapping categories were combined. P-values were corrected for false discovery rate.

Gene name	Cecum/Ileum	Gene description	Gene function
ALS10	Cecum, Ileum	Agglutinin-like sequence 10	Adhesin
ALS3	Cecum, Ileum	Agglutinin-like sequence 3	Adhesin
ENG1	Cecum	Endo-1,3-beta gluconase	Cell separation
FDH1	Ileum	Putative formate dehydrogenase	Uncharacterized
GIT2	Ileum	Putative glycerophosphinositol permease	Uncharacterized
HGT9	Cecum	Putative glucose transporter	Uncharacterized
HSP30	Cecum	Putative heat shock protein 30	Fluconazole down-regulated
HSP31	Ileum	Putative heat shock protein	Uncharacterized
HSP78	Ileum	Putative heat shock protein	Uncharacterized
HSX11	Cecum	UDP-glucose:ceramide glucosyltransferase	Glucosylceramide biosynthesis
HYR1	Cecum, Ileum	Hyphally regulated protein 1	GPI anchored cell wall protein
INO1	Cecum	Inositol-1-phosphate synthase	Inositol biosynthesis
JEN1	Ileum	Lactate transporter	Induced by lactic acid
OPT4	Cecum	Oligopeptide transporter	Phagocytosis induced
PGA10	Cecum	Predicted-GPI-anchored	Plasma membrane protein
PGA23	Ileum	Putative GPI-anchored protein 23	Regulated by *RIM101*
PGA26	Ileum	Putative GPI-anchored protein 26	Cell wall integrity, virulence
PPZ1	Cecum	Protein phosphatase Z	Cell wall integrity
PRP22	Cecum, Ileum	Putative RNA-dependent ATPase	Regulated by weak acid stress
PST2	Cecum, Ileum	Putative NADH:quinone oxidoreductase	Oxidative stress induced
RHD3	Ileum	GPI-anchored cell wall protein	Regulated by iron availability
RIM101	Cecum	Transcription factor	Alkaline pH response, hyphal growth, virulence
SAP4	Cecum, Ileum	Secreted aspartyl protease 4	Aspartic-type endopeptidase
SAP5	Cecum, Ileum	Secreted aspartyl protease 5	Aspartic-type endopeptidase
SAP6	Cecum, Ileum	Secreted aspartyl protease 6	Aspartic-type endopeptidase
SKN1	Cecum	Predicted role in beta-1,6-glucan synthesis	Uncharacterized
SOD5	Cecum, Ileum	Superoxide dismutase 5	Protective against oxidative stress; induced by neutrophil contact
SPC3	Cecum	Signal peptidase complex 3	Essential, ER membrane protein

TEC1	Ileum	Transcription factor	Regulates hyphal specific genes
UME6	Cecum, Ileum	Transcription factor	Required for hyphal formation and virulence

Table 3-10A. Core genes regulated by Efg1p during colonization.

Genes identified as differentially expressed in *efg1*$^{-}$, *efg1*$^{-}$ *efh1-*, and *efg1*$^{-}$ *cph1*$^{-}$ null mutants relative to WT expression levels in colonizing cells. Organ specificity is shown as either cecum-specific, ileum-specific, or present in both organs.

Gene name	Cecum/Ileum	Gene description	Gene function
orf19.1409.2	Cecum, Ileum	Uncharacterized	Uncharacterized
orf19.1409.3	Ileum	Uncharacterized	Uncharacterized
orf19.1774	Cecum	Uncharacterized	Up-regulated during oral candidiasis
orf19.1812	Cecum	Uncharacterized	Putative phosphatase of the PTP family
orf19.1842	Cecum	Uncharacterized	Cellular bud site location
orf19.2168	Ileum	Uncharacterized	Cellular protein localization
orf19.3244	Cecum	Uncharacterized	Mitochondrial respiratory chain complex
orf19.3245	Cecum	Uncharacterized	Uncharacterized
orf19.3263	Cecum	Uncharacterized	Putative transmembrane protein
orf19.3439	Ileum	Uncharacterized	Uncharacterized
orf19.3499	Cecum, Ileum	Uncharacterized	Secreted, stress induced
orf19.3718	Cecum	Uncharacterized	Gene fragment
orf19.3982	Cecum	MAL32	Maltase, induced on sucrose
orf19.4066	Cecum	Uncharacterized	HOG1-downregulated
orf19.4342	Cecum	Uncharacterized	Biofilm induced
orf19.4370	Cecum	Uncharacterized	Induced by oxidative stress
orf19.4524	Cecum	Uncharacterized	Predicted zinc finger protein
orf19.4567	Cecum	Uncharacterized	Degradation of aromatic compounds
orf19.4768	Cecum	Uncharacterized	Uncharacterized
orf19.4883	Cecum	Uncharacterized	Uncharacterized
orf19.4936.1	Cecum	Uncharacterized	Putative adhesion like protein
orf19.5572	Cecum	Uncharacterized	Uncharacterized
orf19.5619	Ileum	Uncharacterized	Alpha-pheromone response
orf19.6277	Cecum	Uncharacterized	Cell communication, predicted phosphatidyl

			inositol binding
orf19.7041	Cecum	Uncharacterized	Putative t-RNA processing
orf19.7215	Cecum	Uncharacterized	Sno-RNA binding activity
orf19.7227	Ileum	Putative protein phosphate inhibitor	Drug sensitivity
orf19.730	Cecum	Uncharacterized	Putative Rho-GTPase
orf19.7341	Ileum	Uncharacterized	Biofilm induced
orf19.7402	Ileum	Uncharacterized	Putative white-opaque switching modulator

Table 3-10B. Unidentified ORFs regulated by Efg1p during colonization.

Genes identified as differentially expressed in *efg1*$^{-}$, *efg1*$^{-}$ *efh1-*, and *efg1*$^{-}$ *cph1*$^{-}$ null mutants relative to WT expression levels in colonizing cells. Organ specificity is shown as either cecum-specific, ileum-specific, or present in both organs.

Sod5p were also co-regulated by all three *efg1*$^{-}$ mutants. The expression of several transcription factors, including Tec1p, Rim101p, and Ume6p, also appeared to be affected by Efg1p. Therefore, in colonizing cells, the regulation of genes involved in host interactions was conserved between these three *efg1*$^{-}$ mutants. However, metabolic gene expression was different between these mutants, and may explain the differential ability of these strains to hyper-colonize in the GI tract.

Altered growth of the *efg1*$^{-}$ null mutant is dependent on carnitine metabolism

When introduced into the GI tract of a naïve mouse, the *efg1*$^{-}$ null mutant reaches a higher population density. This mutant also showed altered metabolic gene expression during colonization compared to WT colonizing cells. To determine whether the mutant strain could grow to higher levels under other conditions, WT and *efg1*$^{-}$ null mutant strains were grown in the laboratory in rich media under yeast growth conditions at 30°C. The concentration of cells in the culture was measured by counting the number of cells/mL with a hemacytometer, as differences in cellular morphology and size alter optical density. During exponential phase growth in glucose-contained medium, both strains grew well and the experimentally determined doubling times during this phase (WT, 58 min; *efg1*$^{-}$, 70 min) were similar to previously published values (WT, 57.6 min; *efg1*$^{-}$, 69 min) [107]. However, at the end of exponential phase growth, wild type cells paused briefly before resuming growth at a slower rate, while *efg1*$^{-}$ null mutant cells continued to grow ($p=0.0036$, 6.25hrs., Student's t test) (Fig. 3-10A). The *efg1*$^{-}$ null mutant strain then reached a higher density during post-exponential phase than wild type *C. albicans* ($p=0.0013$, 48hrs.) (Fig. 3-10B). Therefore, increased growth of the *efg1*$^{-}$

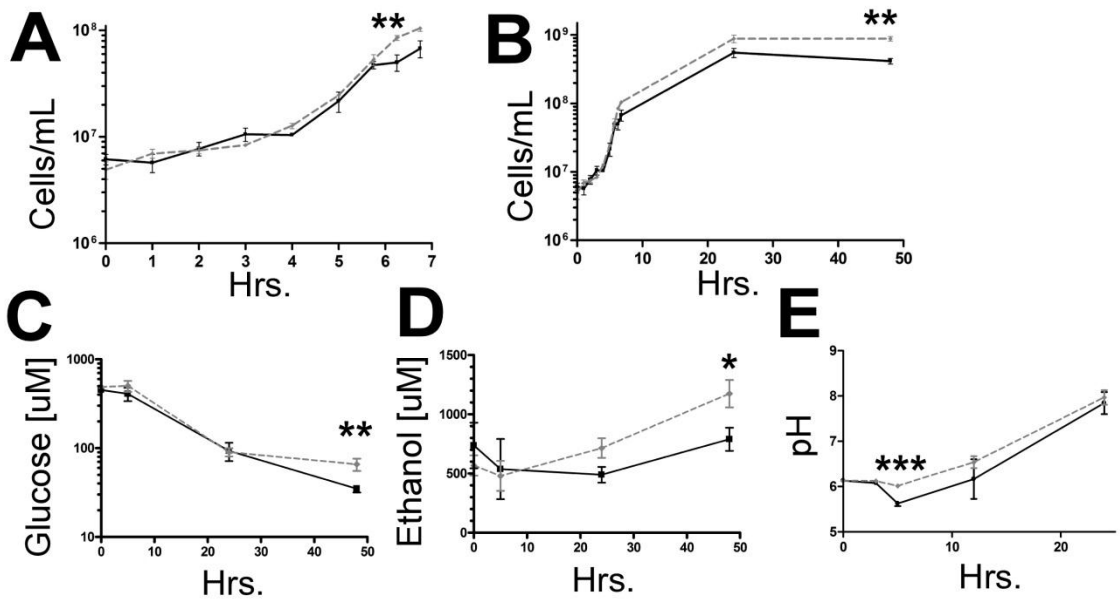


Figure 3-10. Altered growth of the *efg1*$^{-}$ null mutant.

Growth was determined by counting cells with a hemacytometer to determine the number of cells/mL. (A) Growth of WT *C. albicans*, black squares, compared to growth of the *efg1*$^{-}$ null mutant strain, grey triangles, during log phase. (B) Growth of WT and *efg1*$^{-}$ strains in post-exponential phase. (C) Concentration of glucose (µM) during growth of WT and *efg1*$^{-}$ null strains in rich yeast growth media (YPD). Samples were taken from the supernatant of growing cultures in log phase or post-exponential phase. (D) Production of ethanol by WT and *efg1*$^{-}$ null strains during log phase and post-exponential phase growth. Concentration of ethanol (µM) in culture supernatants is shown. (E) pH of the culture supernatant from either WT or *efg1*$^{-}$ null mutant cells grown in YPD in both log phase and post-exponential phase. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; Student's t-test.

mutant was observed under laboratory conditions and during colonization.

To determine if the altered growth observed by the *efg1*$^{-}$ null mutant was due to changes in metabolism, the levels of glucose consumption and ethanol production by this mutant were measured. Supernatants of WT and *efg1*$^{-}$ null mutant cells were collected at various times, and the levels of glucose and ethanol were measured in these samples. After 48 hours of growth, more glucose was present in the supernatant of the *efg1*$^{-}$ null mutant cells than WT cells ($p=0.0079$, 48hrs.) (Fig. 3-10C). Additionally, the *efg1*$^{-}$ null mutant cells produced higher levels of ethanol ($p=0.0350$, 48hrs.) (Fig. 3-10D). These observations suggest that the *efg1*$^{-}$ null mutant strain had reduced utilization of glucose as a carbon source, consistent with the previous demonstration that the *efg1*$^{-}$ null mutant has reduced glycolytic flux relative to WT *C. albicans* [111]. In addition, during log phase WT cells acidified the medium (Fig. 3-10E), while the pH of the supernatant of an *efg1*$^{-}$ null mutant remained more alkaline during log phase growth ($p=0.0003$). Since acidification of the media during log phase is correlated with glucose consumption [226], these results are consistent with altered metabolism in the *efg1*$^{-}$ null mutant. These data confirm that there are significant differences in the metabolism of the WT strain and the *efg1*$^{-}$ null mutant.

To determine the ability of both WT and *efg1*$^{-}$ null mutant cells to grow in nutrient poor conditions, depleted medium was used as a culture medium. In these experiments, filter-sterilized culture supernatant from WT cells grown for 5 days was re-inoculated with either WT or *efg1*$^{-}$ null mutant cells. WT cells were unable to grow in the depleted medium after 4 hours, while the *efg1*$^{-}$ null strain was able to undergo

additional growth (p=0.0087) (Fig. 3-11B). Thus, the *efg1*$^{-}$ null mutant was capable of growth under conditions where the WT strain could not.

During colonization, genes involved in carnitine metabolism (e.g. *AGP2*, *CAT2*, and *CTN1*) were expressed at higher levels in *efg1*$^{-}$ null mutant cells than WT cells (Table 3-6, Figs. 3-4, 3-11A and B). Carnitine is a metabolite that is required for growth on non-fermentable carbon sources, such as ethanol and acetate. Carnitine is used as a shuttle to transfer acetyl-CoA derived from non-fermentable carbon sources to the TCA cycle in order to generate ATP (Fig. 3-11B). While carnitine metabolism is not required for virulence in systemic infections [57], our data suggest that it may be important during colonization. The increased expression of genes involved in carnitine metabolism by the *efg1*$^{-}$ null mutant may contribute to enhanced metabolism during colonization of naïve hosts.

To determine whether addition of carnitine would allow WT cells to grow in depleted medium, carnitine was added to the depleted medium. Under these conditions, WT cells were able to grow (p=0.0181 after 4 hours) (Fig. 3-11A). Similar trends in growth were observed over a period of 24 hours: WT cells were able to grow when carnitine was added to the media (p=0.0009) but not without. In contrast, *efg1*$^{-}$ null mutant cells were able to grow in the absence of additional carnitine (p=0.0007). When carnitine was added, the *efg1*$^{-}$ null mutant was also able to undergo further growth (p=0.0042). These results show that growth of WT cells under these conditions is limited by availability of carnitine. The ability of the *efg1*$^{-}$ null mutant to grow in the absence of additional carnitine may reflect increased levels of carnitine within the cell, which may be due to increased carnitine biosynthesis or increased enzymatic activity.

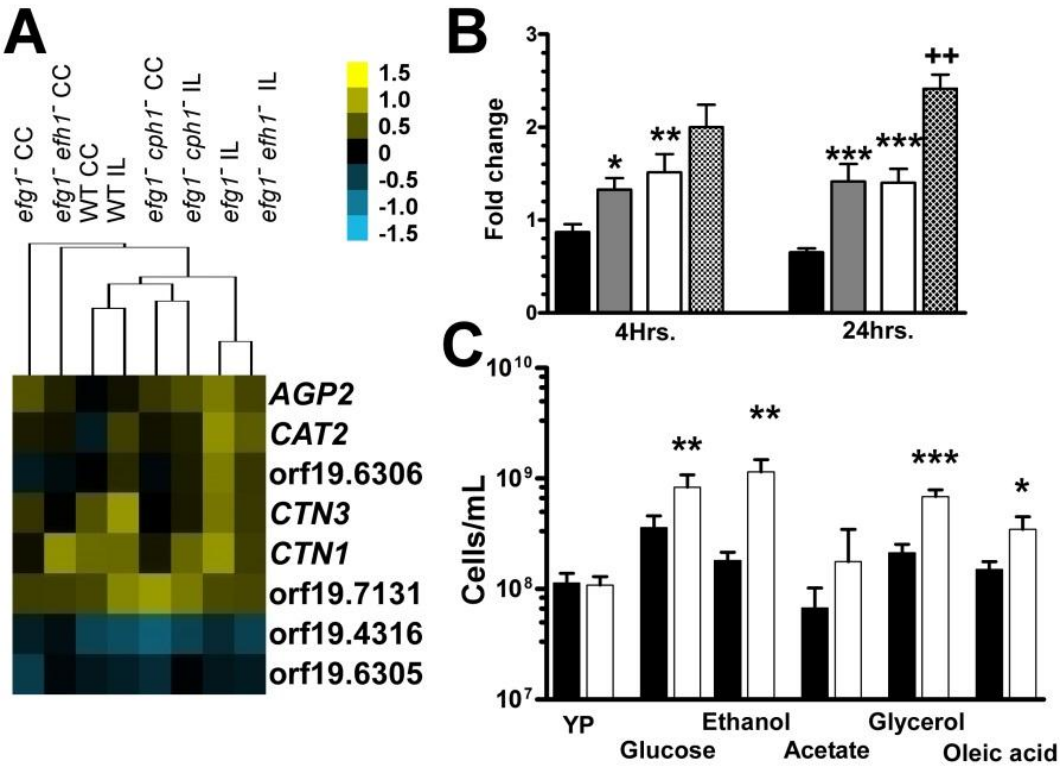


Figure 3-11A. Role of carnitine in increased growth of the *efg1*⁻ null mutant in nutrient-limiting conditions.

(A) Heat map of genes involved in carnitine biosynthesis and utilization in colonizing cells in the cecum and ileum at day 3 post-inoculation. Gene expression is shown relative to laboratory reference levels. Genes which are up-regulated are colored yellow; genes which are down-regulated are colored blue. (B) WT and *efg1*⁻ null mutant strains were grown at 30°C and growth was measured by counting cells using a hemacytometer. Cells were grown in depleted media +/- 10μM carnitine. Growth is shown as fold change after 4 and 24hrs. after inoculation from an overnight culture. WT, black bars; WT + carnitine, grey bars; *efg1*⁻, white bars; *efg1*⁻ + carnitine, cross hatched bars. *p<0.05, **p<0.01, ***p<0.0001; Student's t test comparing either the *efg1*⁻ strain or WT strain + carnitine to the WT strain without carnitine. ++ p<0.01 Student's t test comparing *efg1*⁻ growth with carnitine to *efg1*⁻ without carnitine. (C) Growth of WT and *efg1*⁻ strains on different carbon sources at 30°C at 24hrs. post-inoculation: YP (yeast extract and peptone, no carbon source), glucose, ethanol, acetate, glycerol, oleic acid. WT, black bars; *efg1*⁻, white bars. *p<0.05, **p<0.01, ***p<0.0001; Student's t test.

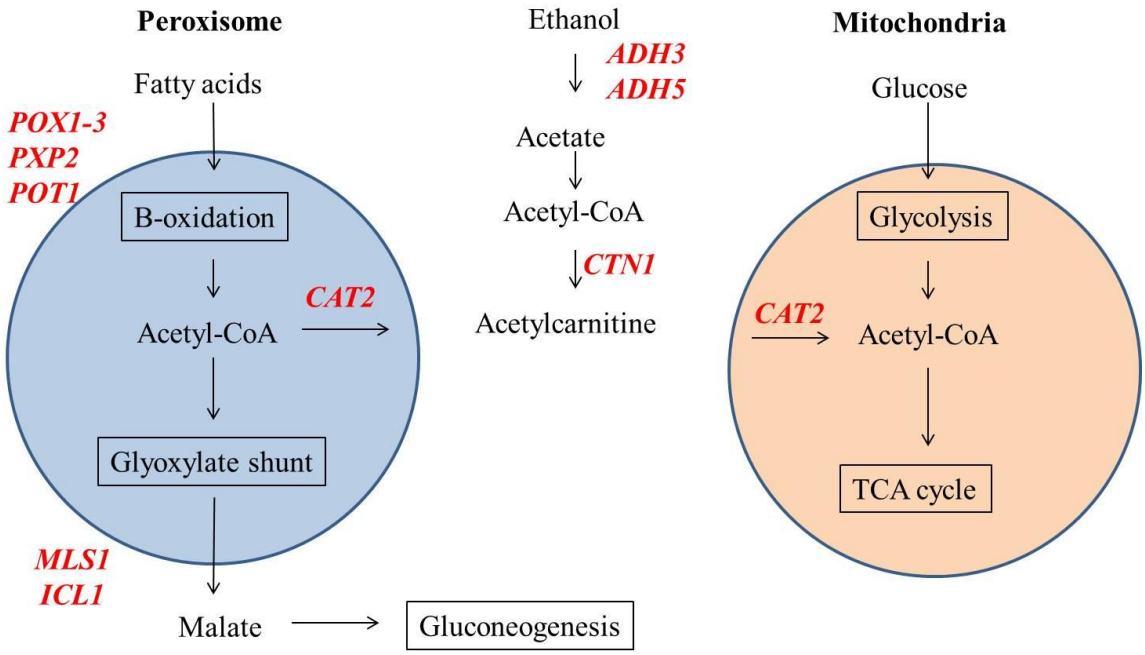


Figure 3-11B. Increased expression of genes involved in the utilization of non-fermentable carbon sources in the absence of *EFG1* during GI tract colonization.

Metabolic pathways required for growth on non-fermentable carbon sources including fatty acids and alcohol. Genes which are expressed at higher levels in the absence of *EFG1* are shown in red. Catabolism of fatty acids and alcohols results in the shuttling of acetyl-CoA from the peroxisome and the cytosol to the mitochondria via the carnitine shuttle. These compounds are then incorporated into the TCA cycle in order to produce ATP.

Growth on certain carbon sources, especially short chain fatty acids, requires carnitine [56, 57]. To determine if the *efg1*$^{-}$ null mutant was able to grow better on alternative carbon sources compared to WT *C. albicans*, growth was measured in the presence of several different carbon sources. As a control, both WT and *efg1*$^{-}$ null cells were grown in YP (yeast extract and peptone) in the absence of any carbon source. Glucose, ethanol, acetate, glycerol, and oleic acid were added to the YP media to a 2% final concentration, and WT and *efg1*$^{-}$ null strains were inoculated at $5x10^{6}$ cells/mL. Cells were counted at 5, 24, and 48 hours post-inoculation (Table 3-11). During log phase growth, the WT and *efg1*$^{-}$ null mutants grew at an equal rate on all carbon sources except oleic acid. Increased growth of the *efg1*$^{-}$ null mutant was observed on oleic acid as early as 5 hours post-inoculation (Table 3-11). After 24 hours of growth, no difference in growth was noted between the WT and *efg1*$^{-}$ null mutant strains in YP. In glucose, the *efg1*$^{-}$ null mutant grew to 2-3 fold higher levels than WT cells (p=0.0005) (Fig. 3-11A) as previously observed. In acetate, glycerol (p=0.0001), and oleic acid (p=0.033), the *efg1* null mutant also grew to 2-3 fold higher levels than WT. In ethanol-containing medium, the increased growth of the *efg1*$^{-}$ was greater: the mutant grew to ~6-fold higher levels than WT (p=0.0013). After 48 hours, growth in acetate was ~4-fold higher for the *efg1* null mutant than WT (p=0.0162), and oleic acid no longer supported growth for either strain (Table 3-11). These data show that *efg1*$^{-}$ null mutant cells grow better than WT cells on alternative carbon sources, which reflects the altered metabolism of carnitine by the *efg1*$^{-}$ null mutant. Decreased signaling through the cAMP/PKA pathway, which activates transcription through Efg1p, is also associated with increased growth on non-fermentable carbon sources, consistent with our results [227, 228]. Thus, the ability of

Carbon source	Strain	5hrs.	24hrs.	48hrs.
YP	WT	2.2×10^7	1.1×10^8	1.2×10^8
	efg1$^-$	1.6×10^7	1.3×10^8	**1.8×10^8 ****
Glucose	WT	1.9×10^7	2.9×10^8	3.1×10^8
	efg1$^-$	2.2×10^7	**6.5×10^8**	**8.7×10^8 *****
Ethanol	WT	1.2×10^7	1.7×10^8	2.8×10^8
	efg1$^-$	1.6×10^7	**1.0×10^9**	**1.7×10^9 ****
Acetate	WT	1.2×10^7	7.8×10^7	1.0×10^8
	efg1$^-$	1.3×10^7	1.8×10^8	**3.7×10^8 ***
Glycerol	WT	1.5×10^7	2.1×10^8	2.3×10^8
	efg1$^-$	1.5×10^7	**6.9×10^8**	**8.0×10^8 ****
Oleate	WT	1.1×10^7	1.5×10^8	1.7×10^8
	efg1$^-$	**1.8×10^7 ***	**3.5×10^8**	1.8×10^8

Table 3-11. Growth of WT and *efg1*$^-$ null mutant strains on non-fermentable carbon sources.

