Identification and Investigation of Dfi1p signaling intermediates and transcriptional targets in *Candida albicans*

A thesis submitted by

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Abstract

The common human commensal, *Candida albicans*, is a polymorphic fungus and a medically significant opportunistic pathogen. *C. albicans* can undergo a morphological shift between a yeast form and a hyphal form in response to many environmental stimuli, including growth in contact with an agar matrix. Wildtype levels of filamentation in response to growth in contact with an agar matrix require the integral membrane protein, Dfi1p, which transmits a signal to the mitogen activated protein kinase (MAPK) Cek1p. The first goal of this work was to identify proteins that participate in signaling to Cek1p during growth in contact with an agar matrix. Activation of Cek1p required proteins predicted to participate in the classical Cek1p MAPK cascade, as well as the GTPase Rac1p and its accessory proteins Dck1p and Lmo1p. Constitutive activation of Rac1p bypassed the Cek1p activation defect of a *dfi1* null strain, demonstrating that Rac1p is epistatic to Dfi1p.

The second goal of this work was to identify transcriptional targets of Dfi1p-dependent Cek1p activation. For this portion we relied upon ionophore activation of Cek1p in a Dfi1p-dependent manner during liquid growth. Transcriptional targets of Dfi1p-dependent Cek1p activation include some members of the Sef1p regulon, which responds to iron starvation. Three genes of interest were chosen for further study: *BMT9*, *SOD4*, and *OPT1*. Dfi1p is not required for the transcriptional response to low iron, but is important for the response to ionophore. *BMT9* and *OPT1* require Dfi1p for a full transcriptional response during growth on agar medium. *SOD4*, however, required Sef1p and not Dfi1p during the same conditions. *OPT1* and *SOD4*

both responded to activation of another transcription factor, Czf1p, which is known for promoting embedded filamentation. We propose that the Dfi1p-dependent transcriptional changes we observed rely on both Sef1p and Czf1p. The connection between iron starvation and embedded growth is a novel and intriguing finding.

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List of Abbreviations

C. albicans	*Candida albicans*
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
MAPK	Mitogen Activated Protein Kinase
PAK	p21-Activated Kinase
cAMP/PKA	Cyclic adenosine monophosphate/Protein kinase A
HIV	Human Immunodeficiency Virus
SAPK	Stress Activated Protein Kinase
GEF	Guanine Nucleotide Exchange Factor
GAP	GTPase activating protein
GDI	Guanine Dissociation Inhibitor
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
DH	Dbl Homology
DHR	Dock Homology Region
CBP	CCAAT binding protein
BPS	Bathophenanthroline disulfonic acid
bZIP	Basic Leucine Zipper
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic acid
CDG	*Candida* Genome Database
YPD	Yeast Peptone Dextrose
YPS	Yeast Peptone Sucrose
CM-U	Complete minimal medium-uridine
ROS	Reactive Oxygen Species
SVG	Sterile Vegetative Growth

Chapter 1: Introduction

Invasive fungal infections have been on the rise since the 1990s (Guinea, 2014). Advancements in modern medicine have opened the door to fungal infections by increasing the population of immunocompromised individuals, including patients with AIDs, patients on immunosuppressive therapies following organ transplants, cancer patients, and premature neonates (Greenberg & Benjamin, 2014; Guinea, 2014). Additionally, antifungal resistance has emerged as an area of increasing concern, especially in the opportunistic pathogen *Candida albicans* (Cowen, Anderson, & Kohn, 2002; Ford *et al*., 2015; Morita, Teixeira, Keniya, & Sanglard, 2016).

The *Candida* genus comprises approximately 150 species. The members of this genus were grouped together based on their ability to form hyphae or pseudohyphae and their lack of apparent sexual cycle, although sexual cycles for several members have subsequently been discovered for several species in this genus, including *C. albicans* (Papon, Courdavault, Clastre, Bennett, & Bennett, 2013; Priest & Lorenz, 2015). Given the relatively vague qualifications for classification as *Candida*, it is not surprising that the *Candida* genus is polyphyletic. For the purposes of studying *C. albicans*, the closest relatives are the CUG clade, named after the codon that in these species is read as serine instead of leucine (Turner & Butler, 2014). The members of the CUG clade comprise most species of *Candida* that can be found in humans. The exceptions are the more-distantly related *C. glabrata* and *C. krusei* (Priest & Lorenz, 2015). Presently, *C. albicans* is the dominant species causing

human disease, but the rising prevalence of *C. glabrata* infections suggests that more research will need to be done in the future concerning these more distantly related species (Bassetti *et al*., 2006).

Candida species are the leading cause of invasive fungal infection, with 92 percent of cases being attributed to five species, the most prevalent of which is *Candida albicans* (Guinea, 2014). *C. albicans* is a common human commensal whose niches include the skin, the oro-pharyngeal cavity, the gastrointestinal tract, and the genitourinary tract. Unlike some fungi, *Candida albicans* has no known significant environmental reservoir, and is almost exclusively associated with warm-blooded animals (Hube, 2004). In healthy individuals, *C. albicans* colonization is typically asymptomatic, with the notable exception of vaginal candidiasis, which is experienced at least once by up to 75 percent of women (P. E. Sudbery, 2011). Other forms of opportunistic infection require some form of immune suppression; based upon the nature of the immune deficiency, *C. albicans* disease can vary in severity from a painful mucosal infection to a potentially deadly blood and deep tissue invasion. T-cell deficiency, caused by HIV infection, famously leads to oral thrush, an overgrowth of *Candida* in the mouth and esophagus. More serious disease states, disseminated candidiasis and candidemia, can result from immunosuppression such as neutropenia in organ transplant patients (J. Kim & Sudbery, 2011; P. Sudbery, Gow, & Berman, 2004). These deeply invasive infections have a mortality rate ranging from 30 to 75 percent, with attributable mortality rates of 10-49 percent (J. Kim & Sudbery, 2011; Lepak & Andes, 2011)

Candida albicans is capable of growth in disparate niches within the host. In order to survive and take full advantage of the host, *C. albicans* must be able to change its gene expression to adjust to whatever environment it encounters. In order to sense, integrate, and respond to the myriad environmental signals, *C. albicans* has many sensory cascades that transduce signals from outside the cell, induce transcriptional changes, and ultimately convert them into responses on the cellular level that allow *C. albicans* to react appropriately to its environment.

One of the important cellular "decisions" that *C. albicans* makes is which growth form to adopt. Many fungi exhibit morphological plasticity, and *C. albicans* is no exception. The Noble group has enumerated nine morphological forms of *C. albicans*, each having its own role in the biology of *C. albicans*. These forms are: yeast (white a/ α), hyphae, pseudohyphae, chlamydospores, opaque (a or α), white (a or α), opaque (a/α), grey (a/α), and gastrointestinally induced transistion (GUT) cells. The role of some of these forms has yet to be demonstrated (Noble, Gianetti, & Witchley, 2016). It is important to note these categories can overlap (typical yeast cells are also white cells), and it is possible that designating nine separate forms is splitting hairs a bit finely. It is, however, undeniable that *C. albicans* is capable of growing in multiple forms. This work will focus on one of the best-established morphological transitions that *C. albicans* undergoes: the shift between yeast and hyphae.

1.1 Filamentation

Many fungi switch between a multicellular mycelial form and a unicellular form. For pathogenic fungi with an environmental reservoir, growth in the mycelial form often occurs at low temperature, with the switch to unicellular growth occurring at higher temperature within the host. These species are termed "thermally dimorphic" (Noble *et al., 2016)*. As discussed above, *C. albicans* is not thought to have an environmental reservoir and, unsurprisingly, behaves differently from fungal pathogens with an environmental reservoir. In fact, in laboratory culture, the *C. albicans* multicellular form is induced at body temperature and the unicellular form is favored at room temperature, in the absence of other inducers (Kadosh & Johnson, 2005).

In *Candida albicans*, the unicellular form is known as the yeast form, and the two most common multicellular forms are the pseudohyphal form and the hyphal form (Figure 1.1). The model fungal organism *Saccharomyces cerevisiae* grows as either yeast or pseudohyphae, and is not capable of forming true hyphae. While many lessons in *Candida* biology can be gleaned from the *S. cerevisiae* literature, the inability of *S. cerevisiae* to grow as true hyphae highlights that there are important differences between the two yeast species as well. This is not surprising, given that these species diverged between 300 and 900 million years ago (Hedges, 2002; Homann, Dea, Noble, Johnson, & Magee, 2009). This work will focus on the yeast form and the hyphal form of *Candida albicans*.