WT and *efg1*$^-$ null mutant strains were inoculated into media containing either no carbon source (YP) or 2% of the carbon source listed at 5×10^6 cells/mL after overnight growth in rich growth media (YPD). Strains were grown at 30°C and growth was determined by counting the cells/mL on a hemacytometer. Significant differences in growth are noted: *$p<0.05$, **$p<0.01$, ***$p<0.001$ at each time point, Student's t test. Differences larger than 2-fold are shown in bold.

the *efg1*$^{-}$ null mutant to grow on non-fermentable carbon sources likely contributes to the increased population density of the *efg1*$^{-}$ null mutant during colonization through the increased expression of carnitine metabolism genes.

Overexpression of S*OD5* increases the fitness of the *efg1*$^{-}$ null mutant

Results of our microarray analysis demonstrated that multiple genes involved in protection against host defenses (e.g., *SOD5*, *CAT1*, *DDR48*) were regulated by Efg1p during colonization of the GI tract. One of these, *SOD5*, was among the most highly up-regulated genes when expression by WT *C. albicans* in the GI tract was compared to expression in laboratory culture. Sod5p is involved in detoxification of reactive oxygen species (ROS) produced by host cells, such as macrophages and neutrophils as part of the innate immune response [142, 143]. Neutropenia is a major risk factor for disseminated candidiasis [34, 172], arguing that production of ROS by neutrophils is an important component of the host immune response during candidiasis. An *efg1*$^{-}$ mutant was more susceptible to phagocytosis by both neutrophils and macrophages and did not produce germ tubes in order to evade the host immune response [25, 107] . During colonization of the GI tract, *C. albicans* up-regulated several ROS defense genes, including *SOD5*, in an Efg1p-dependent manner. Therefore, reduced expression of these genes may contribute to the observed hyper-susceptibility of the *efg1*$^{-}$ null mutant to the host immune response during colonization [165].

To test this hypothesis, *SOD5* was ectopically expressed from the *ACT1* promoter and the construct was integrated into the genome of an *efg1*$^{-}$ null mutant strain (*efg1*$^{-}$ *ACT1pr-SOD5* strain). Expression of *SOD5* from the *ACT1* promoter was not observed

to alter morphology or culture density during post-exponential phase in laboratory medium.

In order to determine the effect of ectopic expression of *SOD5* in the *efg1*$^{-}$ null mutant, the *efg1*$^{-}$ *ACT1pr-SOD5* strain was competed against the *efg1*$^{-}$ null mutant strain (without ectopic expression of *SOD5*), or against a WT strain following oral gavage into BALB/c mice. The *efg1*$^{-}$ null and WT strains carried nourseothricin resistance. Fecal pellets were collected and plated; the number of nourseothricin resistant and sensitive colonies was measured. The competitive index (CI; defined as the ratio of sensitive CFU and resistant CFU divided by their ratio in the inoculum) was measured in fecal pellets at several time points. When the *efg1*$^{-}$ *ACT1pr-SOD5* strain was competed against the *efg1*$^{-}$ null mutant, both strains competed equally at day 1 (Fig. 3-12A and B). Over time, the *efg1*$^{-}$ *ACT1pr-SOD5* strain became increasingly more fit than the *efg1*$^{-}$ null mutant alone and out-competed this strain in both the fecal pellets ($p=0.0006$, Mann Whitney test) and organ contents at day 18 post-inoculation ($p=0.0012$, stomach contents; $p=0.0101$ stomach wall; p=00006, ileum; $p=0.0006$, cecum contents; $p=0.0303$ cecum wall) (Fig. 3-12A and B). Thus, at later times post-inoculation, ectopic expression of *SOD5* in the *efg1*$^{-}$ null mutant conferred a selective advantage relative to the *efg1*$^{-}$ null mutant strain alone.

Previous data showed that when the *efg1*$^{-}$ null mutant strain was competed against WT *C. albicans,* the *efg1*$^{-}$ null mutant had an initial advantage during colonization, but was preferentially lost over time and became less fit than a WT strain (Fig. 3-12A and B, squares) [165]. When the *efg1*$^{-}$ *ACT1pr-SOD5* strain was competed with WT *C. albicans* the results (Fig. 3-12A and B, triangles) were similar to results for the *efg1*$^{-}$/WT

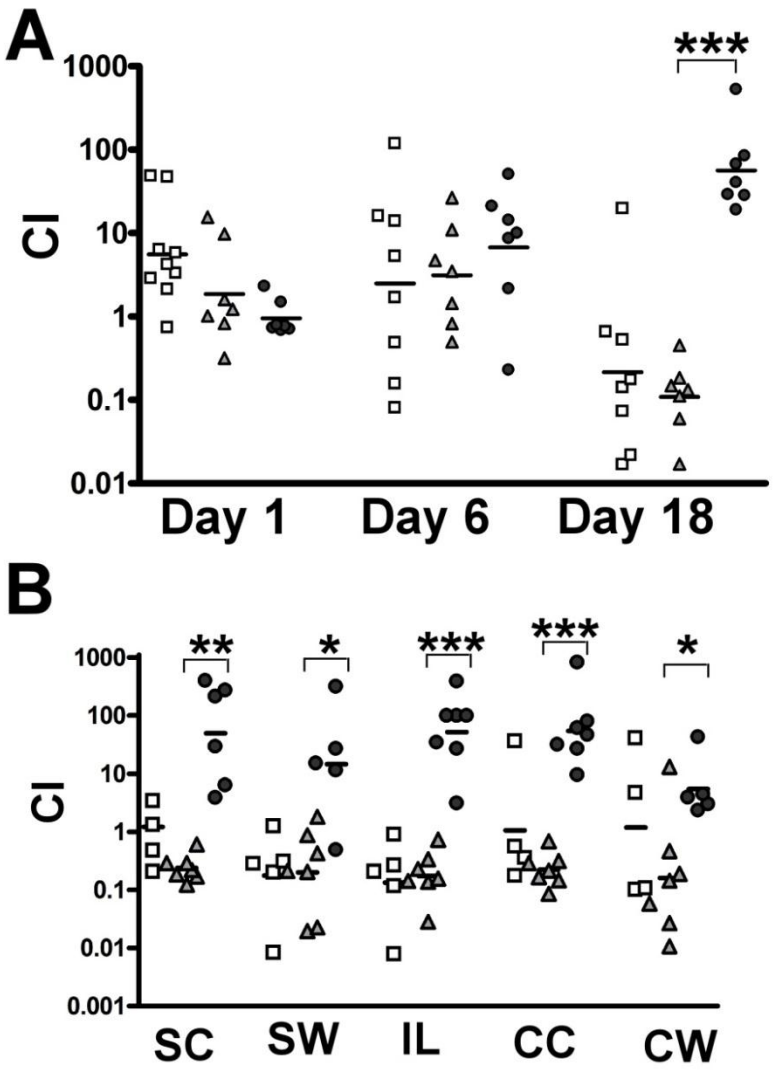


Figure 3-12A. Overexpression of *SOD5* increases the fitness of the *efg1*$^{-}$ null mutant.

Competition experiments were done in BALB/c mice to determine the effect of overexpression of *SOD5* in the absence of *EFG1* during colonization. Strains were inoculated at a 1:1 ratio using a drug resistance marker (nourseothricin, nou) to differentiate between strains. Competitions included: *efg1*$^{-}$ nou^{S}/WT nou^{R} (squares), *efg1*$^{-}$ *ACT1pr-SOD5* nou^{S}/WT nou^{R} (triangles), *efg1*$^{-}$ *ACT1pr-SOD5* nou^{S}/*efg1*$^{-}$ nou^{R} (circles). CI was determined as the ratio of the number of drug sensitive colonies to the number of drug resistance colonies, normalized to the input ratio. Each symbol represents an individual mouse; bars are the geometric mean. (A) CI in fecal pellets samples at days 1, 6, and 18 post-inoculation. (B) CI in GI tract organs at day 18 post-inoculation. SC, stomach contents; SW, stomach wall; IL, ileum; CC, cecum contents; CW, cecum wall. *p<0.05, **p<0.01, ***p<0.0001; Mann whitney test comparing *efg1*$^{-}$ *ACT1pr-SOD5*/WT and *efg1*$^{-}$ *ACT1pr-SOD5*/*efg1*$^{-}$ competitions.

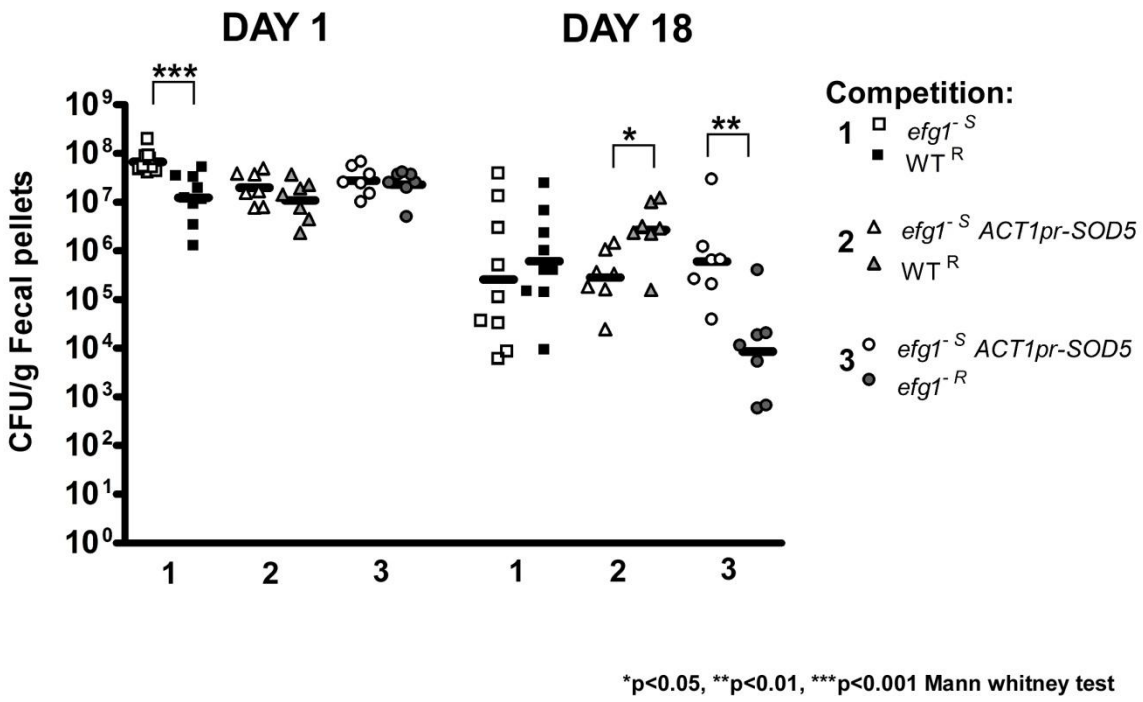


Figure 3-12B. Single strain colonization of an *efg1*$^{-}$ *ACT1pr-SOD5* strain during competition with either WT or *efg1*$^{-}$ strains.

Competition experiments were performed as described in Figure 3-12. Competition 1: *efg1*$^{-\,S}$/WT^{R}, competition 2: *efg1*$^{-}$ *ACT1pr-SOD5* ^{S} /WT^{R}, competition 3: *efg1*$^{-}$ *ACT1pr-SOD5* ^{S} / *efg1*$^{-\,R}$. Symbols for each individual strain are shown in the legend. CFUs for single strains were calculated based on the number of drug resistant and drug sensitive colonies recovered at either day 1 or day 18 post-inoculation from fecal pellets of colonized mice.

competition (Fig. 3-12A and B, squares). Therefore, ectopic expression of *SOD5* in the *efg1*$^{-}$ null mutant did not increase resistance to the host immune response to WT levels, indicating that *SOD5* is not the only determinant of resistance. However, *SOD5* expression was sufficient to increase the resistance of *efg1*$^{-}$ null mutant cells to the immune response. Therefore, *SOD5* contributes to the resistance of *C. albicans* to reactive oxygen species in the GI tract, and defense against this response is important for commensal colonization.

Discussion

Using microarray analysis, we have shown that *EFG1* is a major regulator of gene expression in *C. albicans* cells that are colonizing the gastrointestinal tract. Efg1p is also required for normal GI colonization dynamics [165]. Therefore, in addition to its known role in pathogenesis, *EFG1* plays a key role in commensal colonization. By regulating genes involved in both commensal and pathogenic growth of *C. albicans*, Efg1p is well positioned to play a role in the transition between the two lifestyles and is thus a critical regulator of host-*C. albicans* interactions.

WT fungal cells colonizing both the cecum and the ileum up-regulated genes involved in host interactions. More specifically, genes involved in adhesion, stress responses, and protease secretion had increased expression in colonizing cells relative to laboratory grown cells. These functions contribute to both colonization and pathogenesis. Gene expression in the ileum consistent with increased acetyl-CoA metabolism was noted in colonizing cells. Increased acetyl-CoA metabolism would allow cells to utilize non-fermentable carbon sources thus enhancing GI tract

colonization. In addition, cells colonizing the ileum down-regulated glycosylation genes. Decreased glycosylation in the ileum may be a mechanism by which *C. albicans* evades the host immune response during colonization. Therefore, altered expression of metabolic genes and genes involved in host interactions are major transcriptional responses during colonization by *C. albicans*.

Cells lacking Efg1p or lacking both Efg1p and Efh1p were shown to hyper-colonize the GI tract. These mutant strains also showed increased transcription of genes involved in carnitine metabolism and utilization. In contrast, cells lacking Efg1p and Cph1p neither hyper-colonized nor over-expressed carnitine metabolic genes. These observations suggest that during colonization, *efg1*$^{-}$ null mutant cells are better able to utilize carnitine to metabolize non-fermentable carbon sources than WT *C. albicans*. In support of this hypothesis, we observed that the *efg1*$^{-}$ null mutant was able to grow to a higher density relative to WT *C. albicans* during growth on glucose, and grew to an even higher relative density on non-fermentable carbon sources, such as ethanol and acetate. In nutrient-limiting conditions (depleted medium), the *efg1*$^{-}$ mutant was able to grow, while WT cells could only grow if carnitine was added to the depleted medium. Growth of the *efg1*$^{-}$ null mutant without additional carnitine suggests that the mutant is better able to utilize carnitine than WT *C. albicans*, consistent with the gene expression patterns in colonizing cells. Since altered carnitine metabolism appears to enhance the growth of the *efg1*$^{-}$ null mutant strain, it may influence colonization of the GI tract.

SOD5 was one of the most highly up-regulated genes by WT *C. albicans* during colonization and its expression decreased in the absence of Efg1p. The superoxide dismutase Sod5p is required for the detoxification of reactive oxygen species (ROS)

produced by host immune cells and for virulence in systemic infections [142, 143]. Overexpression of *SOD5* improved colonization of the *efg1*$^{-}$ null mutant strain at later times post-inoculation. When mice deficient in the production of ROS were orally inoculated with *C. albicans*, both the *efg1*$^{-}$ null mutant and WT strains killed mice with equal efficiency [35]. This observation suggests that a major defect of the *efg1*$^{-}$ null mutant strain is its sensitivity to ROS production by the host.

We observed that in a healthy host, the *efg1*$^{-}$ null mutant was more sensitive than WT C. *albicans* to the host immune response that develops over time during colonization [165]. In contrast, in an immunocompromised host, the *efg1*$^{-}$ null mutant strain was able to maintain high levels of colonization [165]. During colonization, neutrophils recruited to the gut produce ROS as an antimicrobial defense in addition to phagocytosis. Thus, ROS production is a major part of the host immune response to *C. albicans* during both colonization and systemic infections.

We have demonstrated that Efg1p regulates growth on non-fermentable carbon sources and sensitivity to reactive oxygen species. ROS can be generated either intracellularly or produced extracellularly by host immune cells. Increased expression of *SOD5* was noted when cells were grown on non-fermentable carbon sources in the laboratory [142]. Higher levels of cellular respiration occur when cells are grown on non-fermentable carbon sources, which in turn increase the levels of intracellular ROS [229, 230]. Increased expression of *SOD5* during growth on non-fermentable carbon sources may therefore be a response to increased intracellular ROS. Here we have demonstrated that the expression of *SOD5* and growth on non-fermentable carbon

sources are inversely regulated by Efg1p activity; however, this regulation may depend on the source of ROS production.

Co-regulation of metabolism and host interactions by the Efg1p transcription factor allows *C. albicans* to choose between two survival strategies: increased growth or protection from the host immune response. In a healthy host, *C. albicans* limits its growth [42] and expresses genes which will protect it against the host immune system. Overgrowth of *C. albicans* may indicate a change towards increased pathogenesis in a colonizing population. Changes in Efg1p activity under different conditions cause global changes in gene expression and alter host-fungal interactions. High Efg1p activity promotes the expression of genes involved in host defense, while low Efg1p activity de-represses metabolic gene expression and leads to increased colonization. Consistent with this, Efg1p expression increases during GI tract colonization of a healthy host, but remains low in an immunocompromised host [165]. Phenotypic variation of Efg1p activity during colonization may produce subpopulations of cells with difference characteristics, enabling host-dependent shaping of the colonizing population and altered expression of genes involved in both metabolism and host defense.

Materials and Methods:

Animal models

5-7week old BALB/c mice (NCI) were treated with antibiotics (tetracycline 1mg/ml, streptomycin 2mg/ml, gentamycin 0.1mg/ml) as described [42]. Mice were tested for fungal contamination prior to each experiment. Mice were inoculated with 0.1ml *C. albicans* cells at $5x10^8$ cells/mL by oral gavage. Colonization was tested over time by collecting fresh fecal pellets and plating homogenates on YPD agar plus streptomycin (100ug/ml) and ampicillin (50ug/ml) (YPD-SA). Mice were sacrificed at 3 days post-inoculation to obtain ceca and ilea contents for RNA isolation. Combined results from at least 2 experiments are shown for all data. Homogenates of kidney, liver, and tongue were plated on YPD-SA. No colonization of these organs was detected in any experiment. All experiments were done in compliance with regulatory guidelines defined by Tufts University IACUC committee.

For competition experiments, colonies on YPD-SA plates were replica plated on YPS-nourseothricin and the ratio of drug sensitive to drug resistant cells was measured. The competitive index= CFU *efg1*$^-$ / CFU WT or CFU *efg1*$^-$ *ACT1pr-SOD5 /* CFU *WT or* CFU *efg1*$^-$ *ACT1*pr-*SOD5* / CFU *efg1*$^-$ colonies, divided by the input ratio. For *efg1*$^-$/WT competitions, the WT strain was nou^R. For *efg1*$^-$ *ACT1*pr-*SOD5* competitions, the *efg1*$^-$ and WT strains were nou^R. At some time points, total colonization levels were below the level of detection and a ratio could not be determined; these samples were not included in the analysis. For samples in which one strain fell below the limit of detection, the CI is shown as 0.01.

RNA isolation

For the reference sample, WT cells were grown in laboratory culture at 37°C in YPD medium to log phase. Due to the high temperature of the culture, some degree of filamentation occurred in the reference sample, and hyphal genes were expressed to some extent. Laboratory grown cells and cecum/ileum samples taken at 3 days post-inoculation were mixed with RNA-Later (Ambion) and frozen at -80°C. RNA was extracted from laboratory grown samples using mechanical disruption and an RNA Easy mini kit (Qiagen) with on column DNaseI digestion. Samples of mouse GI tract contents were filtered through 250um polypropylene mesh (Small Parts, Inc), then pelleted and extracted using the Purelink kit Trizol extraction procedure (Invitrogen) with DNaseI digestion. Two ceca or 4 ilea were combined per sample. Samples were stored at -80C and were sent to the Whiteway lab at the CNRC Montreal, Canada for microarray analysis. RNA concentration was determined by Nanodrop® ND-1000 Spectrophotometer and RNA quality was tested using the Agilent 2100 BioAnalyzer (Agilent Technologies) with the RNA 6000 Nano LabChip®. Reverse Transcription of up to 3ug of RNA using a modified Oligo dT/Oligo T7 dT primer, amplification, and coupling to cy3/cy5 dyes was done using the Amino Allyl MessageAmp™ II RNA Amplification Kit (Ambion® kit cat. AM1753 (now Life technologies™)).

For RT-PCR confirmation, cDNA was generated from 1-10ug RNA with Superscript II or III Reverse transcriptase (Invitrogen) and an oligo-dT primer. Samples were heated to 70°C for 10 min. and the enzyme was added. The sample was then incubated at 42°C or 50°C, respectively, for 1.5 hrs. Following addition of 2.5M NaOH, the reaction was incubated at 37°C for 15 min. 2M Hepes free acid was then added to

neutralize the pH and cDNA was purified using Qiaquick columns (Qiagen). Concentrations were determined using a Nanodrop spectrophotometer and samples were stored at -20°C.

Microarray hybridization

C. albicans microarrays (double-spotted 6,394 intragenic 70-mer oligonucleotides) were obtained from the Biotechnology Research Institute microarray facility center (http://www.nrc cnrc.gc.ca/eng/services/bri/microarray.html, Montreal, Canada). Slides were pre-hybridized for 2hrs. at 42°C and probes were denatured at 96°C. Hybridization was done overnight at 42°C in "Slide Booster" chamber (Advalytix) [231]. Microarray samples were performed with at least 3 biological replicates.

Microarray analysis

Microarray slides were scanned using "ScanArray Gx" scanner (Perkin Elmer) at a 10-micron resolution and quantification was done with QuantArray®software. Data analysis was done with "GeneSpring GX 7.3 Expression Analysis" software (Agilent Technologies) and Microsoft Excel. Additional analysis was done using BioVenn © 2007-2008 Tim Hudson (http://www.cmbi.ru.nl/cdd/biovenn/index.php) and GO (gene ontology) term analysis was done using the Candida Genome Database (http://www.candidagenome.org). Clustering was done using Cluster 3.0 [232] and heat maps generated with Treeview software [233].

Strains

All *C. albicans* strains used in this study are listed in Table 3-12. A CAI-4 strain carrying two copies of chromosome 1 was used. Strains CAI-4 and HLC67 were kindly provided by Drs. W. Fonzi and G. Fink respectively. *RIM101* mutant strains and the

respective WT strain was a helpful gift of Dr. A. Mitchell. *E. coli* strains XL1-Blue or DH5alpha were used for propagating plasmids.

Plasmids

To construct a strain in which *SOD5* was overexpressed, the Clp10 *ACT1*pr-g*LUC*59 plasmid [197] was digested with HindIII and NheI and the g*LUC-PGA59* fusion was replaced with the *SOD5* ORF downstream of the actin promoter (*ACT1pr*). The plasmid was linearized with StuI and transformed into strains CAI-4 and *efg1*$^{-}$ using *URA3* as the selectable marker. Proper integration at the *RPS1* locus was confirmed by PCR.

Drug resistant wild type or *efg1*$^{-}$ null strains were constructed by digesting the plasmid iSAT [42] with BsgI and transforming into CAI-4 or HLC67 using *URA3* as the selectable marker. The resulting strains (JPY105 and JPY106) are resistant to nourseothricin on plates containing sucrose as the carbon source.

Media and Growth conditions

YPD (1% yeast extract, 2% peptone, 2% glucose) and YPS (1% yeast extract, 2% peptone, and 2% sucrose) was used as rich growth media. Minimal CM media lacking uracil was made as previously described [198]. Cells from the GI tract were plated on YPD agar plus streptomycin (100ug/ml) and ampicillin (50ug/ml) (YPD-SA). YPD-SA plates were replica plated onto YP-sucrose plates with nourseothricin (200ug/ml) for competition experiments. Log phase cells were grown to an OD=1 in YPD at 30°C. For mouse inoculations, strains were grown at 37°C in YPD for 21-24 hrs.