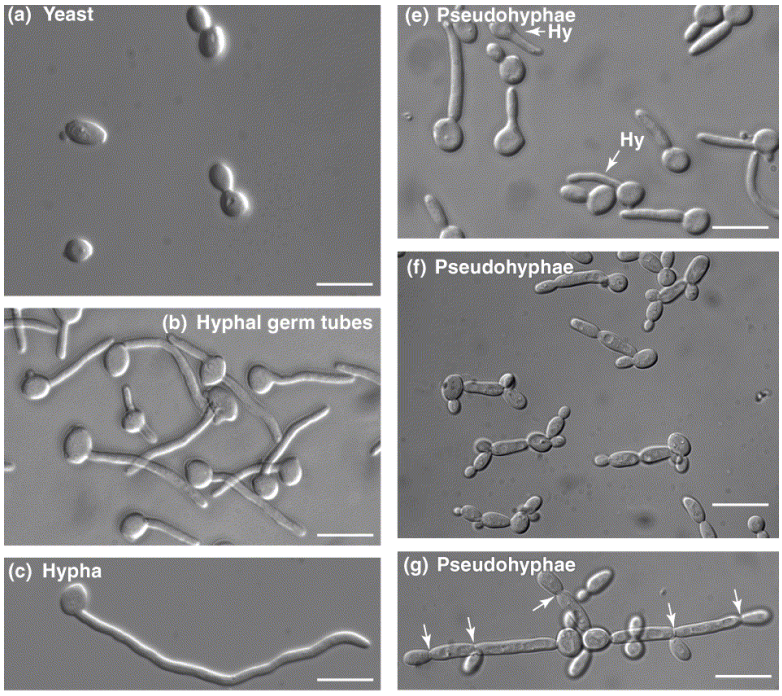

Figure 1.1 Growth forms of *Candida albicans*. Modified from (P. Sudbery *et al.,* 2004). Changes include cropping a multi-panel figure. *Candida albicans* can grow as yeast (a), pseudohyphae (e-g), or hyphae (b and c). Panels e-g show the variable nature of pseudohyphal growth.

Yeast are ovoid cells which divide by budding. During budding, the mother cell does not grow and a septum forms between the mother cell and the daughter cell. Upon cytokinesis, the two resultant cells separate. Hyphae are elongated chains of cells, divided by septa. The hyphal cell is formed by polarized growth, with an evagination of the mother cell, termed a "germ tube" extending (Figure 1, panel b). The first mitosis occurs after germ tube formation, and thus the septum between the mother cell and the daughter cell forms at the tip of the germ tube. Hyphae are smooth, multicellular tube structures with parallel sides and without constrictions at the site of cell division (Noble *et al*., 2016; P. E. Sudbery, 2011).

The ability to shift between morphological states has long been considered a key virulence trait in *C. albicans*. *C. albicans* cells that cannot transition from yeast to

hyphae are defective in their interactions with the host (Lo *et al*., 1997; Murad *et al*., 2001). The yeast form is thought to be conducive to colonization, while the hyphal form is considered the aggressive form needed to invade tissue and cause disseminated disease, although both forms can be found in infected tissue (Cole, Seshan, Phaneuf, & Lynn, 1991).

Filamentation, the transition from budding yeast to hyphae, can occur in response to many stimuli, including temperature, presence of serum, neutral pH, low nitrogen, as well as various synthetic media (P. E. Sudbery, 2011). This work focuses on growth in contact with an agar matrix as a stimulus for filamentation. Growth in agar may approximate contact with a surface within the host, such as the gastrointestinal epithelium or organ tissue.

Different filamentation cues are sensed by different mechanisms. Often there is a plasma membrane-bound sensor protein that transmits the signal via an intracellular signaling cascade. Given the many signals that induce filamentation, removing any one particular membrane sensor usually results in only a partial defect in filamentation.

Contact-dependent filamentation in *Candida albicans* requires the integral membrane protein Dfi1p for optimal response (Zucchi, Davis, & Kumamoto, 2010). This effect is especially pronounced at low temperature (25°C), when the confounding factor of hyphal induction based upon warmer temperatures is reduced. *C. albicans* cells deleted for *dfi1* are capable of forming filaments in response to other stimuli, such as serum, and do eventually form filaments under embedded conditions, so Dfi1p is not absolutely required for filamentation. *dfi1* null

strains are defective for virulence in an intravenous infection model in mice, indicating that Dfi1p plays an important role in *C. albicans* infections (Zucchi *et al*., 2010).