Growth curves

Strains were grown overnight in YPD at 30°C and diluted to $5x10^6$ cells/mL the next morning. Cell density was measured immediately after dilution and at various time points by counting the number of cells/mL using a hemacytometer. Cultures were diluted into either YPD, YP alone, or YP + 2% ethanol, acetate, glycerol, or oleic acid (3:5 ratio of oleate/Tween80) and cells were then grown at 30°C. For depleted media experiments, WT *C. albicans* was grown in YPD for 5 days at 30°C, centrifuged, and the culture supernatant filter-sterilized. The culture supernatant (depleted media) was then re-inoculated with either WT or *efg1*$^-$ null mutant cells. Growth is shown as fold change above the inoculum. Growth curves on various carbon sources were repeated with at least three independent replicates. Growth in glucose and in the depleted media was done in triplicate with at least two replicates. Aliquots of growing cultures were taken at various time points, centrifuged, and the supernatants frozen at -20°C. The supernatants were then used to measure the concentration of glucose and ethanol present in the media of WT and *efg1*$^-$ null mutant cultures using an Ethanol Assay Kit and Glucose Assay Kit (AbCam) using fluorometric analysis. At least 3 biological replicates were tested for each strain.

RT-PCR

Primers used to detect expression of genes are listed in Table 3-13. qRT-PCR reactions were performed with SYBR green reaction mix (Qiagen) with reference dye ROX using an MX3000p (Stratagene). Each sample was tested in triplicate and melting curve analysis in addition to agarose gel electrophoresis was used to confirm primer specificity. Samples tested with no cDNA showed no detectable signal. Standard curves

were generated and all results normalized to the level of actin (*ACT1*) expression in each respective sample. All samples are presented normalized to the level of expression for each respective gene in wild type cells grown in YPD at 30°C to an OD=1.

CAI-4	SC5314 Δ*ura3::imm434/*Δ*ura3::imm434*	Fonzi and Irwin 1993
CKY101	CAI-4 (pDB152)	Brown et al. 1999
HLC67	CAI-4 *efg1*::hisG/*efg1*::hisG	Lo et al. 1997
CKY136	CAI-4 *efg1::*hisG*/efg1::*hisG (pDB152)	Guisani et al. 2002
CKY138	CKY136 *cph1*::hisG/*cph1*::hisG	White et al.
JPY109	HLC67 *efh1::*hisG*/efh1::*hisG (pRC3915)	This study
JPY 105	CAI-4 *ade2*::pISAT	Pierce et al. 2012
JPY 106	HLC67 *ade2*::pISAT	This study
JPY 110	HLC67 *rps1::ACT1pr-SOD5*	This study
DAY185	pARG4::URA3::*arg4*::hisG/*arg4*::hisG pHIS1::*his1*::hisG/*his1*::hisG	Davis et al. 2000
DAY25	*ura3*::*imm434*/*ura3*::*imm434*::*arg4*::hisG/ *arg4*::hisG *his1*::hisG::p*HIS1*/*his1*::hisG:: *rim101*::*ARG4*/*rim101*::*URA3*	Nobile et al. 2008
DAY44	*ura3*::*imm434*/*ura3*::*imm434*::*arg4*::hisG/ *arg4*::hisG *his1*::hisG::p*HIS1RIM101*/*his1*::hisG:: *rim101*::*ARG4*/*rim101*::*URA3*	Nobile et al. 2008

Table 3-12. *C. albicans* strains used in these studies.

Cloning primers		
SOD5	SOD5F1 cloning	5’-CCCAAGCTTGGGATCATGAAGTATTTGTCCAT-3’
SOD5	SOD5R1 cloning	5’-CTAAGCTAGAGCCTCTGGATTCATTTACT-3’
RT-PCR primers		
ACT1	ABAF3	5’-TATGAAAGTTAAGATTATTGCTCCACCAGAAA-3’
ACT1	ABAR3	5’-GGAAAGTAGACAATGAAGCCAAGATAGAAC-3’
CIP1	CIP1F	5’-AAGTTGATGAATATGCTCCAGGGTTTTTAG-3’
CIP1	CIP1R	5’-TCTGGTAATTCTCTTGGATTAGGATTAGTAGCA-3’
CTN1	CTN1F	5’-GTTTCTGCTAATGGGTTTGGTATTGGTTATA-3’
CTN1	CTN1R	5’-GTGTTTTGGTCTAGCTTGTTCCTCTTCTG-3’
HYR1	HYR1F1	5’-GTTGCTAATCCAGTTACTACTTCCACAGA-3’
HYR1	HYR1R1	5’-GGTTTAGGTTTTCCATCAAAGCCAGTCATA-3’
ICL1	ICL1F	5’-AGCATTGGCTGTTGATGACTTCTCTA-3’
ICL1	ICL1R	5’-GGTAACACTGACCATCTTC-3’
INO1	INO1F	5’-GTTGGGTGGTCACAACAAAATAAGTAT-3’
INO1	INO1R	5’-TTCAGTAGCCACCACCAAGTCAATA-3’
PHO89	PHO89F	5’-TGTGTTACGCTGCCGGTGCTTTAGTTG-3’
PHO89	PHO89R	5’-CATTCTCCAGTTGAGGGATTTCCATTCTCT-3’
RBT5	RBT5F	5’-AATCTTTGGCCACTTCCATTTGTTCATCAT-3’
RBT5	RBT5R	5’-AGCGGCAGTAGATTCGGCAGTG-3’
RIM101	RIM101F1	5’-AGCAATACCACGACCATCACGGATACTACTCA-3’
RIM101	RIM101R1	5’-TGCTTGGCCTCTTTTTGTTGTTGTTTCATCA-3’
SOD5	SOD5F1	5’-GAGGGACACGGCAATGCTAACACAACTAT-3’
SOD5	SOD5R1	5’-ACCTGCTGCCAAGAGACCATTTACTACTG-3’
WH11	WH11F	5’-CTCAAAAATCCACTCCAGAACAAT-3’
WH11	WH11R	5’-TGGAGTCAAAAATAGCATCA-3’
WOR1	WOR1F1	5’-AACAACGGTAGCAATGCCCCCACTG-3’
WOR1	WOR1R1	5’-ATTGCCATTACCACCACTAACACTTCCACTG-3’

Table 3-13. Primers used in these studies.

Chapter 4:

Other regulators of *Candida albicans* colonization in the GI tract

Sections of these results were previously published in the journal Eukaryotic Cell [177].

Introduction

Candida albicans is an opportunistic pathogen and colonizing organism of the human gastrointestinal tract [1, 113, 196, 201]. The ability of *C. albicans* to cause disease has been associated with the ability of this microbe to undergo morphological changes from a budding yeast to an invasive hyphal form [3]. Hyphae are often observed at sites of invasion during candidiasis and are thought to be important for dissemination of *C. albicans* across the mucosal epithelium [7, 117, 164].

The yeast to hyphal transition has been widely studied in the laboratory to identify environmental signals responsible for initiating this change, as well as the regulatory pathways involved in controlling hyphal formation [44, 61, 62, 218]. A network of transcription factors has been shown to regulate hyphal formation and these pathways respond to different environmental signals [111, 123, 126, 219]. These signals include: serum, temperature, pH, growth within a matrix, N-acetylglucosamine, and low oxygen [45]. This allows *C. albicans* to alter its morphology and gene expression patterns in a manner which is dependent on environmental signals and allows for integration of multiple signals to uniquely adapt to changes in the environment.

A number of transcription factors which regulate the yeast to hyphal transition under various conditions have been shown to be important for colonization of the GI tract. *EFH1* was up-regulated during GI tract colonization, but not during oral tissue invasion [42]. Cells lacking *EFH1* had increased persistence in the GI tract, while overexpression of *EFH1* caused a defect in GI tract colonization. *EFH1* is a homologue

of *EFG1*, a major regulator of morphology and colonization [111]. While *EFG1* controls hyphal formation under a wide range of conditions as well as virulence, deletion of *EFH1* has little effect on morphogenesis, virulence, and gene expression [107, 111]. *EFG1* is a positive regulator of hyphal formation when induced by a number of signals, including serum and temperature, and also a negative regulator of filamentation during hypoxic and embedded growth conditions (Fig. 1-2). *EFH1* participates in the repression of filamentation under these conditions with *EFG1*. Therefore, while the main role of *EFH1* is in the regulation of GI tract colonization, *EFG1* is important for both colonization and virulence.

EFG1 is regulated at both the transcriptional and post-transcriptional levels. One mechanism of its regulation is the ability to undergo negative auto-regulation soon after hyphal induction [124, 150]. Once hyphal formation has been induced, *EFG1* transcript levels rise, but are quickly repressed. This is caused by the binding of Efg1p to its own promoter along with Sin3p [124]. Sin3p is part of a histone deacetylase complex which is involved in global transcriptional repression and acts as a scaffolding protein, allowing the targeting of the complex to DNA-binding proteins [234, 235]. Other methods of regulation include switching between major and minor promoters of *EFG1* to produce phase-specific transcription and phosphorylation of Efg1p through the activation of the cAMP/PKA pathway [150, 236].

CPH2 and *TEC1*, both transcription factors, were also observed to be up-regulated during GI tract colonization [177]. *CPH2* and *TEC1* positively regulate hyphal formation during laboratory growth, either in Lee's medium or in response to serum, respectively, as well as altering hyphal gene expression under these conditions [130].

CPH2 has been shown to regulate *TEC1* by binding to SRE elements in the *TEC1* promoter. Efg1p also regulated hyphal formation through interactions with *TEC1* [125]. *TEC1* is important for both the ability to escape from phagocytosis by macrophages and cause systemic virulence [131]. Although a *tec1*$^{-}$ strain was defective in forming hyphae in certain conditions and is attenuated for virulence, it was still capable of forming hyphae in both infected murine tissues and in a *Galleria* model of pathogenesis [131, 237] (Fig. 1-2).

Based on these observations, we studied various regulatory factors that affect hyphal formation or *EFG1* expression, and their roles in GI colonization. As *EFG1* and *EFH1* both regulate colonization [42, 165], we examined the regulatory relationship between them during both yeast growth and hyphal induction in the laboratory. We observed that *EFG1* repressed *EFH1* expression during hyphal induction, and that over-expression of *EFH1* expression caused reduced *EFG1* expression.

As a *sin3*$^{-}$ null mutant was reported to be defective in down-regulation of *EFG1* under laboratory conditions [124], we tested a *sin3*$^{-}$ mutant to determine whether *SIN3* was important for the regulation of *EFG1* during colonization. We found that *SIN3* was not required for the observed differences in *EFG1* expression between a healthy host and T cell deficient host in colonizing cells. However, deletion of *SIN3* caused a change in some aspects of colonization.

We also studied the roles of *CPH2* and *TEC1* during GI colonization. A *cph2*$^{-}$ strain of *C. albicans* was defective in establishment of colonization, while a *tec1*$^{-}$ strain colonized at WT levels. Both *CPH2* and *TEC1* altered gene expression by *C. albicans* during colonization. While *CPH2*, *TEC1*, *EFG1,* and *EFH1* all affect the ability of *C.*

albicans to form hyphae during laboratory growth, each of them appears to play a unique role in the regulation of GI tract colonization and there may be novel regulatory relationships between these factors in the GI tract environment.

Results

Interplay between *EFG1* and *EFH1*

To study the relationship between the homologues *EFG1* and *EFH1* during GI colonization, Swiss Webster mice were colonized with an *efg1*$^{-}$ *efh1*$^{-}$ mutant. In the absence of both *EFG1* and *EFH1*, *C. albicans* hyper-colonized at day 1 post-inoculation in the fecal pellets (p=0.0006) (Fig. 4-1A). At day 6 post-inoculation, increased colonization of the *efg1*$^{-}$ mutant was observed relative to WT levels, while the *efg1*$^{-}$ *efh1*$^{-}$ mutant did not hyper-colonize at this time point (Fig. 4-1A). Over time, colonization of this mutant dropped to below WT levels and showed a faster rate of decline than the *efg1*$^{-}$ single null mutant (p=0.0007, day 21). Decreased colonization was also observed in each of the GI tract organs (p=0.0022, stomach contents; p=0.0022, stomach wall; p=0.0022, ileum, p=0.0007, cecum contents) (Fig. 4-1B).

In BALB/c inbred mice, the *efg1*$^{-}$ *efh1*$^{-}$ mutant also colonized similarly to the *efg1*$^{-}$ single null; however, the decline in colonization over time was less dramatic compared to Swiss Webster mice (Fig. 4-2A). BALB/c *nu/nu* mice were also colonized with the *efg1*$^{-}$ *efh1*$^{-}$ strain, and like the *efg1*$^{-}$ single null (Fig. 2-11), the observed decrease in colonization was dependent on the presence of T cells in both the fecal pellets and the cecum contents (Fig. 4-2B). Other organs showed similar patterns of colonization at this

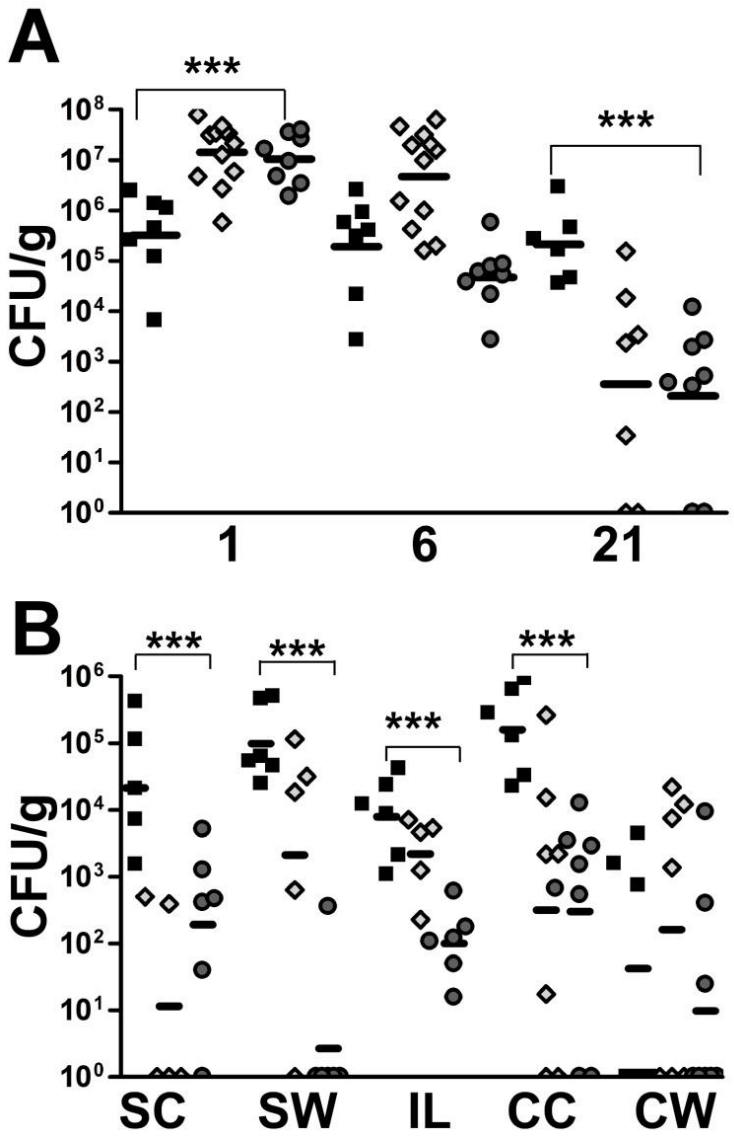


Figure 4-1. GI tract colonization of an *efg1*$^-$ *efh1*$^-$ strain.

Colonization of Swiss Webster mice following oral gavage with WT (squares), *efg1*$^-$ single null mutant (diamonds) or an *efg1*$^-$ *efh1*$^-$ double null mutant (circles) was determined. (A) Fecal pellet colonization at days 1, 6, and 21 post-inoculation. (B) Organ colonization at day 21 post-inoculation. SC, stomach contents; SW, stomach wall; IL, ileum; CC, cecum contents; CW, cecum wall. Each symbol represents an individual mouse; bars are the geometric mean. ***$p<0.001$, Mann whitney test between WT and *efg1*$^-$ *efh1*$^-$ double null strains.

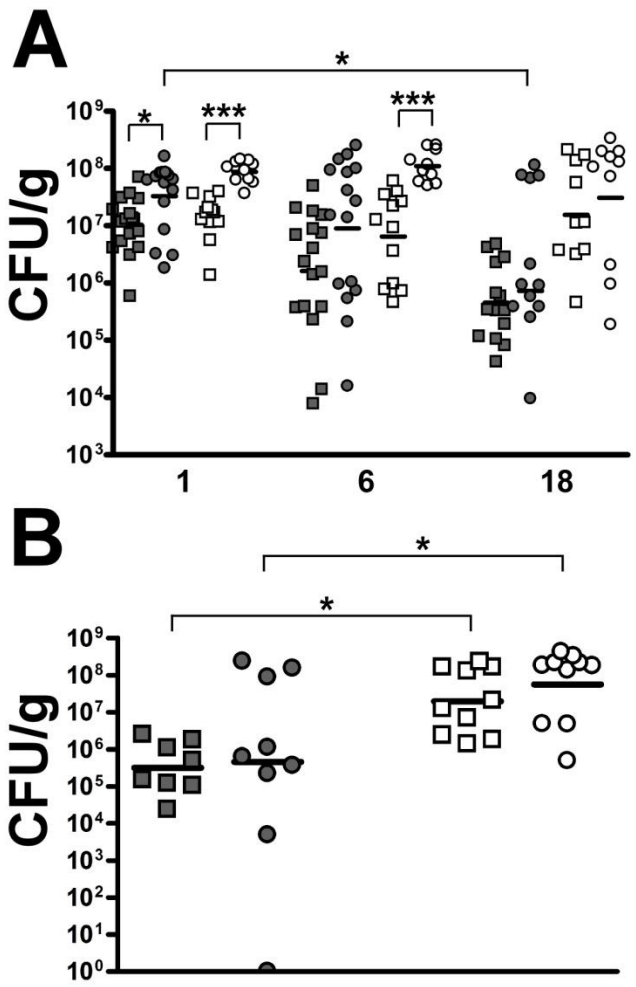


Figure 4-2. Colonization of an *efg1*$^{-}$ *efh1*$^{-}$ strain in healthy and immunocompromised hosts.

(A) Colonization of BALB/c WT (closed symbols) and *nu/nu* mice (open symbols) with WT (squares) and *efg1*$^{-}$ *efh1*$^{-}$ (circles) strains of *C. albicans*. Colonization of fecal pellets is shown at day 1, 6, and 18 post-inoculation. (B) Colonization of cecum contents from BALB/c WT and *nu/nu* mice at day 18 post-inoculation of WT and *efg1*$^{-}$ *efh1*$^{-}$ strains. *$p<0.05$, $p<0.01$ Mann whitney test.

time. Therefore, the *efg1*$^{-}$ mutation is epistatic to the *efh1*$^{-}$ mutation during colonization, as the colonization of the *efg1*$^{-}$ *efh1*$^{-}$ double null mutant is similar to the colonization of the *efg1*$^{-}$ single null mutant.

Histological analysis of tissue was used to assess inflammation during colonization and to detect adherence by *C. albicans* to the tissue following oral gavage of BALB/c mice. No inflammation or adherent fungal cells were detected in the esophagus, ileum wall, or cecum wall during colonization by WT, *efg1*$^{-}$ single null, or *efg1*$^{-}$ *efh1*$^{-}$ double null strains (data not shown). Adherent cells in the presence of host inflammatory cells were detected in association with the stomach wall at various times during colonization (Fig. 4-3).

At day 3, similar levels of inflammation were detected in the presence of the *efg1*$^{-}$ *efh1*$^{-}$ mutant compared to the WT strain, and no adherent *C. albicans* cells were observed in our samples (Fig. 4-3, panels A, B; and Table 4-1). At day 6, high levels of focal inflammation consisting of both neutrophils and lymphocytes were observed and there appeared to be exudates of inflammatory cells at the epithelial barrier (Fig. 4-3, panels C and D; Table 4-1). WT yeast and hyphae, as well as *efg1*$^{-}$ *efh1*$^{-}$ yeast and pseudohyphae were observed in association with the keratinized tissue (Fig. 4-3, panels E and F). This is similar to the effects seen by *efg1*$^{-}$ mutant alone (Fig. 2-13). At day 18, high levels of inflammation in the lamina propria and hyperplasia were observed in the presence of both the WT and *efg1*$^{-}$ *efh1*$^{-}$ strains (Fig. 4-3, panels G and H; Table 4-1). Both WT and *efg1*$^{-}$ *efh1*$^{-}$ cells were observed in association with the stomach tissue at this time as well (Fig. 4-3, panels I and J). These data show that the *efg1*$^{-}$ *efh1*$^{-}$ mutant is capable of interacting with host epithelial cells and generating an inflammatory response during colonization.

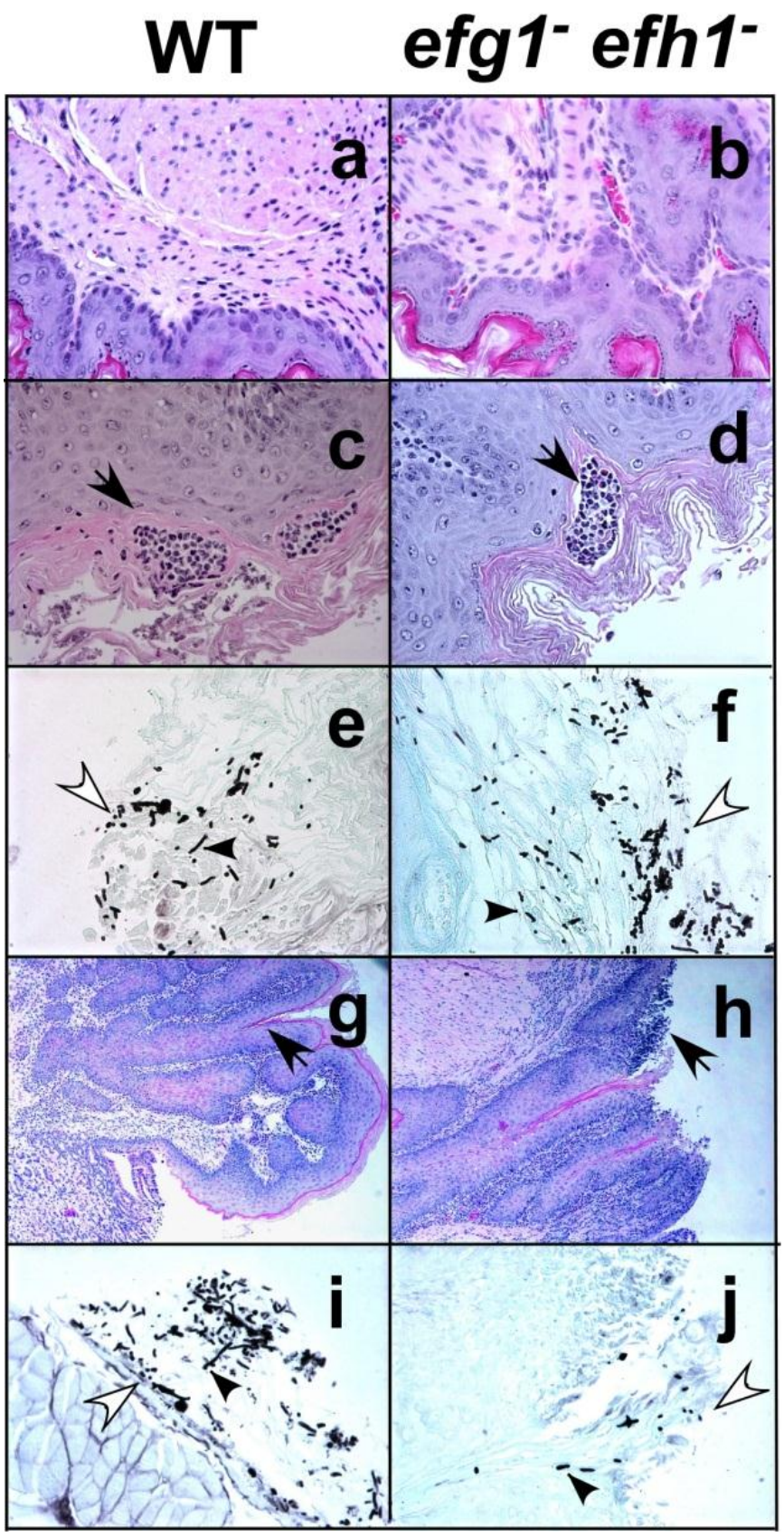

WT
efg1- efh1-
a
b
c
d
e
f
g
h
i
j

Figure 4-3. Inflammation in response to colonization by the *efg1*$^{-}$ *efh1*$^{-}$ mutant.

Histology showing the level of inflammation and presence of adhesive fungi in the stomach wall using H&E and GMS staining, respectively. Panels A, B: day 3 H&E 40X; panels C, D: day 6 H&E 40x; panels E, F: day 6 GMS 40X; panels G, H: day 18 H&E 10X; panels I, J: day 18 GMS 40X. Black arrows indicate host inflammatory infiltrate; black arrowheads indicate hyphae or pseudohyphae; white arrows indicate yeast cells.

Strain	D.p.i	N	Fungal burden	Presence of fungi	Forestomach			Limiting Ridge	Glandular stomach	
					Neutrophils	Mono-nuclear cells	Epidermal hyperplasia			
WT	3	3	2.5×10^4	None	1	0	0	0	1	1
efg1$^-$ *efh1*$^-$	3	4	ND	None	1	0	1	1	1	0
WT	6	3	4.1×10^4	Yeast, hyphae	3	4	4	4	1	0
efg1$^-$ *efh1*$^-$	6	3	ND	Yeast, pseudo-hyphae	3	4	3	4	1	1
WT	18	4	5.2×10^4	Yeast, hyphae	4	2	4	0	2	2
efg1$^-$ *efh1*$^-$	18	4	5.2×10^3	Yeast, pseudo-hyphae	4	2	4	0	1	1

Table 4-1. Inflammation and adherence during colonization by an *efg1*$^-$ *efh1*$^-$ strain.

Histological analysis of stomach wall samples from BALB/c mice colonized with WT and *efg1*$^-$ *efh1*$^-$ strains of *C. albicans*. D.p.i. =days post-inoculation; N=number of individual mice analyzed. The fungal burden in the stomach wall for each condition is listed; ND= not determined. The presence of fungi is noted as either none, yeast, pseudohyphae, or hyphae. Scoring for the level of inflammation is shown for the forestomach, glandular stomach, and limiting ridge was done on a scale of 0-4: 0 indicates no inflammation, 4 is severe inflammation. The level of neutrophils, mononuclear cells, and hyperplasia was noted in the forestomach.

As seen with the *efg1*$^{-}$ mutant, this inflammatory response may contribute to the decrease in colonization by the *efg1*$^{-}$ *efh1*$^{-}$ double null mutant over time.

To study the relationship between *EFG1* and *EFH1* in more detail, the effects of each gene on the expression of the other was measured. RNA was isolated from cells grown either as yeast or hyphae, and cDNA was generated to measure the transcript levels of either *EFG1* or *EFH1* by real-time quantitative RT-PCR (qRT-PCR). Yeast phase cells were grown in YPD at 30°C, and hyphae were induced by diluting cells into serum-containing medium at 37°C. Expression levels were normalized to *ACT1* and expressed relative those present in WT cells during log phase. Samples were taken during log phase for yeast growth and after 6 hours for hyphal growth, as well as at 48 hours post-dilution in both conditions.

EFH1 is expressed at relatively low levels during log phase laboratory growth by WT *Candida albicans* [42]. Expression of *EFH1* increased slightly during post-exponential phase growth (Fig. 4-4A). During yeast phase growth in log phase or post-exponential phase growth, deletion of *EFG1* had little effect on *EFH1* expression ($p=0.7$ Mann whitney test, post-exponential phase). As a control, no *EFH1* transcript was detected in an *efh1*$^{-}$ null strain, and a strain which over-produces *EFH1* had 10-100 fold higher levels of expression relative to WT *C. albicans* (data not shown).

Expression of *EFG1* in yeast cells increased as well during post-exponential phase growth (Fig. 4-4C). WT expression of *EFG1* increased 7.8-fold during post-exponential phase compared to log phase, but decreased ~6-fold when *EFH1* was over-expressed. Therefore, over-expression of *EFH1* caused a decrease in *EFG1* expression during post-exponential phase growth ($p=0.0286$, Mann whitney test). This shows that

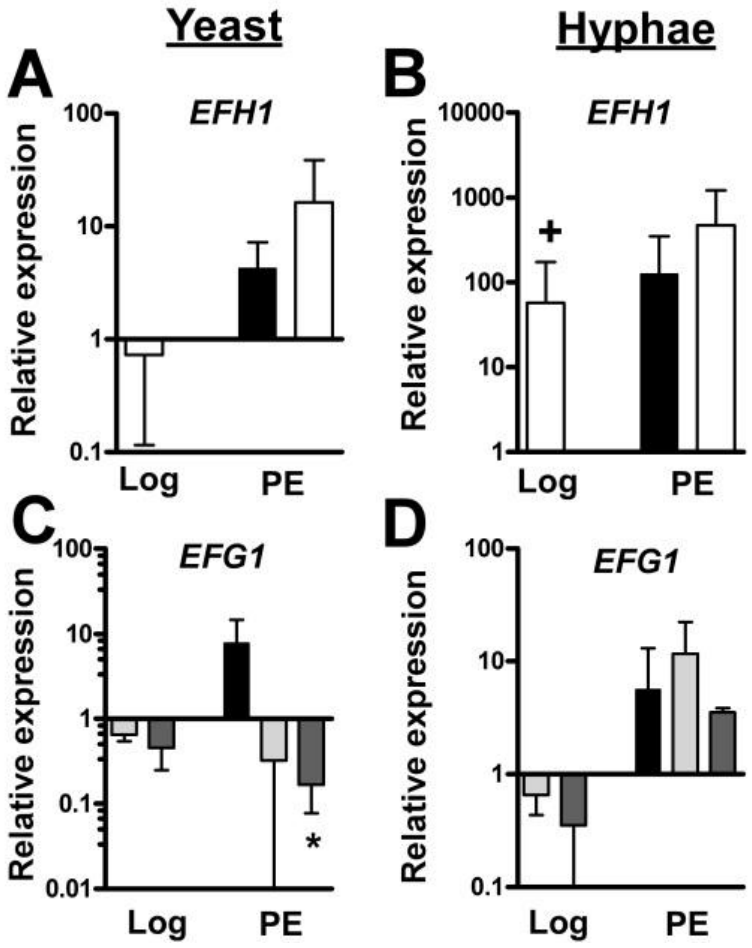


Figure 4-4. Co-regulation of *EFG1* and *EFH1* during yeast and hyphal growth phases.

Cells were grown as either yeast or hyphae after induction with serum at 37°C and samples taken during either log phase (Log) or post-exponential phase (PE). qRT-PCR was used to determine the level of *EFG1* or *EFH1* expression and normalized to the levels of *ACT1* and the level expressed by WT *C. albicans* during log phase for each gene. Data from at least 3 biological replicates is shown; errors bars are the SD. WT, black bars; *efg1*$^{-}$, white bars; *efh1*$^{-}$, light grey bars; *EFH1* over-expressing strain, dark grey bars. (A) *EFH1* expression during yeast growth. (B) *EFH1* expression during hyphal growth. (C) *EFG1* expression during yeast growth. (D) *EFG1* expression during hyphal growth. *$p<0.05$ Mann whitney test, + note fold change relative to WT log phase when normalized to 1.

during yeast phase growth, *EFG1* was not required for normal levels of *EFH1* expression, but over-expression of *EFH1* caused a decrease in *EFG1* levels.

After hyphal induction by dilution into serum, *EFH1* transcript levels were initially low, similar to the effects seen when cells are grown are yeast. *EFH1* transcription increased after 48 hours of growth in serum-containing media (Fig. 4-4B). In the absence of *EFG1*, *EFH1* levels increased ~60 fold relative to WT levels after 6 hours of growth (Fig. 4-4B). After a period of 48 hours, Efg1p no longer significantly repressed *EFH1* expression. Therefore, Efg1p repressed *EFH1* transcription in cells growing in hyphal inducing conditions.

EFG1 expression increased between the 6 and 48 hour time points during hyphal growth as well (Fig. 4-4D). At 6 hours post-induction, *EFG1* levels were ~5 fold lower when *EFH1* was over-expressed. However, at 48 hours post-induction, *EFG1* levels were similar to WT levels in both the absence of *EFH1* and when *EFH1* is over-expressed. These results suggest that over-expression of *EFH1* might also lower *EFG1* levels during growth soon after hyphal induction. These results suggest that Efg1p and Efh1p negatively regulate each other based on growth phase and hyphal inducing conditions. In an *efh1*$^{-}$ strain, which has increased persistence in the GI tract [42], there may be higher levels of *EFG1* expression which would promote enhanced resistance to the host immune response. Therefore, this transcriptional feedback loop may alter the ability of *C. albicans* to persist over time as a colonizing organism in the GI tract.

Effects of Sin3p, a component of a histone deacetylase complex, on GI tract colonization and the expression of *EFG1*

As a central transcription factor important for the physiology of *C. albicans*, *EFG1* undergoes multiple layers of regulation. For example, *SIN3*, a component of a histone deacetylase complex involved in chromatin remodeling, has been shown to repress *EFG1* expression soon after hyphal induction [124, 235]. We sought to determine if *SIN3* was also capable of regulating *EFG1* during GI tract colonization. We constructed a homozygous *sin3*$^-$ null mutant in which both copies of the *SIN3* gene were deleted, and then complemented the mutation by reintroducing one copy of *SIN3* back at its native locus. In media which promotes yeast growth during post-exponential phase at 37°C, mutants lacking *sin3*$^-$ exhibited unusual cellular morphology and grew as rods (Fig. 4-5C). This abnormal morphology was reversed when the mutation was complemented. Previous data showed that this strain grows as budding pseudohyphae and does not form true hyphae when induced by serum [124]. Therefore, deletion of the *SIN3* gene alters cellular morphology.

To determine the effects of *SIN3* on colonization, BALB/c and BALB/c *nu/nu* mice were colonized with WT, *sin3*$^-$, and a complemented *sin3*$^-$ mutant strains (pSIN3/*sin3*$^-$). Overall, as previously observed, colonization was higher in the absence of T cells for all strains when compared to the levels in a WT host [7]. At one day post-inoculation, there was no difference in colonization between WT and the *sin3*$^-$ strain in either a healthy or T cell deficient host. *sin3*$^-$ cells showed higher levels of colonization at day 6 post-inoculation in the fecal pellets of BALB/c *nu/nu* T cell deficient mice ($p<0.012$) (Fig. 4-6A) relative to WT *C. albicans* and this phenotype was

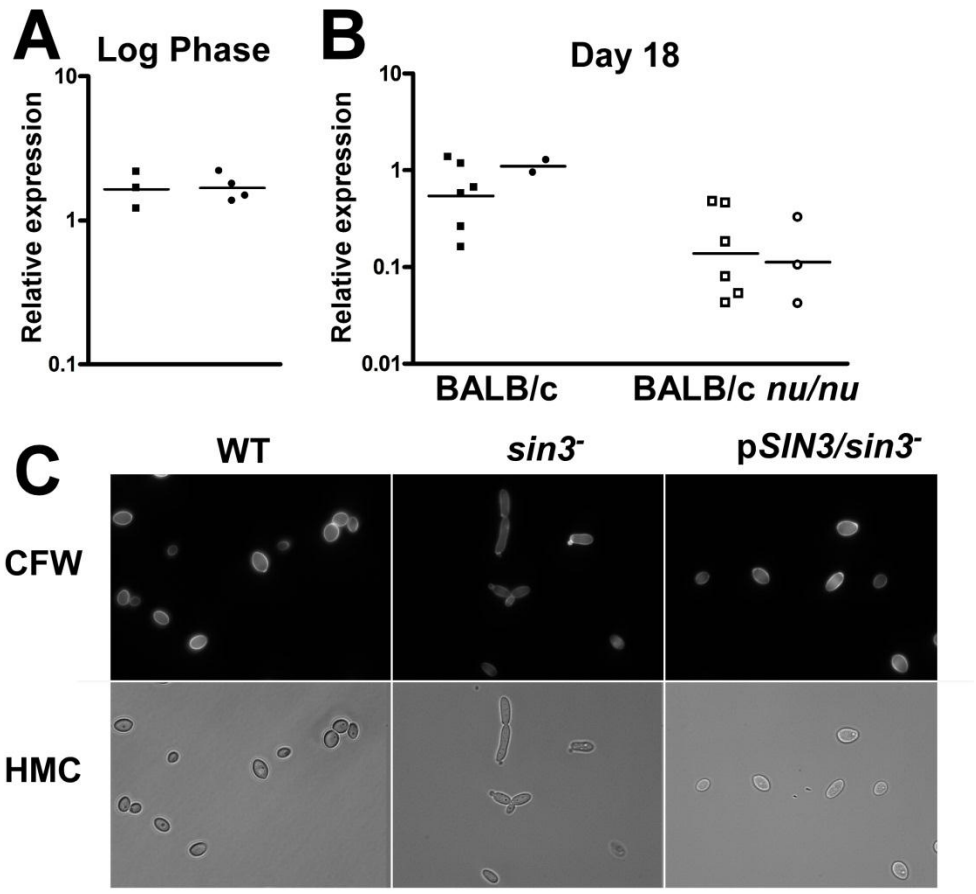


Figure 4-5. Effects of Sin3p on morphology and *EFG1* expression.

A *sin3*$^{-}$ strain and complemented mutant were constructed to observe the effect of *SIN3* on *EFG1* expression. Expression was measured by qRT-PCR and normalized to *ACT1* expression. (A) Expression of *EFG1* during log phase growth in both WT (squares) and *sin3*$^{-}$ strains (circles). (B) Expression of *EFG1* from cells colonizing the cecum either BALB/c (closed symbols) or BALB/c *nu/nu* mice (open symbols) at day 18 post-inoculation from WT (squares) and *sin3*$^{-}$ (circles) strains by RT-PCR. Values are expressed relative to levels present in laboratory grown cells. (C) Effect of *sin3*$^{-}$ deletion on cellular morphology. Cells were grown for 24 hours in rich yeast growth media at 37°C. Cells were stained with Calcofluor white (CFW) to stain chitin on the cell surface (upper panels). HMC, Hoffman modulation contrast (lower panels).

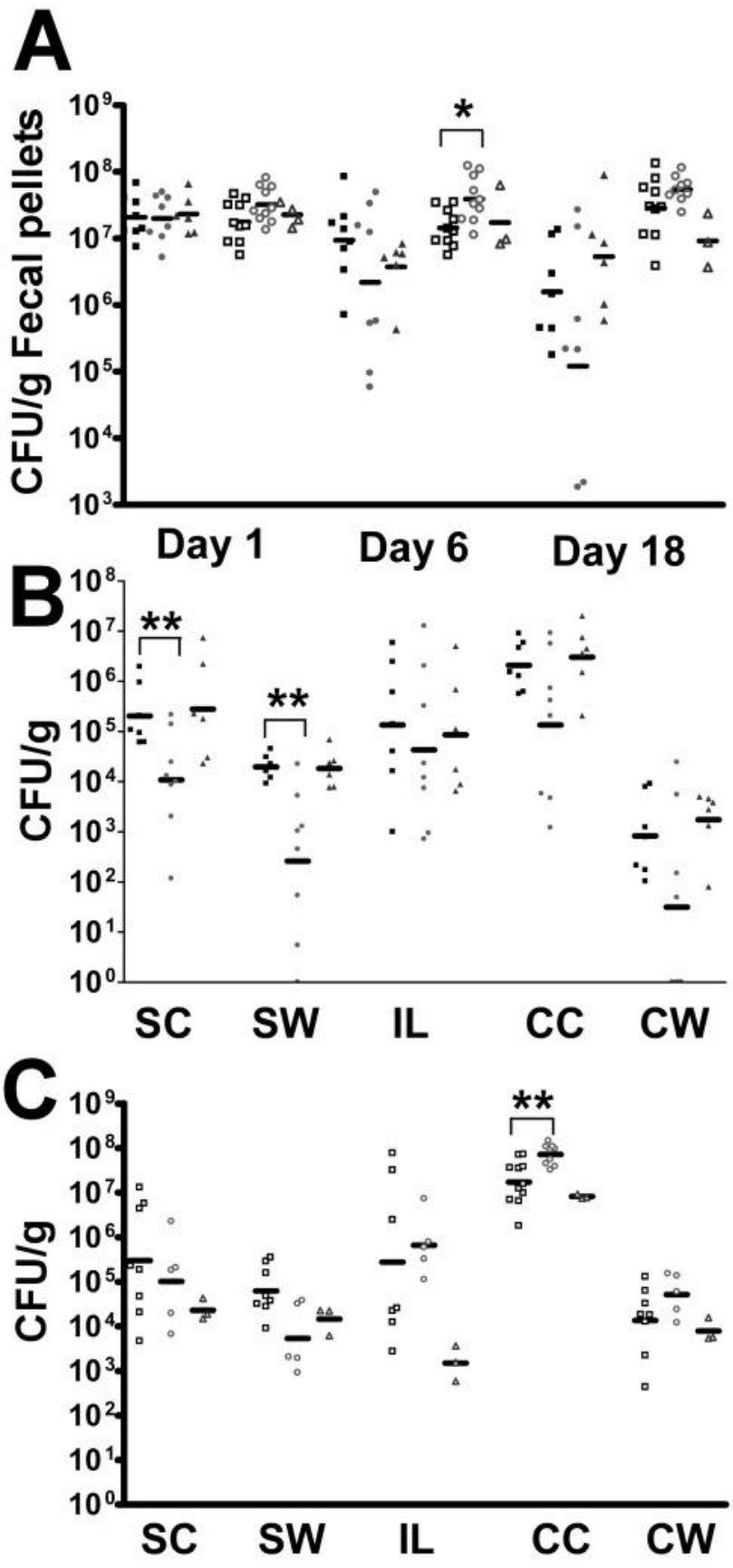

A
CFU/g Fecal pellets
10^9
10^8
10^7
10^6
10^5
10^4
10^3
*
Day 1
Day 6
Day 18
B
CFU/g
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
**
**
SC
SW
IL
CC
CW
C
CFU/g
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
**
SC
SW
IL
CC
CW

Figure 4-6. A *sin3*$^{-}$ deletion causes an increase in colonization over time in the absence of T cells.

Colonization of BALB/c (closed symbols) and BALB/c *nu/nu* mice (open symbols) with WT (squares), *sin3*$^{-}$ (circles), and a *SIN3* complemented strain (triangles). (A) Colonization in the fecal pellets at days 1, 6, and 18 post-inoculation. (B) Colonization of BALB/c organs at day 18 post-inoculation. (C) Colonization of BALB/c *nu/nu* organs at day 18 post-inoculation. SC, stomach contents; SW, stomach wall; IL, ileum; CC, cecum contents; CW, cecum wall. *$p<0.05$, **$p<0.01$ Mann whitney test.

complementable. This hyper-colonization by the *sin3*$^{-}$ mutant strain was not observed in BALB/c WT mice. In the organs at day 18 post-inoculation, the *sin3*$^{-}$ strain showed increased levels of colonization in the cecum contents in the T cell deficient host (Fig. 4-6C), consistent with the hyper-colonization detected in the fecal pellets at day 6. There was also a trend towards higher colonization of the *sin3*$^{-}$ mutant in the fecal pellets at day 18 ($p=0.182$). These observations show that the *sin3*$^{-}$ mutation resulted in a relatively small but detectable increase in colonization in an immunodeficient mouse.

In a healthy host, the *sin3*$^{-}$ strain showed decreased colonization in the stomach contents and stomach wall at day 18 post-inoculation ($p=0.004$; $p=0.03$, respectively) (Fig. 4-6B). There was also a trend towards lower colonization in the stomach wall of the T cell deficient host ($p=0.065$) (Fig. 4-6C). In addition, although the differences were not statistically significant, in the fecal pellets, cecum, and ileum at day 18, the levels of colonization by the *sin3*$^{-}$ strain tended to be lower than WT in a healthy host (fecal pellets, $p=0.336$; cecum, $p=0.121$; ileum, $p=0.397$). These results suggest that, as seen with the *efg1*$^{-}$ null mutant, the *sin3*$^{-}$ null mutant is hyper-susceptible to the host immune response that develops in healthy mice over time, but is able to colonize at higher levels in a T cell deficient host.

The *sin3*$^{-}$ phenotype did not fully recapitulate the effects of an *efg1*$^{-}$ mutant, but did show some similarities. Higher levels of colonization were detected by the *sin3*$^{-}$ strain in the absence of T cells and, in some organs of a healthy host, hyper-susceptibility to the host response was detected. These data also suggest that the *sin3*$^{-}$ mutant may have a specific defect in colonization of the stomach.

To test the effects of the *sin3*$^{-}$ deletion on *EFG1* expression, we analyzed *EFG1* transcript levels in cells grown under different conditions by qRT-PCR. Deletion of *SIN3* had no effect on *EFG1* expression during log phase growth (Fig. 4-5A). Cells were also harvested from the cecum of both WT and T cell deficient mice at day 18 post-inoculation to test the level of *EFG1* expression in the absence of *SIN3*. Previous data showed that in WT cells, *EFG1* expression is higher in a healthy host compared to a T cell deficient host. The deletion of *SIN3* did not affect levels of *EFG1* expression under these conditions: *EFG1* was still observed to be expressed at higher levels in a WT host compared to a T cell deficient host (Fig. 4-5B). During colonization, we were unable to detect a negative effect of *SIN3* on average *EFG1* expression in colonizing cells at day 18 post-inoculation in either a WT or T cell deficient host. However, deletion of *SIN3* allowed an increase in colonization of an immunocompromised host, and a defect in stomach colonization of a healthy host.

Effects of hyphal regulators Tec1p and Cph2p on GI colonization and gene expression

TEC1 and *CPH2* encode transcription factors that regulate hyphal development under particular laboratory conditions [125]. *EFG1* has been shown to regulate *TEC1* cooperatively with *CPH2* to induce hyphal growth [125]. We previously observed that cells colonizing the cecum showed increased expression of *CPH2* and *TEC1* [177]. Cph2p binds to SRE elements in the promoters of genes which it regulates, while Tec1p binds to a CATTCY sequence [130]. These observations suggested that Cph2p and Tec1p might be important for colonization in the GI tract. Therefore, strains lacking

Cph2p, with either a homozygous deletion mutation or UAU insertion mutation, or a strain lacking Tec1p, with a UAU insertion mutation, were tested for their ability to colonize mice following oral inoculation. Fecal pellets collected from mice showed that the *cph2*$^{-}$ null strains were defective in colonization relative to WT or *CPH2*$^{+}$ reconstituted *C. albicans* at day 3 post-inoculation (Fig. 4-7A). The *tec1*$^{-}$ *UAU* mutant showed no defect in colonization and appeared to colonize at WT levels (Fig. 4-7A). Based on these data, *CPH2*, but not *TEC1*, is required for normal levels of colonization in the GI tract.

To determine whether gene expression was altered by the absence of either Cph2p or Tec1p, cells were isolated from the cecum of mice colonized with several strains of *C. albicans*. For these studies, a WT, *cph2*$^{-}$ homozygous strain, *CPH2*$^{+}$ reconstituted strain, and *tec1*$^{-}$*UAU* insertion mutant strain were used. RNA was prepared from these cells, and expression of genes was determined by qRT PCR. Gene expression levels were normalized using the expression of *ACT1*. Expression of *ORF19.4450.1* was strongly up-regulated in cells recovered from the cecum relative to exponential phase growth in laboratory conditions (Fig. 4-7B), and this gene contained an SRE element in its promoter, suggesting it might be regulated by Cph2p. In the *cph2*$^{-}$ null mutant, expression of *ORF19.4450.1* was 100-fold lower than in WT cells; the *CPH2*$^{+}$ reconstituted strain showed expression levels similar to WT (Fig. 4-7B). Therefore, as predicted from the presence of SRE elements in its promoter region, *ORF19.4450.1* was dependent on Cph2p for up-regulation during growth in the cecum.