Based upon sequence analysis, Dfi1p is predicted to have two transmembrane domains, a large extracellular region, and N- and C-terminal intracellular portions. Experimental evidence supports the two-transmembrane region model (Herwald, Zucchi, Tan, & Kumamoto, 2017). The role of the N-terminal intracellular region in embedded filamentation has yet to be established. The C-terminal intracellular portion interacts with calcium-bound calmodulin; this interaction ultimately leads to an increase in dually phosphorylated Cek1p, a mitogen activated protein kinase (MAPK) (Davis, Zucchi,& Kumamoto 2013; Zucchi *et al*., 2010). The MAPK pathways of *C. albicans* will be reviewed in the next section.

In summary, the yeast form and the hyphal form are distinct morphological states with distinct properties. The ability to transition between morphological states aids *C. albicans* in surviving and causing disease inside its host, and *C. albicans* has evolved to respond to many environmental stimuli that promote the transition between the yeast and hyphal forms. The stimulus of interest to this work is growth in contact with an agar matrix, which requires Dfi1p. Dfi1p has been shown to signal to the MAPK Cek1p. Intermediate proteins that participate in this signaling pathway are identified in this work. For background, the next two sections will focus on signaling molecules in *C. albicans*, specifically MAPK cascades and small GTPases.

1.2 MAPK signaling in Yeast

Mitogen activated protein kinases (MAPK) are an important family of serine/threonine protein kinases in eukaryotic organisms and it is well documented that they are responsible for transmitting a variety of signals. These signals govern diverse processes, such as growth, response to hormone, differentiation, and response to stress (Bardwell, Cook, Inouye, & Thorner, 1994; N. Hao *et al.*, 2012; Nan Hao, Zeng, Elston, & Dohlman, 2008). Strong evidence supports that MAPK function as part of core modules, which, at their most basic, consist of three proteins: a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK) (Widmann, Gibson, Jarpe, & Johnson, 1999). The MAPKKK phosphorylates the MAPKK, which phosphorylates the MAPK. The MAPKKK usually receives an upstream signal from a p21-activated kinase (PAK), also referred to as a MAPKKK kinase (MAPKKKK) (Zhao, Mehrabi, & Xu, 2007)

Once activated by dual phosphorylation on a conserved TXY motif, the MAPK often activates a transcription factor, which completes the conversion of the extracellular signal to a change in gene expression, allowing the organism to respond to its environment. The MAPK can, however, have other targets, such as MAPK activated protein kinases (Cargnello & Roux, 2011).

Four MAPK have been identified in *C. albicans*: Hog1p, Mkc1p, Cek1p, and Cek2p (Monge, Román, Nombela, & Pla, 2006). Much of what is known about these MAPK was facilitated by comparisons to their homologs in *Saccharomyces cerevisiae*, and for that reason, the MAPK pathways from both fungal species will be reviewed

here. This comparison is logical because the *S. cerevisiae* MAPK pathways are comparatively well understood. However, it is important to bear in mind that the two organisms are not identical. *S. cerevisiae* and *C. albicans* diverged 300 to 900 million years ago, which is a similar evolutionary distance to that between humans and fish.(Hedges, 2002; Homann, Dea, Noble, Johnson, & Magee, 2009). For a visual reference, the MAPK pathways in *C. albicans* are shown in Figure 1.2. The MAPK pathways of *S. cerevisiae* are shown in Figure 1.3.

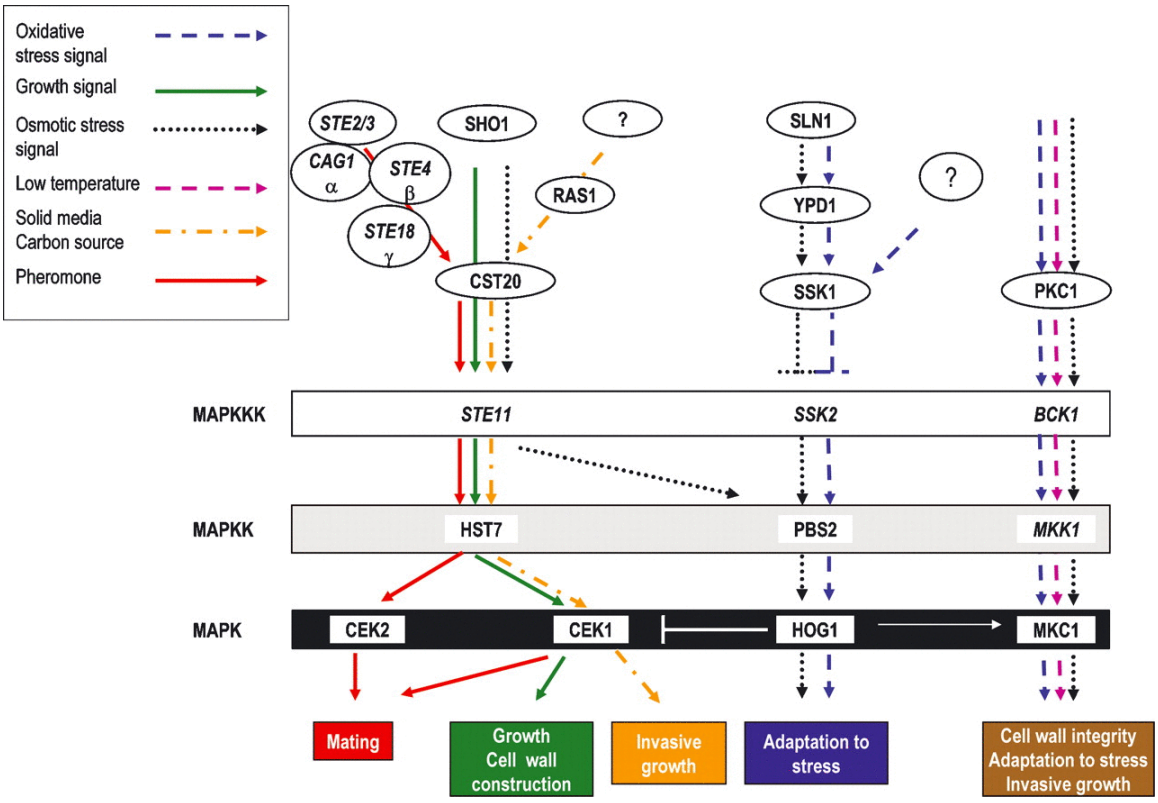

Figure 1.2 Diagram of MAPK pathways of *C. albicans*. Reproduced with permission from (Monge *et al.*, 2006).