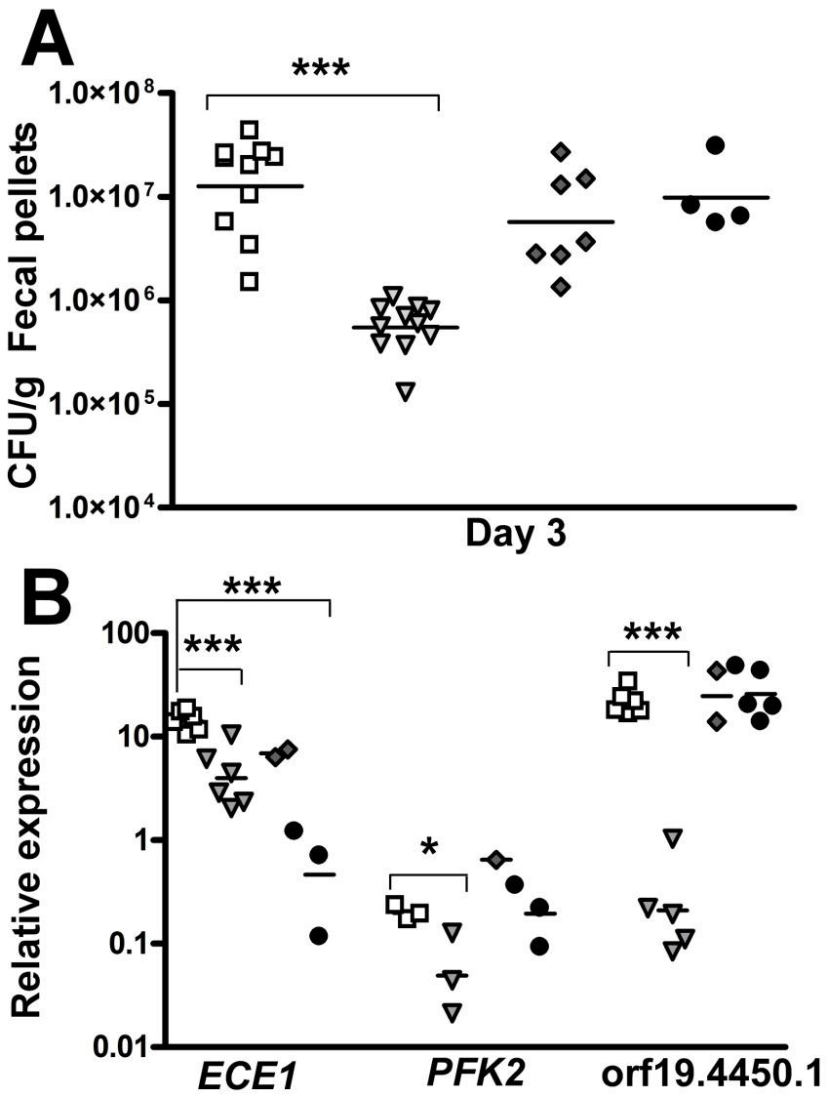


Figure 4-7. A *cph2*$^{-}$ strain is defective for colonization, while both *TEC1* and *CPH2* are required for normal gene expression patterns in colonizing cells.

(A) Colonization of C57/BK6 mice with *C. albicans*. Fecal pellet colonization at day 3 post-inoculation is shown. WT, squares; *cph2*$^{-}$, triangles; *CPH2*$^{+}$, diamonds; *tec1*$^{-}$, circles. (B) RT-PCR was used to determine the expression of *ECE1*, *PFK2*, and orf19.4450.1 from WT, *cph2*$^{-}$, *CPH2*$^{+}$, and *tec1*$^{-}$ strains. Expression was normalized to *ACT1* and expressed relative to laboratory grown cells. *** $p<0.001$ Student's t test.

ECE1 was up-regulated during colonization by WT *C. albicans* (Fig. 4-7B). This gene showed a slight defect in expression in the *cph2*$^{-}$ null mutant, but was expressed at 100-fold lower levels in the strain lacking Tec1p, consistent with the presence of CATTCY sequences in its promoter region [130]. *PFK2* was not highly up-regulated during colonization by WT *C. albicans*, but had decreased expression levels in the absence of *CPH2* (Fig. 4-7B). These results demonstrate that both Cph2p and Tec1p are required for normal patterns of gene expression in *C. albicans* cells growing in the cecum. However, *cph2*$^{-}$ cells were defective in colonization of the cecum, while *TEC1* was not required to maintain WT levels of colonization. These data show that, in addition to Efh1p and Efg1p, other regulators of morphogenesis affect GI tract colonization.

Discussion

In this work, we undertook several studies looking at the relationship of various morphological regulators and their impact on gastrointestinal colonization by *C. albicans*. *EFH1* was previously shown to be important for persistence in the gastrointestinal tract and was highly up-regulated in this specific niche [42]. This suggests that the main role of *EFH1* is as a pro-colonization factor. *EFH1* is a homologue of *EFG1*, a major regulator of both virulence and morphology [107, 111]. Given the importance of Efg1p for other aspects of *C. albicans* biology, we investigated the role of this transcriptional factor in GI tract colonization and showed that Efg1p is also important for colonization (Chapter 2).

We have shown here that Efg1p and Efh1p are involved in a negative feedback loop between yeast and hyphal growth. During hyphal induction, Efg1p negatively regulated *EFH1* expression. Alterations in *EFH1* expression also affect the regulation of *EFG1*: most notably, when *EFH1* is highly expressed, *EFG1* levels are low during yeast growth. This suggests an inverse relationship in the regulation of these two transcription factors: when *EFG1* transcript levels are high, *EFH1* transcript levels are low, and vice versa depending on environmental signals. In support of this model, previous data showed that *EFH1* is highly expressed during colonization [42], while *EFG1* is expressed at low levels during the initial colonization of the GI tract (Fig. 2-15). Therefore, there is a regulatory mechanism(s) that controls the expression of these two transcription factors.

Based on microarray data, Efg1p is active as both an activator and repressor of transcription during colonization. If Efg1p was active as a repressor of *EFH1* under these conditions, we would not expect to see the increased expression of *EFH1* which was previously observed [42]. In the gut, however, most cells are in the yeast form, and *EFH1* did not undergo repression by Efg1p under these conditions during laboratory growth. Therefore, growth in the gut is more similar to yeast growth in which high levels of *EFH1* repressed *EFG1* transcription. We observed that *EFG1* transcription was low during initial colonization. The repression of *EFH1* by Efg1p may require both increased transcript levels of *EFG1* and hyphal-inducing signals present in the environment.

These data suggest a model for the dynamics of *C. albicans* colonization in the GI tract. We propose that when cells are in the GI tract, low *EFG1* expression and high *EFH1* expression represent the initial adaptation to this environmental and colonization. Under these conditions, most of the cells are in the yeast form. In a situation where cues

that promote hyphal formation are detected, *EFG1* activity peaks, causing repression of *EFH1* transcription. This transcriptional program enables potential tissue invasion. Further analysis of the co-regulation of *EFG1* and *EFH1* during colonization and invasive disease would shed light on the regulatory network controlling hyphal formation and the role of these transcription factors in pathogenesis and colonization.

In addition to regulation by other transcription factors, *EFG1* is also regulated through a negative feedback loop upon hyphal induction [124]. This process requires Sin3p, a component of a histone deacetylase complex, which recruits a chromatin remodeling complex and results in global silencing of transcription [238]. As *EFG1* levels rise over time in a healthy host, but remain low in a T cell deficient host, we wanted to determine whether *SIN3* was required for this difference in gene expression. However, it appeared that the absence of *SIN3* did not alter the expression of *EFG1* between a healthy and T cell deficient host. Therefore, the regulation of *EFG1* expression in response to host immune status does not appear to require chromatin remodeling via the Sin3p complex. This does not exclude the possibility that *SIN3* is an important regulator of *EFG1* at other times during colonization or during invasive disease. Much of the regulatory network controlling colonization has yet to be defined.

In a normal host, the levels of colonization by the *sin3*$^{-}$ strain were lower than WT in the stomach, suggesting that this strain may be more sensitive to the stomach environment, which may be due to environmental factors such as low pH and/or the host immune response. The mutant strain was able to colonize to higher levels after about a week post-inoculation relative to WT *C. albicans*, but only in a T cell deficient host. While this increased colonization is similar to that of the *efg1*$^{-}$ mutant, it takes longer to

achieve. At day 18, increased colonization was also observed in the cecum contents, showing that some of the effects of increased colonization persist over time. These results are consistent with the hypothesis that, like the *efg1*$^{-}$ mutant, the *sin3*$^{-}$ mutant is capable of hyper-colonization but is sensitive to the host immune response.

Other transcription factors which regulate morphogenesis have also been shown to play a role in GI tract colonization. Cph2p is necessary for normal colonization of the cecum, and both Cph2p and Tec1p regulate gene expression during colonization. Both transcription factors were required for the expression of *ECE1*. In the laboratory, Cph2p promotes hyphal development during growth in a specific media [130]. Although the mechanism is not known, the activity of Cph2p may be regulated at some level in response to specific nutritional conditions. These observations suggest that the nutritional conditions that exist in the intestinal tract correspond to conditions that regulate Cph2p activity. These data are consistent with the hypothesis that the Cph2p/Tec1p regulatory network plays a specific role during colonization in response to the signals observed during colonization. Studies observing the effects of regulatory genes controlling the yeast to hyphal transition have shown that each factor has a unique role during GI tract colonization, and may be important for sensing and responding to the environmental cues present in this environment.

Materials and Methods

Media and growth conditions

YPD (1% yeast extract, 2% peptone, 2% glucose) and YPS (1% yeast extract, 2% peptone, and 2% sucrose) were used as rich growth media. Minimal dropout (CM) media lacking was made as previously described [198]. Cells from the GI tract were plated on YPD agar plus streptomycin (100ug/ml) and ampicillin (50ug/ml) (YPD-SA). Log phase cells were grown to an OD=1 in YPD at 30°C and post-exponential phase cells were grown for 48hrs. Hyphal induction was done by diluting overnight cultures into RPMI+ 10% serum + 2mM MOPS at pH7. Samples were collected at either 6hrs. (log phase) or 48hrs. (post-exponential phase) post-dilution. For mouse inoculations, strains were grown at 37°C in YPD for 21-24 hrs.

Strains and plasmids

All *C. albicans* strains used in this study are listed in Table 4-2. All strains used were derived from the wild type (WT) clinical strain SC5314. *UAU* mutants with either *CPH2* or *TEC1* deletions, and DAY185 (a WT control) were provided by A. Mitchell (Carnegie Mellon). Strains HLY1928 and HLY1929A, the *cph2*$^{-}$ and *CPH2*$^{+}$ reconstituted strains, were provided by H. Lui (Univ. of California Irving). WT laboratory strain SN100 was used for comparison and provided by A. Johnson (Univ. of California, San Francisco). The *efg1*$^{-}$ *efh1*$^{-}$ double null mutant was provided by J. Ernst. CAI-4 strain carrying two copies of chromosome 1 was used. Strains CAI-4 and HLC67 were kindly provided by Drs. W. Fonzi and G. Fink respectively. *E. coli* strains XL1-Blue or DH5alpha were used for propagating plasmids.

RT-PCR

Laboratory grown cells or contents of cecum (2 ceca/sample) taken at 3 days post-inoculation were mixed with RNA-Later (Ambion) and frozen at -80°C. RNA was extracted from laboratory grown samples using mechanical disruption and an RNA Easy mini kit (Qiagen) with on column DNaseI digestion. Samples of mouse GI tract contents were filtered through 250um polypropylene mesh (Small Parts, Inc), then pelleted and extracted using the Purelink kit Trizol extraction procedure (Invitrogen) with DNaseI digestion. Two ceca were combined per sample. RNA concentration was determined by Nanodrop® ND-1000 Spectrophotometer.

For RT-PCR confirmation, cDNA was generated from 1-10ug RNA with Superscript II or III Reverse transcriptase (Invitrogen) and an oligo-dT primer. Samples were heated to 70°C for 10 min. and the enzyme was added. The sample was then incubated at 42°C or 50°C, respectively, for 1.5 hrs. Following addition of 2.5M NaOH, the reaction was incubated at 37°C for 15 min. 2M Hepes free acid was then added to neutralize the pH and cDNA was purified using Qiaquick columns (Qiagen). Concentrations were determined using a Nanodrop spectrophotometer and samples were stored at -20°C.

Primers used to detect expression of genes are listed in Table 4-3. qRT-PCR reactions were performed with SYBR green reaction mix (Qiagen) with reference dye ROX using an MX3000p (Stratagene). Each sample was tested in triplicate and melting curve analysis in addition to agarose gel electrophoresis was used to confirm primer specificity. Samples tested with no cDNA showed no detectable signal. Standard curves were generated and all results normalized to the level of actin (*ACT1*) expression in each

respective sample. Expression from colonizing cells are presented normalized to the level of expression for each respective gene in wild type cells grown in YPD at 30°C to an OD=1. Laboratory grown cells are normalized to WT levels of expression during log phase.

Microscopy

Strains were grown to post-exponential phase at 37°C in YPD for 24hrs. Cells were resuspended in a 1mg/ml solution of Calcofluor white for 5 min., and then washed twice in PBS. Cells were visualized using a Nikon Eclipse TE2000-U inverted microscope and Roper Scientific CCD black and white camera using NIS-elements-BR software. Calcofluor white staining was detected with a UE-2E/C filter to detect fluorescence. Photographs were taken at 90X magnification. Hoffman modulation contrast (HMC) was used to image cells.

Animal models

5-7week old female BALB/c mice or BALB/c *nu/nu* (NCI) were treated with antibiotics (tetracycline 1mg/ml, streptomycin 2mg/ml, gentamycin 0.1mg/ml) as described [42]. For *CPH2* and *TEC1* experiments, female C57/BK6 mice (18-20 g, Charles River) were used. Mice were tested for fungal contamination prior to each experiment. Mice were inoculated with 0.1ml *C. albicans* cells at $5x10^8$ cells/mL by oral gavage. Colonization was tested over time by collecting fresh fecal pellets and plating homogenates on YPD agar plus streptomycin (100ug/ml) and ampicillin (50ug/ml) (YPD-SA). Mice were sacrificed at 3 or 18 days post-inoculation to obtain ceca contents for RNA isolation. Combined results from at least 2 experiments are shown for all data. Homogenates of kidney, liver, and tongue were plated on YPD-SA. No colonization of

these organs was detected in any experiment. All experiments were done in compliance with regulatory guidelines defined by Tufts University IACUC committee. Tissue for histology was preserved in 10% formalin and embedded in paraffin for sectioning by the DLAM histology lab. H&E staining was used to identify inflammation; GMS staining was used to identify fungi. Analysis of sections and imaging was done in part by Lauren Richey in a blinded study. The most severe regions of inflammation were scored on a scale of 0-4, where 0 is no inflammation and 4 is severe inflammation. Scoring was done for the glandular regions, forestomach and limiting ridge. In the forestomach, individual scores were given for particular types of inflammation: the presence of either neutrophils or monocytes, as well as epidermal hyperplasia was noted.

SC5314	Prototrophic, clinical isolate	Fonzi 1993
CAI-4	SC5314 *ura3*::imm434/*ura3*::imm434	Fonzi 1993
CKY101	CAI-4 (pDB152)	Brown et al. 1999
BWP17	SC5314 *ura3*::imm434 *his1*::hisG/*his1*::hisG *arg4*::hisG/*arg4*::hisG	Wilson 1999
DAY185	pARG4::URA3::*arg4*::hisG/*arg4*::hisG pHIS1::*his1*::hisG/*his1*::hisG	Davis 2000
SN100	*his1*::*his1* URA3/*ura3*::imm434	Noble 2005
HLY1928	*cph2*::ARG4/*cph2*::HIS1 *ura3*::1 imm434/*ura3*::1 imm434 *his1*::hisG/*his1*::hisG *arg4*::hisG/*arg4*::hisG ADE2/*ade2*::URA3	Lane 2001
HLY1929	*cph2*::ARG4/*cph2*::HIS1 *ura3*::1 imm434/*ura3*::1 imm434 *his1*::hisG/*his1*/hisG *arg4*::hisG/*arg4*::hisG ADE2/*ade2*::*CPH2*-URA3	Lane 2001
Cph2UAU	*his1*::hisG/*his1*::hisG *cph2-Tn7*::UAU1/*cph2-Tn7*::URA3	Enloe 2000
Tec1UAU	*his1*::hisG/*his1*::hisG *tec1*-Tn7::UAU/*tec1*-Tn7::URA3	Enloe 2000
JPY111	CAI-4 *sin3*::hisG/*sin3*::hisG	This study
JPY112	CAI-4 *sin3*::hisG/*sin3*::hisG *SIN3*::URA3	This study
CKY366	BWP17 *efh1*::ARG4/*efh1*::HIS1 leu2::pRD3915 (URA3)	White
HLC67	CAI-4 *efg1*::hisG/*efg1*::hisG	Lo et al. 1997
CKY136	CAI-4 *efg1::*hisG/*efg1::*hisG (pDB152)	Guisani et al. 2002
CKY364	SN100 *adh1*::pCK74 (Padh1-*EFH1*, HIS1 URA3)	White
JPY109	CKY136 *efh1::*hisG/*efh1::*hisG pRC3915::URA3	This study

Table 4-2. *C. albicans* strains used in these studies.

ACT1	ABAF3	5’-TATGAAAGTTAAGATTATTGCTCCACCAGAAA -3’
ACT1	ABAR3	5’-GGAAAGTAGACAATGAAGCCAAGATAGAAC-3’
ECE1	CRT9I	5’-CTTCAAAGACTCCCACAACTCAT-3’
ECE1	AB9R2	5’-A CTTTTCCGAAATATTCTTCAATCA-3’
EFG1	EFG1F1	5’-CAGTATGGTCAGTATAATGCTGGTAAGAA-3’
EFG1	EFG1R1	5’-TTGTTGTTGCTGTTGGTATGGATATGATGATG-3’
EFH1	AB53F1	5’-TATATCAACAATTTACAGCGTCTTACC-3’
EFH1	AB53R1	5’-GTTGTTGCTTTGCTCCGAATGCTTGAA-3’
Orf19.4450.1	FFFPF1	5’-CTGGTACTGCTATATTAATTGGAGGTGTG-3’
Orf19.4450.1	FFFPR1	5’-CATACTGTATCTTTTGCATTGGGTGA

Table 4-3. Primers used in these studies.

Chapter 5:

Discussion and conclusions

In these studies, we sought to further our understanding of how *C. albicans*, a diploid fungus, is capable of colonizing the GI tract and how this process is regulated. While the physiology of *C. albicans* has been well studied in laboratory conditions and multiple virulence factors have been identified, relatively little is known about how *C. albicans* colonizes its major environmental niche, the GI tract. Key to our understanding of *C. albicans* pathogenesis is how this microbe transitions from a colonizing organism to a pathogen when a host becomes immunocompromised. We have focused our studies on the contribution of transcription factors which govern morphogenesis to the regulation of GI tract colonization. In addition, we have investigated how one member of this group of proteins, Efg1p, affects the behavior of this colonizing microbe in healthy and immunocompromised (IC) hosts. We have demonstrated that Efg1p is subject to phenotypic variation and generates host-dependent gene expression in colonizing cells to produce populations which are primed for either colonization in a healthy host or pathogenesis in an IC host.

Phenotypic variation of Efg1p

Phenotypic variation is a common mechanism among microbes and may increase the fitness of individuals in the environment in the absence of genetic mutation [182, 239]. Bistability of gene expression in *Bacillus subtilis*, genetic competence, phage-dependent lysis of bacteria, and epigenetic inheritance are all examples of phenotypic variation within a population [240-242]. The ability to generate phenotypic variants increases diversity within a population and gives a subset of cells a survival advantage under stressful conditions, ensuring the survival of the population.

Antibiotic treatment of both bacterial and *C. albicans* populations generates subpopulations of cells with increased antibiotic resistance, called 'persisters' [243-245]. Persisters are not genetic mutants; they are genetically identical to non-persisters, but exhibit increased antibiotic resistance. When persister cells are isolated and propagated, the population will eventually revert back to its original composition: a majority of the cells will be sensitive to antibiotics, and a small number of cells will be persisters.

In the enteric pathogen *Salmonella typhimurium*, expression of the type three secretion system (TTSS) is variable due to stochasticity [246]. The production of phenotypic variants increased the overall fitness of the population by creating subpopulations in the gut which differed in their expression of the TTSS. In the gastrointestinal lumen, about 15% of *S. typhimurium* cells expressed the TTSS; in the gut tissue, however, almost all the bacteria expressed the TTSS. Bacteria which expressed the TTSS were more invasive compared to bacteria which did not, and the $TTSS^{+}$ bacteria produced high levels of gut inflammation. High levels of inflammation then caused a decrease in the number of commensal bacteria present in the gut. This effect then allowed for the proliferation of bacteria which did not express the TTSS. Thus, production of two phenotypic variants (+/- TTSS) increased the overall fitness of the microbial population. In *C. albicans*, variation in Efg1p may also produce cells which are more capable of invasion, or cells which colonize the GI tract better through increased metabolic capacity. Therefore, phenotypic variation is a common strategy allowing microbes to increase their fitness by altering the capacity of the population for virulence or colonization.

We demonstrated that Efg1p undergoes phenotypic variation, and that the level of its transcription is broadly distributed throughout the population. Varying levels of Efg1p transcript in individual cells leads to differential activity of Efg1p as a transcriptional repressor. Fdh1p, a putative formate dehydrogenase, was also expressed at varying levels within a population in an Efg1p-dependent manner. As *EFG1* regulates the expression of a large number of genes in the cell, differences in Efg1p activity are expected to have large effects on global gene expression. The *EPA1* gene of *C. glabrata*, encoding an important adhesin, was also recently demonstrated to exhibit phenotypic variation which persisted over several generations, showing that this phenomenon is not limited to Efg1p [184]. Phenotypic variants of *EPA1* differed in their ability to adhere to epithelial cells. Identification of genes with a similar pattern of heterogeneity in their expression would allow further characterization of the potential function of phenotypic variants during colonization.

As a wild type population was composed of cells with both high and low levels of Efg1p expression, we modeled the dynamics of such a population in a host using competition experiments. Mice were co-inoculated at a 1:1 ratio with strains which either lack *EFG1* (*efg1*$^-$) or over-express *EFG1* (Efg1p$^+$). In this scenario, the *efg1*$^-$ cells initially out-competed the Efg1p$^+$ strain. Over time, however, the over-expressing strain was more fit than the *efg1*$^-$ mutant. The out-competition of the Efg1p$^+$ strain occurred despite the fact that this strain is defective in colonization relative to WT *C. albicans* during a single strain inoculation. This biphasic phenotype was more dramatic than that of an *efg1*$^-$/WT competition. In this modeling experiment, the population was not capable of switching between either high or low Efg1p activity, and switching may alter

the dynamics of the colonization. Cells lacking Efg1p activity have an initial advantage in colonization, but cells with high Efg1p activity are more fit over time. These results support our model that cells with either high or low *EFG1* expression, generated through phenotypic variation, both contribute to normal colonization of the GI tract. Using mathematical models to predict the production of phenotypic variants and the relative fitness of these variants will allow us to more accurately characterize the population dynamics observed in the colonization of *C. albicans*.

Efg1p has been associated with other types of phenotypic variation, including the ability to switch between yeast and hyphal morphologies, as well as between white and opaque states [111, 153, 159]. Switching between white and opaque phases alters the mating capacity and virulence of *C. albican*s [153, 155, 247]. We observed that *WOR1*, the major transcription factor which regulates the generation of opaque cells, was up-regulated during GI colonization. Up-regulation of *WOR1* occurred with the concomitant down-regulation of *EFG1* and *WH11*, both white phase-specific genes, at early time points post-inoculation [159, 248]. This observation suggests that the production of opaque cells or cells with characteristics may be promoted during GI tract colonization. Therefore, in addition to the observed variability in Efg1p activity, white-opaque phase switching may also play a role in determining the fitness of colonizing populations.

Generation of phenotypic variation is controlled by both stochastic and regulated processes [182]. Positive feedback loops, such as those involved in opaque cell formation by *WOR1* and bistability in *Bacillus*, have been shown to generate phenotypic variation [106, 157, 240, 242]. Simulations and modeling show that negative auto-regulatory loops can also promote oscillations in protein abundance [249, 250]. The p53

and Hes1 transcription factors exhibit phenotypic variation through negative feedback loops [251, 252]. Hes1 negatively regulates its own expression, and this negative feedback leads to oscillations in Hes1 protein abundance. In this situation, high protein abundance is thought to cause transcriptional repression, followed by protein degradation. Once protein abundance decreases below a certain threshold, transcription is activated. In a more complex system involving at least two proteins, the human tumor suppressor p53 and its negative regulator Mdm2 both show oscillatory behavior following activation of p53 in response to DNA damage [253]. *EFG1* undergoes negative auto-regulation through the binding of Efg1p to its own promoter after the induction of hyphal growth [124]. Efg1p activity could vary in a manner similar to Hes1p, and utilize negative feedback loops to produce phenotypic diversity. Thus, oscillatory expression of *EFG1* in non-synchronized cells could generate cell-to-cell variation in Efg1p activity, contributing to the responsiveness of *C. albicans* populations to host immune status.