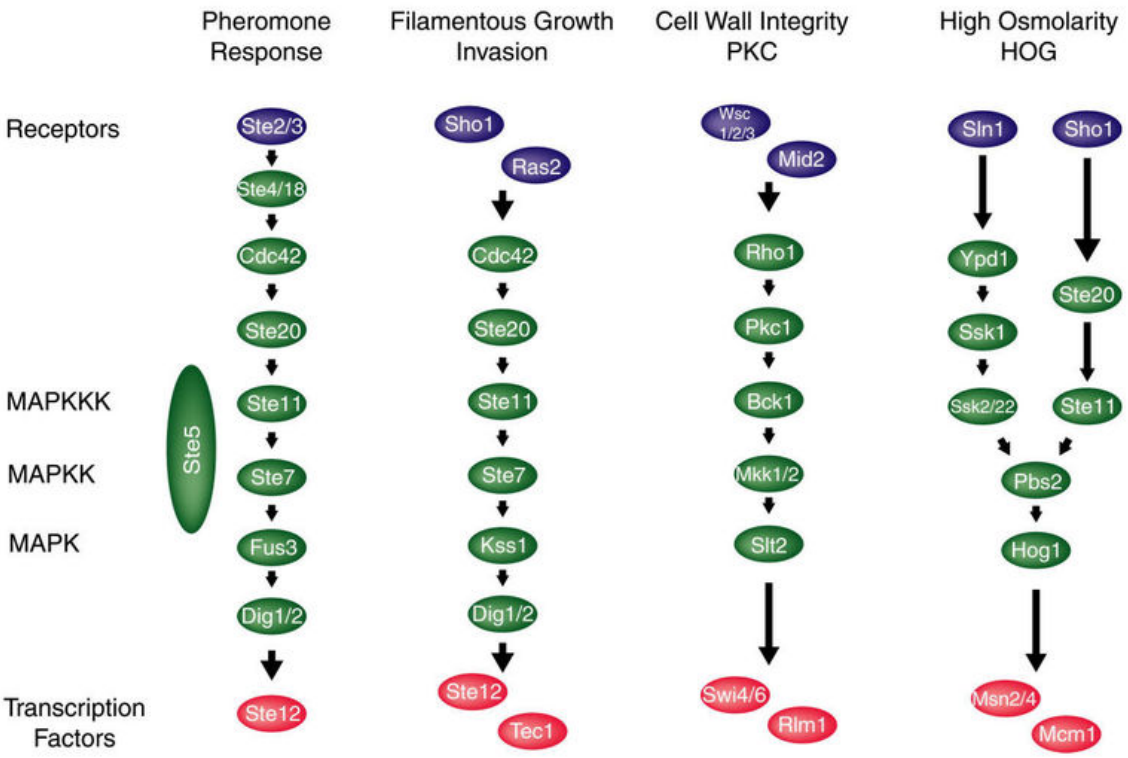

Figure 1.3 MAPK cascades of *Saccharomyces cerevisiae*. Reproduced with permission from (Steffen, Petti, Aach, D'haeseleer, & Church, 2002).

1.2.1 Hog1

The high osmolarity glycerol (Hog1) MAPK is the *C. albicans* and *S. cerevisiae* homolog of the mammalian Stress Activated Protein Kinase (SAPK). Hog1p in *S. cerevisiae* (ScHog1) is one of the best-studied MAPK pathways. Interestingly, the *C. albicans* Hog1p cascade differs from that found in *S. cerevisiae*.

In *S. cerevisiae,* Hog1p is activated by three MAPKKK: ScSsk2p, ScSsk22, and ScSte11p. These three proteins function in two pathways, one relying on the redundant ScSsk2p and ScSsk22p and the other relying on ScSte11p. The ScSsk2p and ScSsk22p branch of the ScHog1p pathway are activated by a histidine kinase two-component system comprising Sln1p, Ypd1p, and Ssk1p. The ScSte11p pathway

is activated downstream of the adaptor membrane protein Sho1p and Opy2, acting with either Msb2p or Hkr1p (Nishimura *et al*., 2016). Although Msb2p can signal to other MAPK pathways, Hkr1p is specific to Hog1p (Pitoniak, Birkaya, Dionne, Vadaie, & Cullen, 2009). Downstream of either the ScSho1p/ScMsb2p or ScSho1p/ScHkr1p complex, is the G-protein ScCdc42 and the PAK ScSte20p. ScSte20p then activates ScSte11p. Input from either the ScSsk2p/ScSsk22p or ScSte11p branch is enough to support survival on high-osmolarity medium (Tatebayashi *et al*., 2007). ScSsk2p/ScSsk22p or ScSte11p branches converge at ScPbs2p, a MAPKK, which then phosphorylates ScHog1p.

In *C. albicans*, Hog1p responds to osmotic stress, as well as oxidative and heavy metal stress. Homologs of the *S. cerevisiae* pathway components exist in *C. albicans,* although a few more membrane sensors exist in *C. albicans*. Upstream sensor proteins Sho1p, Sln1p, Hkr1p, Nik1p, Chk1p, and Msb2p sense oxidative or osmotic stress. Like in *S. cerevisiae*, Hkr1p and Msb2p are somewhat redundant inputs into the Sho1p branch of the Hog1p pathway. The other proteins are upstream of the Ssk2p branch. As in *S. cerevisiae*, all signals pass through the MAPKK Pbs2p. Although homologues of proteins from both the ScSsk2p and ScSte11p branches are present in *C. albicans*, Pbs2p activation in *C. albicans* appears to require only the Ssk2p branch (Cheetham *et al*., 2007). This observation is not universally accepted, and while the evidence supporting the understanding of upstream signaling leading to ScHog1p activation is strong, there is only limited literature at this point concerning the relative importance of the Ste11p pathway

and the Ssk2p pathway to Hog1p activation (Cheetham et al., 2007; Nishimura et al., 2016).

Deletion of *hog1* is deleterious to virulence and the resulting strain is nearly avirulent. Hog1p also plays a role in regulating hyphal morphogenesis. Hog1p appears to repress filamentation, as *hog1* null strains are filamentous under non-inducing conditions (Alonso-Monge *et al*., 2003). Filamentation is thought to occur because removal of Hog1p derepresses *BRG1*. Brg1p is a transcription factor that promotes hyphal elongation and is downstream of the Tor1 pathway (Su, Lu, & Liu, 2013).