Genes regulated by chromatin remodeling processes have also been shown to have a high degree of noise (stochasticity) in their expression [180, 181]. Chromatin-mediated variation has been shown to be heritable for several generations [239]. Efg1p negatively regulates its own transcription through a histone deacetylase dependent mechanism [124]. The negative regulation of *EFG1* by Sin3p [124], a scaffold for histone deacetylases, suggests that histone modification may be a mechanism that contributes to the observed variation in *EFG1* expression [124, 234]. We showed that, in the absence of *SIN3*, *C. albicans* exhibited increased colonization of an immunocompromised host. This result may be due to aberrant regulation of *EFG1* that allows the accumulation of cells with low *EFG1* expression in a *sin3*$^{-}$ strain. Interestingly, the *sin3*$^{-}$ null mutant was also defective

at colonizing the stomach wall in a healthy host, the site where *C. albicans* typically invades tissue in murine models [6]. These observations suggest that Sin3p may positively regulate invasion of the stomach wall in a healthy host, but inhibit high levels of colonization through chromatin remodeling in an IC host.

Several other histone modification complexes have been shown to play a role in both virulence and morphogenesis. *EFG1* has been shown to recruit the Swi/Snf histone acetylase complex to the promoters of genes expressed during hyphal growth [148]. The Swi/Snf complex is required for systemic infections and the regulation of morphology. These observations suggest that positive regulation of gene expression by histone modification is important for invasive disease [254]. Others studies have shown that the histone deacetylase Set3p/Hos2p complex is required for virulence and the regulation of hyphal growth as well [255]. Therefore, chromatin remodeling at key genes may promote pathogenesis and limit colonization through both positive and negative regulation by histone-modifying complexes.

Although Sin3p did not appear to alter *EFG1* transcription during colonization, whether Sin3p contributes to the heterogeneity of *EFG1* expression or phenotypic switching during colonization remains to be determined. Interactions between Efg1p and histone modification proteins, such as Sin3p and the Swi/Snf complex, may contribute to the observed phenotypic variation in Efg1p activity. Understanding the mechanism driving the broad distribution of *EFG1* expression and the rate of change between these states will further our understanding of how *C. albicans* regulates both colonization and pathogenesis.

In addition to specific mechanisms which generate noise in gene expression, stochasticity in cellular processes is a major source of biological noise [180, 182, 256]. An important source of stochasticity in the cell is the rate of mRNA production/destruction [180]. Functional groups of proteins exhibit varying degrees of noise. For example, proteins which are regulated by chromatin remodeling have high levels of noise, while proteins involved in translation have low levels of noise [180]. Transcription factors are generally expressed at low levels, which can create variability in their activity [241]. Cell-to-cell variability in levels of transcription factors creates phenotypically diverse populations, increasing the fitness of the population by allowing some members of the group to adapt rapidly to changes in the environment.

Further experiments demonstrating the production of cells with both high and low Efg1p activity in the GI tract will be increase our understanding of the role of Efg1p in colonization. Isolation of cells with different Efg1p activity on a large scale would allow the assessment of different phenotypic properties of these cells, such as increased invasion or altered metabolism. In addition, the mechanism controlling the phenotypic variation of Efg1p and the stability of these states remains to be elucidated. Possible mechanisms include chromatin remodeling, regulatory feedback loops, or stochastic events in the cell.

Immunosensing by *C. albicans*

Using a model of gastrointestinal colonization, we have shown that colonizing *C. albicans* populations differ in their gene expression patterns based on the immune status of their host, specifically through the altered expression of Efg1p. Efg1p was initially

down-regulated in the gut relative to the level present in the inoculum. After this initial adaptation period, the average expression of Efg1p increased in a healthy host, but remained low in an immunocompromised host. Additionally, we observed that Efg1p activity as a transcriptional repressor of *FDH1* was differential between these two hosts. *FDH1* was expressed at higher levels in an IC host than a healthy host, consistent with negative regulation by Efg1p. Based on these studies, we expect that other genes regulated by Efg1p will also be differentially expressed in hosts of differing immune status, but these genes have yet to be identified. These observations are the first demonstration that colonizing populations alter their gene expression based on the immune status of the host. These events may produce populations with different capacities for either colonization or pathogenesis.

Thus, changes in the host induce changes in the gene expression within the colonizing microbial population, and we propose that these changes then lead to either colonization or disease, a process which we term 'immunosensing' [166]. Through phenotypic variation inherent in the expression of some genes, such as *EFG1*, we propose that *C. albicans* produces heterogeneous populations composed of cells which differ in their overall fitness [184, 246]. *C. albicans* initiates a gene expression program in a healthy host to suppress population size and thereby minimize its stimulation of the host immune system and promote colonization [42]. In an IC host, altered gene expression by the colonizing microbe can lead to overgrowth and the initiation of disease.

Differences in gene expression within a population may allow a portion of the cells to take advantage of different environmental conditions, which may be local (lumen vs. the epithelium) or global (changes in the host immune system) [239]. Environmental

or host-dependent selection can therefore alter the composition of a population to change its global gene expression. Alternatively, signaling between subpopulations of cells in response to local changes could cause the conversion of cells from one population to the other through altered gene expression. This strategy could therefore be used to sense the status of the immune response and control gene expression patterns to promote either colonization or pathogenesis [166]. Similar strategies may be employed by other opportunistic pathogens to allow them to sense changes in the host immune system, promoting either a colonizing or pathogenic lifestyle.

Deficiencies in the host immune response promote candidiasis

For these studies, we utilized BALB/c *nu/nu* mice, which are athymic, lack T cells, and show a partial defect in B cells, as a model of an immunocompromised host. During GI tract colonization, neither WT nor *nu/nu* mice exhibited signs of disseminated disease. As previously shown [7], we observed that BALB/c *nu/nu* mice developed an increased fungal burden over time in the GI tract, an early indicator of potential disease. In this model, T cells may control the levels of *C. albicans* colonization through the recruitment of neutrophils to the gut, which results in the killing of *C. albicans* cells through both phagocytosis and production of ROS. This model allowed us to detect differences in colonizing populations that are dependent on the host immune response prior to the development of systemic disease. The results from this model suggest that early detection of patients at risk for disseminated candidiasis may be feasible based on gene expression changes in the endogenous colonizing *C. albicans* populations.

Utilization of other models of immunodeficiency would allow for a better understanding of how *C. albicans* interacts with various components of the host immune system in the GI tract. T cells have been shown to play a major role in protection against mucosal disease, but are less important in controlling systemic infections. Further studies in understanding the contributions of specific subsets of T cells, such as Th1/Th2 and Treg cells, during colonization by *C. albicans* would allow us to better understand these responses in the gut [26, 174]. *C. albicans* has been shown to promote a pro-inflammatory Th17 response, which is essential for controlling mucosal infections [257-259]. In the GI tract, Th17 cells development is induced by commensal flora which can be protective against invading pathogens [27, 195]. Studies of the interactions between colonizing *C. albicans* cells and Th17 cells could be addressed using mice which lack the IL17 receptor, or by changing the microbial flora to alter the development of the Th17 response in the gut.

To further understand how fungal cells progress along the axis from commensal to pathogen, other models which promote invasion from the GI tract will be required. These models would allow the assessment of various stages of disease: from increased invasion of the GI tract mucosa, to dissemination to the bloodstream, and finally to invasion of deep organs such as the kidneys and liver [260]. Disruption of the GI tract mucosa and neutrophil depletion promotes disseminated disease from the GI tract [34]. Infant mouse models also promote invasion through the GI tract after oral inoculation with *C. albicans* [261]. These models would allow the assessment of further changes in gene expression by *C. albicans* as cells transition from colonization to invasion, and potentially identify factors important for regulating each stage of systemic disease.

Interaction of *C. albicans* with the microbiota

Colonization of the gastrointestinal tract by *C. albicans* is influenced not only by host functions, but also by the presence of hundreds of bacterial species, and the interactions between them. The microbiome is important in promoting colonization resistance against *C. albicans* [65]. After antibiotic treatment, *C. albicans* prevented the regrowth of *Lactobacilli* and promoted growth of *Enterococcus* species [67]. These disturbances to the microbiota were persistent over a period of weeks after antibiotic treatment was ceased. Bacterial re-colonization after antibiotic treatment also decreased the level of gastritis caused by *C. albicans*. Whether specific commensal species present in select mouse colonies, such as segmented filamentous bacteria, affect the ability of *C. albicans* to colonize the GI tract of mice remains unknown.

In addition to the commensal flora, invading pathogens can also alter the GI tract environment. Co-infection of *C. elegans* with the enteric pathogen *Salmonella Typhimurium* was shown to inhibit *C. albicans* hyphal formation and viability [80]. This was dependent on expression of *sopB*, an effector of the type III secretion system (TTSS) [79]. Thus, both pathogens and commensal bacteria can alter interactions between *C. albicans* and the host.

Growth of microbes in the GI tract is dependent on their ability to metabolize nutrients made available either by the host or as secondary metabolites produced by the microbiota. Metabolism in the gut is affected by commensal bacteria which breakdown undigestable polysaccharides into smaller carbon molecules, such as short chain fatty acids (SCFAs) [68, 262]. SCFAs, such as acetate, propionate, and butyrate, are non-fermentable carbon sources [57]. Both *Lactobacilli* and *Bacteroides* produce SCFAs as

major metabolic by-products of growth in the gut [60, 69]. The production of metabolites by bacteria, such as lactic acid and SCFAs, has been shown to inhibit filamentation by *C. albicans* [69, 263]. Other studies have shown that *C. albicans* is capable of growth on non-fermentable carbon sources, including SCFAs, in nutrient limiting conditions [57]. Therefore, SCFAs may be a carbon source for *C. albicans* in the gut, in addition to promoting yeast morphology. Carnitine is required for growth on non-fermentable carbon sources and mutations in genes involved in carnitine metabolism lead to growth defects on certain carbon sources, such as ethanol and acetate [56, 58]. Carnitine-dependent metabolism by *C. albicans* was not required for systemic virulence, but the ability to utilize non-fermentable carbon sources may allow for increased colonization by *C. albicans* [57]. Whether mutants with defects in carnitine metabolism affect the ability of *C. albicans* to colonize the GI tract has yet to be tested. It is possible that inhibitors of carnitine metabolism could be developed that would decrease *C. albicans* colonization in the gut.

Efg1p has been shown to repress metabolic gene expression during laboratory growth [111]. We observed that the *efg1*$^{-}$ mutant had both increased expression of genes involved in carnitine metabolism in the gut and higher colonization of the gut relative to WT cells. In addition, the *efg1*$^{-}$ mutant exhibited altered growth in laboratory conditions. *efg1*$^{-}$ cells were capable of reaching a higher population density in post-exponential phase growth compared to WT cells. Increased growth of the *efg1*$^{-}$ mutant was carbon source dependent; its growth was particularly enhanced in the presence of certain non-fermentable carbon sources.

We determined that the increased growth of the *efg1*$^{-}$ mutant in nutrient poor conditions reflected carnitine limitation in WT cells. Therefore, increased utilization of SCFAs through a carnitine-dependent mechanism in the gut may contribute to the hyper-colonization of the *efg1*$^{-}$ mutant. Indeed, the hyper-colonization of the *efg1*$^{-}$ null mutant is first observed in organs of the distal GI tract such as the cecum, potentially due to the higher levels of short chain fatty acids in this environment. Therefore, altered metabolism of the *efg1*$^{-}$ null mutant, particularly in carnitine utilization, likely contributes to the hyper-colonization of this mutant in the GI tract. Phenotypic variation in Efg1p activity may therefore alter the metabolic capacity in a percentage of the colonizing population, altering interactions between *C. albicans* and the bacterial microflora. Further studies demonstrating that *C. albicans* is capable of growth on bacterial metabolic by-products would increase our knowledge of how these microbes interact in the gut environment.

Role of morphology in colonization

In addition to regulating metabolic gene expression, Efg1p is a central regulator of both virulence and morphology [107, 111]. The ability to change from the yeast to hyphal morphology has been recognized as a major virulence factor of *C. albicans* [3]. The majority of the *C. albicans* cells observed in the GI tract environment exhibit yeast morphology, although hyphal cells are also present [42]. Yeast cells have been shown to colonize better than hyphal cells, and may increase the ability of *C. albicans* to disseminate through the bloodstream [81, 83]. Roles for hyphal cells in disease may include invasion of tissue or destruction of host cells leading to increased lethality during

an infection. The prevalence of yeast cells in the GI tract lumen may reflect their increased colonization ability. The presence of hyphae may generate greater diversity within the colonizing population, allowing for a rapid response to changes in the environment.

Fungal cells in association with the epithelium at the limiting ridge of the stomach, where *C. albicans* has been observed to invade in murine models, exhibit both hyphal and yeast morphologies [7]. Mutants which do not produce true hyphae, such as the *efg1*$^{-}$ strain, are capable of binding to this region, and can be observed as both yeast and pseudohyphae. Strains which produced either hyphae or pseudohyphae were both capable of inducing an inflammatory response, consisting in large part of the recruitment of neutrophils to the epithelial layer and extruding into the lumen. Other studies have shown that *efg1*$^{-}$ cells are capable of producing invasive lesions, and can be more invasive in hypoxic conditions [114, 115].

Yeast and hyphae have been shown to produce different immune responses when interacting with host cells (reviewed in [21]). Whether the nature of the immune response during colonization is affected by the morphology or gene expression of the *efg1*$^{-}$ mutant remains to be determined. *efg1* $^{-}$cells may interact differently with host cell receptors based on altered exposure of cell surface components or expression of cell surface proteins in this mutant. We observed that *efg1*$^{-}$ cells adhered earlier than WT *C. albicans* during colonization and evoked an earlier immune response, suggesting there may be some differences in the immune response to these strains. The specific interactions of the immune system with phenotypic variants consisting of cells with either high or low Efg1p activity require further elucidation.

Transcription factors which regulate morphogenesis have specific roles in colonization

Much study has focused on the role of transcription factors in responding to environmental signals to regulate morphology. Efh1p was initially identified as a homologue of Efg1p, and is a putative transcription factor [111]. Under laboratory conditions, Efh1p co-repressed filamentation during matrix embedding and hypoxia along with Efg1p [111]. Previous work identified Efh1p as important for persistence of colonization in the GI tract [42]. *EFH1* expression was induced in colonizing cells, but not during an oral infection model [42]. We observed that Efg1p and Efh1p are involved in a regulatory feedback loop, depending on whether cells are grown as yeast or hyphae. In the GI tract, initial changes in gene expression showed that Efh1p expression was induced [42], while Efg1p expression decreased. Efg1p was also shown to be epistatic to Efh1p during colonization. Both Efg1p and Efh1p contribute to the regulation of colonization, and interactions between these two transcription factors may promote either colonization or pathogenesis.

Multiple other transcription factors have been shown to regulate hyphal growth in response to specific growth conditions [123], but whether these proteins were also required for colonization was undetermined. The transcription factor Cph2p is required for filamentation in specific media, and the nutrient-dependent conditions which activate Cph2p may also be present in the GI tract [130]. We observed that Cph2p was required for WT levels of colonization and for normal gene expression patterns in colonizing cells [177]. In contrast, Tec1p did not alter the ability of *C. albicans* to colonize the GI tract, but did affect gene expression under these conditions. Cph2p, along with Efg1p, signals

through Tec1p to activate transcription under laboratory hyphal-inducing conditions, but whether this regulation is present in the GI tract environment is unknown [130].

Cph1p may alter the metabolic activity of *C. albicans* cells in the GI tract, as deletion of this gene caused a reduction in colonization of the *efg1*$^{-}$ mutant. Microarray data also implicated the importance of the transcription factors *UME6* and *WOR1* during colonization, both of which were up-regulated by WT *C. albicans* during colonization relative to laboratory grown cells. However, not every transcription factor involved in regulating the yeast to hyphal transition was required for GI tract colonization: deletions of *TEC1* and *RIM101* did not affect colonization levels. *RIM101* expression was induced in the GI tract, and, like *TEC1*, may be important for the regulation of gene expression in the GI tract.

Specific components of the transcriptional regulatory network that governs morphogenesis are responsible for the determination of population size and gene expression patterns during GI tract colonization. Based on our data, a subset of the regulatory pathways involved in the control of morphogenesis also responds to signals in the gastrointestinal tract. Further analysis of these transcription factors, as well as the signaling components upstream of them, will help identify the essential signaling network involved in colonization and the relevant environmental signals which govern this response.

Multiple signaling pathways are responsible for communicating changes in the environment to the network of transcription factors which promote hyphal growth. The upstream signaling components which might be involved in transcriptional responses during colonization have yet to be identified in this environment. These signaling

pathways may include components of the cAMP/PKA pathway, such as Ras1p and Tpk1/2p, which have been shown to signal through Efg1p under laboratory conditions [124]. Efg1p also signals downstream of the matrix embedding pathway and GlcNAc sensing pathway [190], both of which were up-regulated by WT *C. albicans* during colonization. These data suggest that these may be relevant signaling pathways which alter gene expression during colonization and involve Efg1p. Analysis of mutants in these pathways will determine their relative contribution to signaling during colonization and help identify relevant signals in this environment.

In response to signaling through the cAMP/PKA pathway, Efg1p activates gene expression during hyphal growth [124]. During colonization, Efg1p was required for the expression of a subset of these genes, including *ALS3*, *SAPs4-6*, and *HYR1*. However, not all genes regulated by Efg1p under laboratory conditions required *EFG1* for their expression during colonization (*ECE1*, *HWP1*, and *RBT5*), consistent with previous data [42]. This shows that in colonizing cells, Efg1p is only required for the expression of a subset of hyphal-regulated genes, while others are expressed independently of Efg1p. Therefore, the regulation of genes expressed during filamentation is different from their regulation in colonizing cells. In colonizing cells, Efg1p regulates the expression of a subset of genes associated with hyphal formation, despite the fact that these cells are mainly yeast.

Among the genes regulated by Efg1p, *SOD5*, a superoxide dismutase, was highly up-regulated by WT *C. albicans* in colonizing cells. *SOD5* is induced in the presence of macrophages and neutrophils, and is protective against reactive oxygen species production [142, 143]. This suggests that an important component of the host immune

response during colonization by *C. albicans* is the production of reactive oxygen species (ROS). Therefore, a major defect of the *efg1*$^{-}$ mutant in colonization is its sensitivity to ROS. *efg1*$^{-}$ cells have been shown to be more susceptible to phagocytosis by both macrophages and neutrophils [25, 107], and this may be due to both increased ROS sensitivity and their inability to escape phagocytosis through hyphal formation. The *efg1*$^{-}$ strain was capable of dissemination and disease when orally inoculated into a host deficient in ROS production [35]. These results support the model that *efg1*$^{-}$ cells have reduced virulence in a WT host due to their sensitivity to the production of ROS. Variable activity of Efg1p within a population would then alter the susceptibility of some cells to the immune response based on the sensitivity of cells with low Efg1p activity to ROS production.

Efg1p acts as an immunosensor by altering gene expression

In colonizing cells, Efg1p regulated the expression of genes involved in both the defense against the host immune response and metabolism. Efg1p was required for the expression of genes, involved in protection against the host immune response, such as the super oxide dismutase *SOD5*. Metabolic gene expression, specifically carnitine metabolism, was negatively regulated by Efg1p during colonization. Therefore, the expression of either host-defense genes or metabolic genes depends on the level of Efg1p activity. As Efg1p exhibits phenotypic variation, this could produce subpopulations with different capacities for growth or defense against the immune response.

The ability of *C. albicans* to colonize at higher levels is balanced with its ability to protect itself against the host immune response through the activity of Efg1p. Our data

show that in the absence of Efg1p, cells were capable of hyper-colonization, potentially through the increased utilization of non-fermentable carbon sources like short chain fatty acids. However, the *efg1*$^{-}$ mutant was more sensitive to the host immune response and was defective in colonization over time. The increased susceptibility of the *efg1*$^{-}$ mutant to the host immune response was due in part to decreased expression of *SOD5*. This suggests that sensitivity to reactive oxygen species production during colonization contributed to the defect in colonization of the *efg1*$^{-}$ mutant. By regulating the expression of Efg1p, *C. albicans* can either promote increased colonization or defense against the immune response through the regulation of metabolism and defense against the host immune response.

Increased colonization is observed in the GI tract of immunocompromised hosts [7] and this is considered a risk factor for systemic infections in susceptible patient populations [113]. In a healthy host, *C. albicans* suppresses its growth during colonization, potentially limiting inflammation, while also activating the expression of genes involved in defense against the host immune response [42]. These two lifestyles are thus regulated by the level of Efgp1 activity. Low Efg1p activity de-represses metabolic genes to increase colonization in an IC host, while higher Efg1p activity limits colonization and promotes expression of genes involved in immune response resistance in a healthy host. Therefore, low Efg1p activity is characteristic of cells capable of initiating disease, while increased activity is representative of cells in a colonizing state. Inter-conversion between these two states could therefore indicate a switch between global transcriptional patterns which promote either colonization or pathogenesis.

Distinct regulatory circuits for colonization and pathogenesis by *C. albicans* were also observed in the regulation of iron uptake [104]. The transcription factor Sef1p actively regulated iron uptake in the presence of plasma, similar to the conditions observed during systemic infections. A second transcription factor, Sfu1p repressed iron uptake in colonizing cells. Sef1p was required for systemic virulence, while a *sfu1*$^{-}$ mutant was more defective in colonization in the GI tract. Sef1p and Sfu1p co-regulated each other via a negative feedback loop that was dependent on the presence of iron in the environment. Therefore, there are distinct regulatory mechanisms active in either colonizing or pathogenic populations based on their gene expression patterns. We propose that the regulation of Efg1p acts in a similar fashion to produce phenotypically diverse populations with different capacities for pathogenesis based on the host immune system environment.

Therefore, through the phenotypic variation of Efg1p, *C. albicans* is able to regulate its gene expression in a manner which allows it to adapt to changes in its environment, specifically the host immune status. Efg1p-dependent regulation of metabolism and host defense produces cells which have either the ability to colonize at high levels, or protect themselves from the host immune system. When changes in the host immune system occur, the global gene expression of the population is able to respond through Efg1p activity. Therefore, Efg1p activity could be used as a measure of the pathogenic potential of the population. Understanding the differences between colonizing and pathogenic populations and how these differences are regulated may lead to better identification of patients at risk for developing disease, as well as novel drug targets. Modulation of Efg1p activity or its downstream targets may represent new

strategies for anti-fungal drug development. The production of phenotypic variants during colonization may also alter the drug sensitivity of cells in that environment. Thus, analysis of gene expression in a colonizing microbial population may help to identify patients at the highest risk for developing candidiasis and in the development of better treatment methods.

We have shown that a colonizing *C. albicans* population is able to alter its gene expression based on the immune status of the host through the activity of Efg1p. Efg1p undergoes phenotypic variation which may produce subpopulations with altered capabilities for either growth or defense against the host. These characteristics would then promote increased gastrointestinal colonization by *C. albicans* in an IC host, or protect colonizing fungal cells against the immune response of a healthy host. Therefore, Efg1p can act as an immunosensor to promote either colonization or pathogenesis through the regulation of gene expression by controlling both metabolism and host interactions.