1.2.2 Mkc1

Mkc1p is the *Candida albicans* cell wall integrity MAPK; its homolog in *S. cerevisiae* is Mpk1/Slt2 (Navarro-Garcia, Sanchez & Pla, 1995). As the name suggests, this pathway responds to several forms of cell wall stressing conditions. Unlike Hog1p, the Mkc1p pathway in *C. albicans* is quite similar to that in *S. cerevisiae*.

In *S. cerevisiae*, the pathway is activated by ScPkc1, a protein kinase c homologue, which phosphorylates the MAPKKK ScBck1p. Bck1p then phosphorylates ScMkk1p or ScMkk2p, redundant MAPKK, which transfer a phosphate group to Mpk1p. In *C. albicans*, Pkc1p activates the pathway by phosphorylating Bck1p, which phosphorylates Mkk1p, resulting in phosphorylation of Mkc1p (Steffen *et al*., 2002).

The cell wall integrity pathway has been found to respond to several stimuli, including oxidative stress, low temperature, and osmotic stress (Navarro-Garcia,

2005). In the laboratory, Mkc1p responds to cell wall stress produced by treatment with Nikkomycin Z, as well as stress produced by membrane perturbation, produced by chlorpromazine, a drug that causes membrane curvature (Kumamoto, 2005).

Additionally, Mkc1p plays a role in regulating morphogenesis and interactions with surfaces. When heterologously overexpressed in *S. cerevisiae*, Mkc1p results in enhanced pseudohyphal morphogenesis in response to low nitrogen (F. Navarro-Garcia et al., 1998). In *C. albicans*, Mkc1p promotes filamentous invasion in response to growth in contact with a semisolid matrix. Mkc1p is also required for normal biofilm formation on a plastic surface (Kumamoto, 2005).

The cell wall of an *mkc1* null strain is altered subtly from that of a wildtype strain. One noticeable difference is the increase in O-glycosylated mannoproteins exposed in an *mkc1* null strain (F. Navarro-Garcia et al., 1998). This altered cell wall is defective in a way that is not completely understood and confers temperature sensitivity. An *mkc1* null strain can grow normally at 37°C, but loses viability at 42°C. This mutant is also more susceptible to high calcium or caffeine (Monge *et al*., 2006). Unsurprisingly, like Hog1p, Mkc1p is required for wildtype virulence in a mouse model of intravenous infection (Diez-orejas *et al*., 1997).

1.2.3 Cek1

The Cek1p MAP kinase is the MAPK of most interest to this work. Identified for its ability to interfere with cell cycle arrest in *S. cerevisiae* induced mating factor exposure, Cek1p is well known for its role in promoting filamentous growth

(Whiteway, Dignard, & Thomas, 1992). The role of Cek1p in promoting embedded filamentation is of particular interest to this work.

Cek1p is homologous to the *S. cerevisiae* MAPK ScKss1p. The MAPK cascade leading to ScKss1p involves the MAPKK ScSte7p, the MAPKKK ScSte11p, and the PAK ScSte20p. ScKss1p phosphorylates ScSte12p, a transcription factor (Herskowitz, 1995). In *C. albicans*, the MAPKK is Hst7p, the MAPKKK is CaSte11p, and the PAK is Cst20p. Cek1p, like ScKss1p, phosphorylates a transcription factor. ScKss1p activates ScSte12p, and Cek1p is required for Ste11p-mediated activation of the Ste12 homolog Cph1p (Lee & Elion, 1999; Ramírez-Zavala *et al*., 2013).