Bibliography

1. Odds, F.C., *Candida infections: an overview.* Crit Rev Microbiol, 1987. **15**(1): p. 1-5.
2. Kauffman, C.A., *Fungal infections.* Proc Am Thorac Soc, 2006. **3**(1): p. 35-40.
3. Calderone, R.A. and W.A. Fonzi, *Virulence factors of Candida albicans.* Trends Microbiol, 2001. **9**(7): p. 327-35.
4. Woodmansey, E.J., et al., *Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects.* Appl Environ Microbiol, 2004. **70**(10): p. 6113-22.
5. Bendel, C.M., *Colonization and epithelial adhesion in the pathogenesis of neonatal candidiasis.* Semin Perinatol, 2003. **27**(5): p. 357-64.
6. Cole, G.T., et al., *Chlamydospore-like cells of Candida albicans in the gastrointestinal tract of infected, immunocompromised mice.* Can J Microbiol, 1991. **37**(8): p. 637-46.
7. Balish, E., et al., *Colonization of congenitally athymic, gnotobiotic mice by Candida albicans.* Appl Environ Microbiol, 1984. **47**(4): p. 647-52.
8. Thewes, S., et al., *In vivo and ex vivo comparative transcriptional profiling of invasive and non-invasive Candida albicans isolates identifies genes associated with tissue invasion.* Mol Microbiol, 2007. **63**(6): p. 1606-28.
9. Walker, L.A., et al., *Genome-wide analysis of Candida albicans gene expression patterns during infection of the mammalian kidney.* Fungal Genet Biol, 2009. **46**(2): p. 210-9.
10. Netea, M.G., et al., *Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors.* J Clin Invest, 2006. **116**(6): p. 1642-50.
11. Gross, O., et al., *Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence.* Nature, 2009. **459**(7245): p. 433-6.
12. Pivarcsi, A., et al., *Expression and function of Toll-like receptors 2 and 4 in human keratinocytes.* Int Immunol, 2003. **15**(6): p. 721-30.
13. Roeder, A., et al., *Toll-like receptors and innate antifungal responses.* Trends Microbiol, 2004. **12**(1): p. 44-9.
14. Gow, N.A., et al., *Immune recognition of Candida albicans beta-glucan by dectin-1.* J Infect Dis, 2007. **196**(10): p. 1565-71.
15. McGreal, E.P., et al., *The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose.* Glycobiology, 2006. **16**(5): p. 422-30.
16. Szolnoky, G., et al., *A mannose-binding receptor is expressed on human keratinocytes and mediates killing of Candida albicans.* J Invest Dermatol, 2001. **117**(2): p. 205-13.
17. Cambi, A., et al., *Dendritic cell interaction with Candida albicans critically depends on N-linked mannan.* J Biol Chem, 2008. **283**(29): p. 20590-9.
18. Cambi, A., et al., *The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells.* Eur J Immunol, 2003. **33**(2): p. 532-8.

19. Cheng, S.C., et al., *The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of Candida albicans.* J Leukoc Biol, 2011. **90**(2): p. 357-66.
20. Gantner, B.N., R.M. Simmons, and D.M. Underhill, *Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments.* Embo J, 2005. **24**(6): p. 1277-86.
21. Gow, N.A., et al., *Candida albicans morphogenesis and host defence: discriminating invasion from colonization.* Nat Rev Microbiol, 2012. **10**(2): p. 112-22.
22. Villar, C.C. and A. Dongari-Bagtzoglou, *Immune defence mechanisms and immunoenhancement strategies in oropharyngeal candidiasis.* Expert Rev Mol Med, 2008. **10**: p. e29.
23. Tuite, A., A. Mullick, and P. Gros, *Genetic analysis of innate immunity in resistance to Candida albicans.* Genes Immun, 2004. **5**(7): p. 576-87.
24. Romani, L., *Innate and adaptive immunity in Candida albicans infections and saprophytism.* J Leukoc Biol, 2000. **68**(2): p. 175-9.
25. Korting, H.C., et al., *Reduced expression of the hyphal-independent Candida albicans proteinase genes SAP1 and SAP3 in the efg1 mutant is associated with attenuated virulence during infection of oral epithelium.* J Med Microbiol, 2003. **52**(Pt 8): p. 623-32.
26. De Luca, A., et al., *Functional yet balanced reactivity to Candida albicans requires TRIF, MyD88, and IDO-dependent inhibition of Rorc.* J Immunol, 2007. **179**(9): p. 5999-6008.
27. Ivanov, II, et al., *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. **139**(3): p. 485-98.
28. van de Veerdonk, F.L., B.J. Kullberg, and M.G. Netea, *Pathogenesis of invasive candidiasis.* Curr Opin Crit Care, 2010. **16**(5): p. 453-9.
29. Wu, H.J., et al., *Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells.* Immunity, 2010. **32**(6): p. 815-27.
30. Huang, W., et al., *Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice.* J Infect Dis, 2004. **190**(3): p. 624-31.
31. Puel, A., et al., *Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity.* Science, 2011. **332**(6025): p. 65-8.
32. van de Veerdonk, F.L., et al., *STAT1 mutations in autosomal dominant chronic mucocutaneous candidiasis.* N Engl J Med, 2011. **365**(1): p. 54-61.
33. Westwater, C., et al., *Susceptibility of gnotobiotic transgenic mice (Tgepsilon26) with combined deficiencies in natural killer cells and T cells to wild-type and hyphal signalling-defective mutants of Candida albicans.* J Med Microbiol, 2007. **56**(Pt 9): p. 1138-44.
34. Koh, A.Y., et al., *Mucosal damage and neutropenia are required for Candida albicans dissemination.* PLoS Pathog, 2008. **4**(2): p. e35.
35. Balish, E., et al., *Susceptibility of germfree phagocyte oxidase- and nitric oxide synthase 2-deficient mice, defective in the production of reactive metabolites of both oxygen and nitrogen, to mucosal and systemic candidiasis of endogenous origin.* Infect Immun, 2005. **73**(3): p. 1313-20.

36. Bozza, S., et al., *A crucial role for tryptophan catabolism at the host/Candida albicans interface.* J Immunol, 2005. **174**(5): p. 2910-8.
37. Romani, L., F. Bistoni, and P. Puccetti, *Adaptation of Candida albicans to the host environment: the role of morphogenesis in virulence and survival in mammalian hosts.* Curr Opin Microbiol, 2003. **6**(4): p. 338-43.
38. Cheng, S.C., et al., *Candida albicans dampens host defense by downregulating IL-17 production.* J Immunol, 2010. **185**(4): p. 2450-7.
39. Noverr, M.C., et al., *Pathogenic yeasts Cryptococcus neoformans and Candida albicans produce immunomodulatory prostaglandins.* Infect Immun, 2001. **69**(5): p. 2957-63.
40. Villamon, E., et al., *Toll-like receptor 2 mediates prostaglandin E(2) production in murine peritoneal macrophages and splenocytes in response to Candida albicans.* Res Microbiol, 2005. **156**(1): p. 115-8.
41. Erb-Downward, J.R. and M.C. Noverr, *Characterization of prostaglandin E2 production by Candida albicans.* Infect Immun, 2007. **75**(7): p. 3498-505.
42. White, S.J., et al., *Self-regulation of Candida albicans population size during GI colonization.* PLoS Pathog, 2007. **3**(12): p. e184.
43. Davis, D., et al., *Candida albicans RIM101 pH response pathway is required for host-pathogen interactions.* Infect Immun, 2000. **68**(10): p. 5953-9.
44. Hameed, S., et al., *Iron deprivation induces EFG1-mediated hyphal development in Candida albicans without affecting biofilm formation.* FEMS Yeast Res, 2008. **8**(5): p. 744-55.
45. Brown, A.J. and N.A. Gow, *Regulatory networks controlling Candida albicans morphogenesis.* Trends Microbiol, 1999. **7**(8): p. 333-8.
46. Ernst, J.F. and D. Tielker, *Responses to hypoxia in fungal pathogens.* Cell Microbiol, 2009. **11**(2): p. 183-90.
47. He, G., et al., *Noninvasive measurement of anatomic structure and intraluminal oxygenation in the gastrointestinal tract of living mice with spatial and spectral EPR imaging.* Proc Natl Acad Sci U S A, 1999. **96**(8): p. 4586-91.
48. Fallingborg, J., et al., *Measurement of gastrointestinal pH and regional transit times in normal children.* J Pediatr Gastroenterol Nutr, 1990. **11**(2): p. 211-4.
49. Wang, Y. and X.L. Xu, *Bacterial peptidoglycan-derived molecules activate Candida albicans hyphal growth.* Commun Integr Biol, 2008. **1**(2): p. 137-9.
50. Johnson, L., *Iron and siderophores in fungal-host interactions.* Mycol Res, 2008. **112**(Pt 2): p. 170-83.
51. McLaughlin, H.P., C. Hill, and C.G. Gahan, *The impact of iron on Listeria monocytogenes; inside and outside the host.* Curr Opin Biotechnol, 2011. **22**(2): p. 194-9.
52. Pieracci, F.M. and P.S. Barie, *Iron and the risk of infection.* Surg Infect (Larchmt), 2005. **6 Suppl 1**: p. S41-6.
53. Miwa, T., et al., *Gpr1, a putative G-protein-coupled receptor, regulates morphogenesis and hypha formation in the pathogenic fungus Candida albicans.* Eukaryot Cell, 2004. **3**(4): p. 919-31.
54. Midkiff, J., et al., *Small molecule inhibitors of the Candida albicans budded-to-hyphal transition act through multiple signaling pathways.* PLoS One, 2011. **6**(9): p. e25395.

55. Askew, C., et al., *Transcriptional regulation of carbohydrate metabolism in the human pathogen Candida albicans.* PLoS Pathog, 2009. **5**(10): p. e1000612.
56. Strijbis, K., et al., *Carnitine-dependent transport of acetyl coenzyme A in Candida albicans is essential for growth on nonfermentable carbon sources and contributes to biofilm formation.* Eukaryot Cell, 2008. **7**(4): p. 610-8.
57. Zhou, H. and M.C. Lorenz, *Carnitine acetyltransferases are required for growth on non-fermentable carbon sources but not for pathogenesis in Candida albicans.* Microbiology, 2008. **154**(Pt 2): p. 500-9.
58. Strijbis, K., et al., *Contributions of carnitine acetyltransferases to intracellular acetyl unit transport in Candida albicans.* J Biol Chem, 2010. **285**(32): p. 24335-46.
59. Cummings, J.H., et al., *Short chain fatty acids in human large intestine, portal, hepatic and venous blood.* Gut, 1987. **28**(10): p. 1221-7.
60. Macfarlane, S. and G.T. Macfarlane, *Regulation of short-chain fatty acid production.* Proc Nutr Soc, 2003. **62**(1): p. 67-72.
61. Brown, D.H., Jr., et al., *Filamentous growth of Candida albicans in response to physical environmental cues and its regulation by the unique CZF1 gene.* Mol Microbiol, 1999. **34**(4): p. 651-62.
62. Giusani, A.D., M. Vinces, and C.A. Kumamoto, *Invasive filamentous growth of Candida albicans is promoted by Czf1p-dependent relief of Efg1p-mediated repression.* Genetics, 2002. **160**(4): p. 1749-53.
63. Lorenz, M.C., J.A. Bender, and G.R. Fink, *Transcriptional response of Candida albicans upon internalization by macrophages.* Eukaryot Cell, 2004. **3**(5): p. 1076-87.
64. Stecher, B. and W.D. Hardt, *Mechanisms controlling pathogen colonization of the gut.* Curr Opin Microbiol, 2011. **14**(1): p. 82-91.
65. van der Waaij, D., *The digestive tract in immunocompromised patients: importance of maintaining its resistance to colonization, especially in hospital in-patients and those taking antibiotics.* Antonie Van Leeuwenhoek, 1984. **50**(5-6): p. 745-61.
66. Clark, J.D., *Influence of antibiotics or certain intestinal bacteria on orally administered Candida albicans in germ-free and conventional mice.* Infect Immun, 1971. **4**(6): p. 731-7.
67. Mason, K.L., et al., *Interplay between the gastric bacterial microbiota and Candida albicans during postantibiotic recolonization and gastritis.* Infect Immun, 2012. **80**(1): p. 150-8.
68. Cummings, J.H. and G.T. Macfarlane, *Role of intestinal bacteria in nutrient metabolism.* JPEN J Parenter Enteral Nutr, 1997. **21**(6): p. 357-65.
69. Noverr, M.C. and G.B. Huffnagle, *Regulation of Candida albicans morphogenesis by fatty acid metabolites.* Infect Immun, 2004. **72**(11): p. 6206-10.
70. Round, J.L. and S.K. Mazmanian, *Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota.* Proc Natl Acad Sci U S A, 2010. **107**(27): p. 12204-9.
71. Mazmanian, S.K., et al., *An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system.* Cell, 2005. **122**(1): p. 107-18.

72. Lee, Y.K. and S.K. Mazmanian, *Has the microbiota played a critical role in the evolution of the adaptive immune system?* Science, 2010. **330**(6012): p. 1768-73.
73. Mazmanian, S.K., J.L. Round, and D.L. Kasper, *A microbial symbiosis factor prevents intestinal inflammatory disease.* Nature, 2008. **453**(7195): p. 620-5.
74. Peleg, A.Y., D.A. Hogan, and E. Mylonakis, *Medically important bacterial-fungal interactions.* Nat Rev Microbiol, 2010. **8**(5): p. 340-9.
75. Hogan, D.A. and R. Kolter, *Pseudomonas-Candida interactions: an ecological role for virulence factors.* Science, 2002. **296**(5576): p. 2229-32.
76. Peleg, A.Y., et al., *Prokaryote-eukaryote interactions identified by using Caenorhabditis elegans.* Proc Natl Acad Sci U S A, 2008. **105**(38): p. 14585-90.
77. Hogan, D.A., A. Vik, and R. Kolter, *A Pseudomonas aeruginosa quorum-sensing molecule influences Candida albicans morphology.* Mol Microbiol, 2004. **54**(5): p. 1212-23.
78. Bauernfeind, A., et al., *Qualitative and quantitative microbiological analysis of sputa of 102 patients with cystic fibrosis.* Infection, 1987. **15**(4): p. 270-7.
79. Kim, Y. and E. Mylonakis, *Killing of Candida albicans filaments by Salmonella enterica serovar Typhimurium is mediated by sopB effectors, parts of a type III secretion system.* Eukaryot Cell, 2011. **10**(6): p. 782-90.
80. Tampakakis, E., A.Y. Peleg, and E. Mylonakis, *Interaction of Candida albicans with an intestinal pathogen, Salmonella enterica serovar Typhimurium.* Eukaryot Cell, 2009. **8**(5): p. 732-7.
81. Bendel, C.M., et al., *Comparative virulence of Candida albicans yeast and filamentous forms in orally and intravenously inoculated mice.* Crit Care Med, 2003. **31**(2): p. 501-7.
82. Mukherjee, P.K., et al., *Reintroduction of the PLB1 gene into Candida albicans restores virulence in vivo.* Microbiology, 2001. **147**(Pt 9): p. 2585-97.
83. Saville, S.P., et al., *Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of Candida albicans during infection.* Eukaryot Cell, 2003. **2**(5): p. 1053-60.
84. Qian, Q. and J.E. Cutler, *Gamma interferon is not essential in host defense against disseminated candidiasis in mice.* Infect Immun, 1997. **65**(5): p. 1748-53.
85. Naglik, J.R., et al., *Quantitative expression of the Candida albicans secreted aspartyl proteinase gene family in human oral and vaginal candidiasis.* Microbiology, 2008. **154**(Pt 11): p. 3266-80.
86. Tampakakis, E., I. Okoli, and E. Mylonakis, *A C. elegans-based, whole animal, in vivo screen for the identification of antifungal compounds.* Nat Protoc, 2008. **3**(12): p. 1925-31.
87. Fuchs, B.B., et al., *Methods for using Galleria mellonella as a model host to study fungal pathogenesis.* Virulence, 2010. **1**(6): p. 475-82.
88. Glittenberg, M.T., et al., *Wild-type Drosophila melanogaster as an alternative model system for investigating the pathogenicity of Candida albicans.* Dis Model Mech, 2011. **4**(4): p. 504-14.
89. Chamilos, G., et al., *Drosophila melanogaster as a facile model for large-scale studies of virulence mechanisms and antifungal drug efficacy in Candida species.* J Infect Dis, 2006. **193**(7): p. 1014-22.

90. Naglik, J.R., S.J. Challacombe, and B. Hube, *Candida albicans secreted aspartyl proteinases in virulence and pathogenesis.* Microbiol Mol Biol Rev, 2003. **67**(3): p. 400-28, table of contents.
91. Naglik, J.R., et al., *In vivo analysis of secreted aspartyl proteinase expression in human oral candidiasis.* Infect Immun, 1999. **67**(5): p. 2482-90.
92. Lermann, U. and J. Morschhauser, *Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by Candida albicans.* Microbiology, 2008. **154**(Pt 11): p. 3281-95.
93. Kretschmar, M., et al., *Individual acid aspartic proteinases (Saps) 1-6 of Candida albicans are not essential for invasion and colonization of the gastrointestinal tract in mice.* Microb Pathog, 2002. **32**(2): p. 61-70.
94. Correia, A., et al., *Limited role of secreted aspartyl proteinases Sap1 to Sap6 in Candida albicans virulence and host immune response in murine hematogenously disseminated candidiasis.* Infect Immun, 2010. **78**(11): p. 4839-49.
95. Hube, B., et al., *The role and relevance of phospholipase D1 during growth and dimorphism of Candida albicans.* Microbiology, 2001. **147**(Pt 4): p. 879-89.
96. Dolan, J.W., et al., *Candida albicans PLD I activity is required for full virulence.* Med Mycol, 2004. **42**(5): p. 439-47.
97. Gale, C., et al., *Cloning and expression of a gene encoding an integrin-like protein in Candida albicans.* Proc Natl Acad Sci U S A, 1996. **93**(1): p. 357-61.
98. Gale, C.A., et al., *Linkage of adhesion, filamentous growth, and virulence in Candida albicans to a single gene, INT1.* Science, 1998. **279**(5355): p. 1355-8.
99. Bendel, C.M., et al., *Systemic infection following intravenous inoculation of mice with Candida albicans int1 mutant strains.* Mol Genet Metab, 1999. **67**(4): p. 343-51.
100. Kinneberg, K.M., et al., *Effect of INT1 gene on Candida albicans murine intestinal colonization.* J Surg Res, 1999. **87**(2): p. 245-51.
101. Lan, C.Y., et al., *Regulatory networks affected by iron availability in Candida albicans.* Mol Microbiol, 2004. **53**(5): p. 1451-69.
102. Haley, K.P. and E.P. Skaar, *A battle for iron: host sequestration and Staphylococcus aureus acquisition.* Microbes Infect, 2012. **14**(3): p. 217-27.
103. Bensen, E.S., et al., *Transcriptional profiling in Candida albicans reveals new adaptive responses to extracellular pH and functions for Rim101p.* Mol Microbiol, 2004. **54**(5): p. 1335-51.
104. Chen, C., et al., *An iron homeostasis regulatory circuit with reciprocal roles in Candida albicans commensalism and pathogenesis.* Cell Host Microbe, 2011. **10**(2): p. 118-35.
105. Homann, O.R., et al., *A phenotypic profile of the Candida albicans regulatory network.* PLoS Genet, 2009. **5**(12): p. e1000783.
106. Zordan, R.E., et al., *Interlocking transcriptional feedback loops control white-opaque switching in Candida albicans.* PLoS Biol, 2007. **5**(10): p. e256.
107. Lo, H.J., et al., *Nonfilamentous C. albicans mutants are avirulent.* Cell, 1997. **90**(5): p. 939-49.
108. Staab, J.F., et al., *Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1.* Science, 1999. **283**(5407): p. 1535-8.

109. Sonneborn, A., et al., *Protein kinase A encoded by TPK2 regulates dimorphism of Candida albicans.* Mol Microbiol, 2000. **35**(2): p. 386-96.
110. Lorenz, M.C. and G.R. Fink, *The glyoxylate cycle is required for fungal virulence.* Nature, 2001. **412**(6842): p. 83-6.
111. Doedt, T., et al., *APSES proteins regulate morphogenesis and metabolism in Candida albicans.* Mol Biol Cell, 2004. **15**(7): p. 3167-80.
112. Gow, N.A., *Germ tube growth of Candida albicans.* Curr Top Med Mycol, 1997. **8**(1-2): p. 43-55.
113. Cole, G.T., A.A. Halawa, and E.J. Anaissie, *The role of the gastrointestinal tract in hematogenous candidiasis: from the laboratory to the bedside.* Clin Infect Dis, 1996. **22 Suppl 2**: p. S73-88.
114. Kim, A.S., et al., *Hypoxia and extraintestinal dissemination of Candida albicans yeast forms.* Shock, 2003. **19**(3): p. 257-62.
115. Riggle, P.J., et al., *Invasive lesions containing filamentous forms produced by a Candida albicans mutant that is defective in filamentous growth in culture.* Infect Immun, 1999. **67**(7): p. 3649-52.
116. Braun, B.R., et al., *Identification and characterization of TUP1-regulated genes in Candida albicans.* Genetics, 2000. **156**(1): p. 31-44.
117. Andrutis, K.A., et al., *Intestinal lesions associated with disseminated candidiasis in an experimental animal model.* J Clin Microbiol, 2000. **38**(6): p. 2317-23.
118. Park, Y.N. and J. Morschhauser, *Tetracycline-inducible gene expression and gene deletion in Candida albicans.* Eukaryot Cell, 2005. **4**(8): p. 1328-42.
119. Braun, B.R., D. Kadosh, and A.D. Johnson, *NRG1, a repressor of filamentous growth in C.albicans, is down-regulated during filament induction.* Embo J, 2001. **20**(17): p. 4753-61.
120. Song, W., H. Wang, and J. Chen, *Candida albicans Sfl2, a temperature-induced transcriptional regulator, is required for virulence in a murine gastrointestinal infection model.* FEMS Yeast Res, 2011. **11**(2): p. 209-22.
121. Spiering, M.J., et al., *Comparative transcript profiling of Candida albicans and Candida dubliniensis identifies SFL2, a C. albicans gene required for virulence in a reconstituted epithelial infection model.* Eukaryot Cell, 2010. **9**(2): p. 251-65.
122. Stoldt, V.R., et al., *Efg1p, an essential regulator of morphogenesis of the human pathogen Candida albicans, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi.* Embo J, 1997. **16**(8): p. 1982-91.
123. Liu, H., *Transcriptional control of dimorphism in Candida albicans.* Curr Opin Microbiol, 2001. **4**(6): p. 728-35.
124. Tebarth, B., et al., *Adaptation of the Efg1p morphogenetic pathway in Candida albicans by negative autoregulation and PKA-dependent repression of the EFG1 gene.* J Mol Biol, 2003. **329**(5): p. 949-62.
125. Lane, S., et al., *DNA array studies demonstrate convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in Candida albicans.* J Biol Chem, 2001. **276**(52): p. 48988-96.
126. Braun, B.R. and A.D. Johnson, *TUP1, CPH1 and EFG1 make independent contributions to filamentation in candida albicans.* Genetics, 2000. **155**(1): p. 57-67.

127. Liu, H., J. Kohler, and G.R. Fink, *Suppression of hyphal formation in Candida albicans by mutation of a STE12 homolog.* Science, 1994. **266**(5191): p. 1723-6.
128. El Barkani, A., et al., *Dominant active alleles of RIM101 (PRR2) bypass the pH restriction on filamentation of Candida albicans.* Mol Cell Biol, 2000. **20**(13): p. 4635-47.
129. Bauer, J. and J. Wendland, *Candida albicans Sfl1 suppresses flocculation and filamentation.* Eukaryot Cell, 2007. **6**(10): p. 1736-44.
130. Lane, S., et al., *The basic helix-loop-helix transcription factor Cph2 regulates hyphal development in Candida albicans partly via TEC1.* Mol Cell Biol, 2001. **21**(19): p. 6418-28.
131. Schweizer, A., et al., *The TEA/ATTS transcription factor CaTec1p regulates hyphal development and virulence in Candida albicans.* Mol Microbiol, 2000. **38**(3): p. 435-45.
132. Noffz, C.S., et al., *Functional mapping of the Candida albicans Efg1 regulator.* Eukaryot Cell, 2008. **7**(5): p. 881-93.
133. Cao, F., et al., *The Flo8 transcription factor is essential for hyphal development and virulence in Candida albicans.* Mol Biol Cell, 2006. **17**(1): p. 295-307.
134. Gimeno, C.J. and G.R. Fink, *Induction of pseudohyphal growth by overexpression of PHD1, a Saccharomyces cerevisiae gene related to transcriptional regulators of fungal development.* Mol Cell Biol, 1994. **14**(3): p. 2100-12.
135. Leng, P., et al., *Efg1, a morphogenetic regulator in Candida albicans, is a sequence-specific DNA binding protein.* J Bacteriol, 2001. **183**(13): p. 4090-3.
136. Lassak, T., et al., *Target specificity of the Candida albicans Efg1 regulator.* Mol Microbiol, 2011. **82**(3): p. 602-18.
137. Nantel, A., et al., *Transcription profiling of Candida albicans cells undergoing the yeast-to-hyphal transition.* Mol Biol Cell, 2002. **13**(10): p. 3452-65.
138. Wang, A., et al., *Hyphal chain formation in Candida albicans: Cdc28-Hgc1 phosphorylation of Efg1 represses cell separation genes.* Mol Cell Biol, 2009. **29**(16): p. 4406-16.
139. Sohn, K., et al., *EFG1 is a major regulator of cell wall dynamics in Candida albicans as revealed by DNA microarrays.* Mol Microbiol, 2003. **47**(1): p. 89-102.
140. Argimon, S., et al., *Developmental regulation of an adhesin gene during cellular morphogenesis in the fungal pathogen Candida albicans.* Eukaryot Cell, 2007. **6**(4): p. 682-92.
141. Saville, S.P., D.P. Thomas, and J.L. Lopez Ribot, *A role for Efg1p in Candida albicans interactions with extracellular matrices.* FEMS Microbiol Lett, 2006. **256**(1): p. 151-8.
142. Martchenko, M., et al., *Superoxide dismutases in Candida albicans: transcriptional regulation and functional characterization of the hyphal-induced SOD5 gene.* Mol Biol Cell, 2004. **15**(2): p. 456-67.
143. Frohner, I.E., et al., *Candida albicans cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance.* Mol Microbiol, 2009. **71**(1): p. 240-52.

144. Felk, A., et al., *Candida albicans hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs.* Infect Immun, 2002. **70**(7): p. 3689-700.
145. Staib, P., et al., *Transcriptional regulators Cph1p and Efg1p mediate activation of the Candida albicans virulence gene SAP5 during infection.* Infect Immun, 2002. **70**(2): p. 921-7.
146. Setiadi, E.R., et al., *Transcriptional response of Candida albicans to hypoxia: linkage of oxygen sensing and Efg1p-regulatory networks.* J Mol Biol, 2006. **361**(3): p. 399-411.
147. Ramanan, N. and Y. Wang, *A high-affinity iron permease essential for Candida albicans virulence.* Science, 2000. **288**(5468): p. 1062-4.
148. Lu, Y., et al., *Efg1-mediated recruitment of NuA4 to promoters is required for hypha-specific Swi/Snf binding and activation in Candida albicans.* Mol Biol Cell, 2008. **19**(10): p. 4260-72.
149. Maidan, M.M., et al., *The G protein-coupled receptor Gpr1 and the Galpha protein Gpa2 act through the cAMP-protein kinase A pathway to induce morphogenesis in Candida albicans.* Mol Biol Cell, 2005. **16**(4): p. 1971-86.
150. Bockmuhl, D.P. and J.F. Ernst, *A potential phosphorylation site for an A-type kinase in the Efg1 regulator protein contributes to hyphal morphogenesis of Candida albicans.* Genetics, 2001. **157**(4): p. 1523-30.
151. Jones, D.L., et al., *Transcriptome profiling of a Saccharomyces cerevisiae mutant with a constitutively activated Ras/cAMP pathway.* Physiol Genomics, 2003. **16**(1): p. 107-18.
152. Cannon, J.F., R. Gitan, and K. Tatchell, *Yeast cAMP-dependent protein kinase regulatory subunit mutations display a variety of phenotypes.* J Biol Chem, 1990. **265**(20): p. 11897-904.
153. Liu, H., *Co-regulation of pathogenesis with dimorphism and phenotypic switching in Candida albicans, a commensal and a pathogen.* Int J Med Microbiol, 2002. **292**(5-6): p. 299-311.
154. Slutsky, B., et al., *"White-opaque transition": a second high-frequency switching system in Candida albicans.* J Bacteriol, 1987. **169**(1): p. 189-97.
155. Morschhauser, J., *Regulation of white-opaque switching in Candida albicans.* Med Microbiol Immunol, 2010. **199**(3): p. 165-72.
156. Lockhart, S.R., et al., *In Candida albicans, white-opaque switchers are homozygous for mating type.* Genetics, 2002. **162**(2): p. 737-45.
157. Huang, G., et al., *Bistable expression of WOR1, a master regulator of white-opaque switching in Candida albicans.* Proc Natl Acad Sci U S A, 2006. **103**(34): p. 12813-8.
158. Zordan, R.E., D.J. Galgoczy, and A.D. Johnson, *Epigenetic properties of white-opaque switching in Candida albicans are based on a self-sustaining transcriptional feedback loop.* Proc Natl Acad Sci U S A, 2006. **103**(34): p. 12807-12.
159. Sonneborn, A., B. Tebarth, and J.F. Ernst, *Control of white-opaque phenotypic switching in Candida albicans by the Efg1p morphogenetic regulator.* Infect Immun, 1999. **67**(9): p. 4655-60.

160. Kvaal, C., et al., *Misexpression of the opaque-phase-specific gene PEP1 (SAP1) in the white phase of Candida albicans confers increased virulence in a mouse model of cutaneous infection.* Infect Immun, 1999. **67**(12): p. 6652-62.
161. Kvaal, C.A., T. Srikantha, and D.R. Soll, *Misexpression of the white-phase-specific gene WH11 in the opaque phase of Candida albicans affects switching and virulence.* Infect Immun, 1997. **65**(11): p. 4468-75.
162. Lan, C.Y., et al., *Metabolic specialization associated with phenotypic switching in Candidaalbicans.* Proc Natl Acad Sci U S A, 2002. **99**(23): p. 14907-12.
163. Soll, D.R., *High-frequency switching in Candida albicans.* Clin Microbiol Rev, 1992. **5**(2): p. 183-203.
164. Nucci, M. and E. Anaissie, *Revisiting the source of candidemia: skin or gut?* Clin Infect Dis, 2001. **33**(12): p. 1959-67.
165. Pierce, J.V. and C.A. Kumamoto, *Variation in Candida albicans EFG1 Expression Enables Host-Dependent Changes in Colonizing Fungal Populations.* MBio, 2012. **3**(4).
166. Kumamoto, C.A. and J.V. Pierce, *Immunosensing during colonization by Candida albicans: does it take a village to colonize the intestine?* Trends Microbiol, 2011. **19**(6): p. 263-7.
167. Lay, K.M. and C. Russel, *Candida species and yeasts in mouths of infants from a special care unit of a maternity hospital.* Arch Dis Child, 1977. **52**(10): p. 794-6.
168. Wisplinghoff, H., et al., *Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study.* Clin Infect Dis, 2004. **39**(3): p. 309-17.
169. Macphail, G.L., et al., *Epidemiology, treatment and outcome of candidemia: a five-year review at three Canadian hospitals.* Mycoses, 2002. **45**(5-6): p. 141-5.
170. Kibbler, C.C., et al., *Management and outcome of bloodstream infections due to Candida species in England and Wales.* J Hosp Infect, 2003. **54**(1): p. 18-24.
171. Pfeiffer, C.D., et al., *Quantitation of Candida CFU in initial positive blood cultures.* J Clin Microbiol, 2011. **49**(8): p. 2879-83.
172. Ruping, M.J., J.J. Vehreschild, and O.A. Cornely, *Patients at high risk of invasive fungal infections: when and how to treat.* Drugs, 2008. **68**(14): p. 1941-62.
173. Romani, L., *Immunity to Candida albicans: Th1, Th2 cells and beyond.* Curr Opin Microbiol, 1999. **2**(4): p. 363-7.
174. Cenci, E., et al., *T helper cell type 1 (Th1)- and Th2-like responses are present in mice with gastric candidiasis but protective immunity is associated with Th1 development.* J Infect Dis, 1995. **171**(5): p. 1279-88.
175. Cole, G.T., et al., *Retrovirus-induced immunodeficiency in mice exacerbates gastrointestinal candidiasis.* Infect Immun, 1992. **60**(10): p. 4168-78.
176. Challacombe, S.J. and J.R. Naglik, *The effects of HIV infection on oral mucosal immunity.* Adv Dent Res, 2006. **19**(1): p. 29-35.
177. Rosenbach, A., et al., *Adaptations of Candida albicans for growth in the mammalian intestinal tract.* Eukaryot Cell, 2010. **9**(7): p. 1075-86.
178. Bendel, C.M., et al., *Cecal colonization and systemic spread of Candida albicans in mice treated with antibiotics and dexamethasone.* Pediatr Res, 2002. **51**(3): p. 290-5.

179. Sundstrom, P., E. Balish, and C.M. Allen, *Essential role of the Candida albicans transglutaminase substrate, hyphal wall protein 1, in lethal oroesophageal candidiasis in immunodeficient mice.* J Infect Dis, 2002. **185**(4): p. 521-30.
180. Newman, J.R., et al., *Single-cell proteomic analysis of S. cerevisiae reveals the architecture of biological noise.* Nature, 2006. **441**(7095): p. 840-6.
181. Bar-Even, A., et al., *Noise in protein expression scales with natural protein abundance.* Nat Genet, 2006. **38**(6): p. 636-43.
182. Pilpel, Y., *Noise in biological systems: pros, cons, and mechanisms of control.* Methods Mol Biol, 2011. **759**: p. 407-25.
183. Cohen, A.A., et al., *Dynamic proteomics of individual cancer cells in response to a drug.* Science, 2008. **322**(5907): p. 1511-6.
184. Halliwell, S.C., et al., *Heterogeneous expression of the virulence-related adhesin Epa1 between individual cells and strains of the pathogen Candida glabrata.* Eukaryot Cell, 2012. **11**(2): p. 141-50.
185. Cormack, B.P., et al., *Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in Candida albicans.* Microbiology, 1997. **143 (Pt 2)**: p. 303-11.
186. Barelle, C.J., et al., *GFP as a quantitative reporter of gene regulation in Candida albicans.* Yeast, 2004. **21**(4): p. 333-40.
187. Chen, X. and C.A. Kumamoto, *A conserved G protein (Drg1p) plays a role in regulation of invasive filamentation in Candida albicans.* Microbiology, 2006. **152**(Pt 12): p. 3691-700.
188. Dantuma, N.P., et al., *Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells.* Nat Biotechnol, 2000. **18**(5): p. 538-43.
189. Keppler-Ross, S., C. Noffz, and N. Dean, *A new purple fluorescent color marker for genetic studies in Saccharomyces cerevisiae and Candida albicans.* Genetics, 2008. **179**(1): p. 705-10.
190. Sudbery, P.E., *Growth of Candida albicans hyphae.* Nat Rev Microbiol, 2011. **9**(10): p. 737-48.
191. Hazan, I., M. Sepulveda-Becerra, and H. Liu, *Hyphal elongation is regulated independently of cell cycle in Candida albicans.* Mol Biol Cell, 2002. **13**(1): p. 134-45.
192. Farkas, B., et al., *BALB/c mice genetically susceptible to proteoglycan-induced arthritis and spondylitis show colony-dependent differences in disease penetrance.* Arthritis Res Ther, 2009. **11**(1): p. R21.
193. Eckburg, P.B., et al., *Diversity of the human intestinal microbial flora.* Science, 2005. **308**(5728): p. 1635-8.
194. Stecher, B., et al., *Like will to like: abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria.* PLoS Pathog, 2010. **6**(1): p. e1000711.
195. Ivanov, II, et al., *Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine.* Cell Host Microbe, 2008. **4**(4): p. 337-49.
196. Castagnola, E. and S. Buratti, *Clinical aspects of invasive candidiasis in paediatric patients.* Drugs, 2009. **69 Suppl 1**: p. 45-50.

197. Enjalbert, B., et al., *A multifunctional, synthetic Gaussia princeps luciferase reporter for live imaging of Candida albicans infections.* Infect Immun, 2009. **77**(11): p. 4847-58.
198. Sherman, F., *Getting started with yeast.* Methods Enzymol, 1991. **194**: p. 3-21.
199. Zucchi, P.C., T.R. Davis, and C.A. Kumamoto, *A Candida albicans cell wall-linked protein promotes invasive filamentation into semi-solid medium.* Mol Microbiol, 2010. **76**(3): p. 733-48.
200. Fonzi, W.A. and M.Y. Irwin, *Isogenic strain construction and gene mapping in Candida albicans.* Genetics, 1993. **134**(3): p. 717-28.
201. Bouza, E. and P. Munoz, *Epidemiology of candidemia in intensive care units.* Int J Antimicrob Agents, 2008. **32 Suppl 2**: p. S87-91.
202. Launay, O., et al., *Candidemia: a nosocomial complication in adults with late-stage AIDS.* Clin Infect Dis, 1998. **26**(5): p. 1134-41.
203. Odds, F.C., et al., *Epidemiology of vaginal Candida infection: significance of numbers of vaginal yeasts and their biotypes.* Eur J Obstet Gynecol Reprod Biol, 1987. **25**(1): p. 53-66.
204. Fallingborg, J., *Intraluminal pH of the human gastrointestinal tract.* Dan Med Bull, 1999. **46**(3): p. 183-96.
205. Hao, W.L. and Y.K. Lee, *Microflora of the gastrointestinal tract: a review.* Methods Mol Biol, 2004. **268**: p. 491-502.
206. Ashburner, M., et al., *Gene ontology: tool for the unification of biology. The Gene Ontology Consortium.* Nat Genet, 2000. **25**(1): p. 25-9.
207. Leuker, C.E., et al., *Sequence and promoter regulation of the PCK1 gene encoding phosphoenolpyruvate carboxykinase of the fungal pathogen Candida albicans.* Gene, 1997. **192**(2): p. 235-40.
208. De la Rosa, J.M., T. Ruiz, and L. Rodriguez, *Cloning and characterization of a Candida albicans gene homologous to fructose-1,6-bisphosphatase genes.* Curr Microbiol, 2000. **41**(6): p. 384-7.
209. Gerami-Nejad, M., et al., *Cassettes for the PCR-mediated construction of regulatable alleles in Candida albicans.* Yeast, 2004. **21**(5): p. 429-36.
210. Singh, V., et al., *The key enzyme in galactose metabolism, UDP-galactose-4-epimerase, affects cell-wall integrity and morphology in Candida albicans even in the absence of galactose.* Fungal Genet Biol, 2007. **44**(6): p. 563-74.
211. Barelle, C.J., et al., *Niche-specific regulation of central metabolic pathways in a fungal pathogen.* Cell Microbiol, 2006. **8**(6): p. 961-71.
212. Bailey, D.A., et al., *The Candida albicans HYR1 gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins.* J Bacteriol, 1996. **178**(18): p. 5353-60.
213. Luo, G., et al., *Candida albicans Hyr1p confers resistance to neutrophil killing and is a potential vaccine target.* J Infect Dis, 2010. **201**(11): p. 1718-28.
214. Fonzi, W.A., *PHR1 and PHR2 of Candida albicans encode putative glycosidases required for proper cross-linking of beta-1,3- and beta-1,6-glucans.* J Bacteriol, 1999. **181**(22): p. 7070-9.
215. Sharkey, L.L., et al., *HWP1 functions in the morphological development of Candida albicans downstream of EFG1, TUP1, and RBF1.* J Bacteriol, 1999. **181**(17): p. 5273-9.

216. Yin, Z., et al., *A proteomic analysis of the salt, cadmium and peroxide stress responses in Candida albicans and the role of the Hog1 stress-activated MAPK in regulating the stress-induced proteome.* Proteomics, 2009. **9**(20): p. 4686-703.
217. Wysong, D.R., et al., *Cloning and sequencing of a Candida albicans catalase gene and effects of disruption of this gene.* Infect Immun, 1998. **66**(5): p. 1953-61.
218. De Bernardis, F., et al., *The pH of the host niche controls gene expression in and virulence of Candida albicans.* Infect Immun, 1998. **66**(7): p. 3317-25.
219. Davis, D., R.B. Wilson, and A.P. Mitchell, *RIM101-dependent and-independent pathways govern pH responses in Candida albicans.* Mol Cell Biol, 2000. **20**(3): p. 971-8.
220. Wang, H., et al., *Alkaline stress triggers an immediate calcium fluctuation in Candida albicans mediated by Rim101p and Crz1p transcription factors.* FEMS Yeast Res, 2011. **11**(5): p. 430-9.
221. Murciano, C., et al., *Candida albicans cell wall glycosylation may be indirectly required for activation of epithelial cell proinflammatory responses.* Infect Immun, 2011. **79**(12): p. 4902-11.
222. McKenzie, C.G., et al., *Contribution of Candida albicans cell wall components to recognition by and escape from murine macrophages.* Infect Immun, 2010. **78**(4): p. 1650-8.
223. Buurman, E.T., et al., *Molecular analysis of CaMnt1p, a mannosyl transferase important for adhesion and virulence of Candida albicans.* Proc Natl Acad Sci U S A, 1998. **95**(13): p. 7670-5.
224. Munro, C.A., et al., *Mnt1p and Mnt2p of Candida albicans are partially redundant alpha-1,2-mannosyltransferases that participate in O-linked mannosylation and are required for adhesion and virulence.* J Biol Chem, 2005. **280**(2): p. 1051-60.
225. Prill, S.K., et al., *PMT family of Candida albicans: five protein mannosyltransferase isoforms affect growth, morphogenesis and antifungal resistance.* Mol Microbiol, 2005. **55**(2): p. 546-60.
226. Northrop, F.D., S. Ljubojevic, and J.M. Davies, *Influence of Na+ and anions on the dimorphic transition of Candida albicans.* Microbiology, 1997. **143 (Pt 12)**: p. 3757-65.
227. Dumortier, F., J.C. Arguelles, and J.M. Thevelein, *Constitutive glucose-induced activation of the Ras-cAMP pathway and aberrant stationary-phase entry on a glucose-containing medium in the Saccharomyces cerevisiae glucose-repression mutant hex2.* Microbiology, 1995. **141 (Pt 7)**: p. 1559-66.
228. Mbonyi, K., et al., *Glucose-induced hyperaccumulation of cyclic AMP and defective glucose repression in yeast strains with reduced activity of cyclic AMP-dependent protein kinase.* Mol Cell Biol, 1990. **10**(9): p. 4518-23.
229. Ito-Kuwa, S., et al., *Oxidative stress sensitivity and superoxide dismutase of a wild-type parent strain and a respiratory mutant of Candida albicans.* Med Mycol, 1999. **37**(5): p. 307-14.
230. Rikhvanov, E.G., et al., *Sodium azide reduces the thermotolerance of respiratively grown yeasts.* Curr Microbiol, 2002. **45**(6): p. 394-9.
231. Nantel A, R.T., Hogues H, Whiteway M, *Microarrays for studying pathology in Candida albicans*2006, Hoboken, NJ: Wiley Press

232. Eisen, M.B., et al., *Cluster analysis and display of genome-wide expression patterns.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 14863-8.
233. Saldanha, A.J., *Java Treeview--extensible visualization of microarray data.* Bioinformatics, 2004. **20**(17): p. 3246-8.
234. Grzenda, A., et al., *Sin3: master scaffold and transcriptional corepressor.* Biochim Biophys Acta, 2009. **1789**(6-8): p. 443-50.
235. Kadosh, D. and K. Struhl, *Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters.* Cell, 1997. **89**(3): p. 365-71.
236. Lachke, S.A., T. Srikantha, and D.R. Soll, *The regulation of EFG1 in white-opaque switching in Candida albicans involves overlapping promoters.* Mol Microbiol, 2003. **48**(2): p. 523-36.
237. Fuchs, B.B., et al., *Role of filamentation in Galleria mellonella killing by Candida albicans.* Microbes Infect, 2010. **12**(6): p. 488-96.
238. Kadosh, D. and K. Struhl, *Targeted recruitment of the Sin3-Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo.* Mol Cell Biol, 1998. **18**(9): p. 5121-7.
239. Avery, S.V., *Microbial cell individuality and the underlying sources of heterogeneity.* Nat Rev Microbiol, 2006. **4**(8): p. 577-87.
240. Dubnau, D. and R. Losick, *Bistability in bacteria.* Mol Microbiol, 2006. **61**(3): p. 564-72.
241. Veening, J.W., W.K. Smits, and O.P. Kuipers, *Bistability, epigenetics, and bet-hedging in bacteria.* Annu Rev Microbiol, 2008. **62**: p. 193-210.
242. Avery, S.V., *Cell individuality: the bistability of competence development.* Trends Microbiol, 2005. **13**(10): p. 459-62.
243. Lewis, K., *Persister cells, dormancy and infectious disease.* Nat Rev Microbiol, 2007. **5**(1): p. 48-56.
244. Lafleur, M.D., Q. Qi, and K. Lewis, *Patients with long-term oral carriage harbor high-persister mutants of Candida albicans.* Antimicrob Agents Chemother, 2010. **54**(1): p. 39-44.
245. LaFleur, M.D., C.A. Kumamoto, and K. Lewis, *Candida albicans biofilms produce antifungal-tolerant persister cells.* Antimicrob Agents Chemother, 2006. **50**(11): p. 3839-46.
246. Ackermann, M., et al., *Self-destructive cooperation mediated by phenotypic noise.* Nature, 2008. **454**(7207): p. 987-90.
247. Miller, M.G. and A.D. Johnson, *White-opaque switching in Candida albicans is controlled by mating-type locus homeodomain proteins and allows efficient mating.* Cell, 2002. **110**(3): p. 293-302.
248. Srikantha, T. and D.R. Soll, *A white-specific gene in the white-opaque switching system of Candida albicans.* Gene, 1993. **131**(1): p. 53-60.
249. Pigolotti, S., S. Krishna, and M.H. Jensen, *Oscillation patterns in negative feedback loops.* Proc Natl Acad Sci U S A, 2007. **104**(16): p. 6533-7.
250. Lewis, J., *Autoinhibition with transcriptional delay: a simple mechanism for the zebrafish somitogenesis oscillator.* Curr Biol, 2003. **13**(16): p. 1398-408.
251. Lahav, G., *Oscillations by the p53-Mdm2 feedback loop.* Adv Exp Med Biol, 2008. **641**: p. 28-38.

252. Hirata, H., et al., *Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop.* Science, 2002. **298**(5594): p. 840-3.
253. Lahav, G., et al., *Dynamics of the p53-Mdm2 feedback loop in individual cells.* Nat Genet, 2004. **36**(2): p. 147-50.
254. Mao, X., et al., *The Swi/Snf chromatin remodeling complex is essential for hyphal development in Candida albicans.* FEBS Lett, 2006. **580**(11): p. 2615-22.
255. Hnisz, D., et al., *The Set3/Hos2 histone deacetylase complex attenuates cAMP/PKA signaling to regulate morphogenesis and virulence of Candida albicans.* PLoS Pathog, 2010. **6**(5): p. e1000889.
256. Chang, H.H., et al., *Transcriptome-wide noise controls lineage choice in mammalian progenitor cells.* Nature, 2008. **453**(7194): p. 544-7.
257. van de Veerdonk, F.L., et al., *The macrophage mannose receptor induces IL-17 in response to Candida albicans.* Cell Host Microbe, 2009. **5**(4): p. 329-40.
258. Hernandez-Santos, N. and S.L. Gaffen, *Th17 Cells in Immunity to Candida albicans.* Cell Host Microbe, 2012. **11**(5): p. 425-35.
259. Conti, H.R., et al., *Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis.* J Exp Med, 2009. **206**(2): p. 299-311.
260. Szabo, E.K. and D.M. MacCallum, *The contribution of mouse models to our understanding of systemic candidiasis.* FEMS Microbiol Lett, 2011. **320**(1): p. 1-8.
261. Field, L.H., et al., *Persistence and spread of Candida albicans after intragastric inoculation of infant mice.* Infect Immun, 1981. **31**(2): p. 783-91.
262. Child, M.W., et al., *Studies on the effect of system retention time on bacterial populations colonizing a three-stage continuous culture model of the human large gut using FISH techniques.* FEMS Microbiol Ecol, 2006. **55**(2): p. 299-310.
263. Yamaguchi, N., et al., *Gastric colonization of Candida albicans differs in mice fed commercial and purified diets.* J Nutr, 2005. **135**(1): p. 109-15.