The most well-studied activator of the Cek1p MAPK pathway is the mucin-like protein Msb2p, acting in concert with Sho1p. Activation of Cek1p by Msb2p is transmitted through the G-protein Cdc42 and its guanine nucleotide exchange factor (GEF) Cdc24 (Román, Cottier, Ernst, & Pla, 2009). More recently, another GTPase, Rac1p, has been implicated in signaling to Cek1p. Deletion of Rac1p or its associated proteins, Dck1p and Lmo1p, leads to a defect in embedded filamentation, which can be overcome by overexpression of *CEK1* (Hope, Schmauch, Arkowitz, & Bassilana, 2010). This is especially interesting as this suggests a divergence between activation of the Cek1p pathway in the two species (Bassilana & Arkowitz, 2006; Hope, Bogliolo, Arkowitz, & Bassilana, 2008; Hope *et al*., 2010). With recent research revealing potential Dck1p, Lmo1p, and Rac1p homologues in *S. cerevisiae*, more research is needed to determine whether this is a true divergence between the two species, or a case of *C. albicans* research informing research in *S. cerevisiae*, as has, thus far, not often been the case. Also needed for embedded filamentation, as

discussed previously, is the integral membrane protein Dfi1p. Dfi1p promotes embedded filamentation via Cek1p (Zucchi *et al*., 2010). Strong evidence supports that Rac1p and Dfi1p are needed for embedded filamentation, and that Dfi1p promotes Cek1p signaling during embedded filamentation. This work will present evidence that Rac1p, Dck1p, and Lmo1p also signal via this MAPK.

In addition to activating MAPK, it is important for cells to be able to turn off the signal. This is done by de-phosphorylation, and, in the case of Cek1p is done by Cpp1p. Cpp1p is a member of the VH1 family of dual specificity phosphatases (C Csank *et al*., 1997). Deletion of one allele of *CPP1* rescues the embedded filamentation defect of a *dfi1* null strain, demonstrating the importance of activated Cek1p to this process (Zucchi *et al*., 2010).

The role of Cek1p is not restricted to promoting filamentation. Deletion of *cek1*, *hst7*, or *sho1* leads to sensitivity to cell wall targeting drugs, such as Congo Red (Elvira Román *et al*., 2009). Additionally, treatment with tunicamycin, which, based upon its effect on *S. cerevisiae* glycosylation, is expected to cause cell wall damage, activates Cek1p (Cantero *et al*., 2007). This suggests that Cek1p plays a role in sensing and responding to cell wall damage. Cek1p may also play a role in cell wall biogenesis, as a *cek1* or *hst7* null strain shows an altered cell wall structure, resulting in increased exposure of β-1,3-glucan exposure and increased α-1,2 and β-1,2-mannosides. Exposure of β-1,3-glucan is particularly interesting because exposure of β-1,3-glucan is recognized by the receptor Dectin-1, an important step in immune recognition of fungi (Gow *et al*., 2007; E Román *et al*., 2016). Deletion of

cek1 results in decreased virulence, but this observation is complicated by the fact that cells deleted for *cek1* are defective for growth at 37°C (Csank *et al.*, 1998a).

Along with posttranslational regulation by phosphorylation, Cek1p is subject to regulation by proteasomal degradation. MAPK regulation by proteasomal degradation in *C. albicans* appears to be specific to Cek1p. The short-lived nature of the Cek1p protein is important for growth phase regulation. Cek1p is activated during the first hour of growth resumption after a stationary phase culture is diluted, but is subsequently decreased. Cek1p activation during growth resumption is caused by relief of repression of Cek1p by the quorum-sensing molecule farnesol (Elvira Román *et al.*, 2009). Farnesol in a stationary phase culture is diluted out with new culture medium, allowing Cek1p to be activated. The following decrease in activated Cek1p is facilitated by proteasomal degradation (Elvira Román *et al.*, 2009).

1.2.4 Cek2

Cek2p in *C. albicans* and its homolog ScFus3p in *S. cerevisiae* are the MAPK that respond to mating pheromone. ScFus3p shares the upstream components of the ScKss1p MAPK cascade, as well as an additional scaffold protein ScSte5 (Bardwell, 2005). The same is true of Cek2p and Cek1p. A *cek2 cek1* double null strain is completely defective for mating, while the single null mutants are reduced, suggesting that there is some redundancy between the two MAPK for this function. Cek2p is only expressed in mating competent cells, which are homozygous at the mating locus (*MTLa/MTLa* or *MTLα/MTLα*. *CEK2* expression is positively regulated

by Cph1p ((Jiangye Chen, Chen, Lane, & Liu, 2002). All the cells in this study are MTLa/α, and, therefore, do not express Cek2p.

1.2.5 MAPK crosstalk

Although each MAPK has a distinct function, they can respond to similar stimuli, creating the possibility for crosstalk between the signaling cascades. For example, Mkc1p and Hog1p both respond to oxidative stress, and Mkc1p activation requires activation of Hog1p, as a *hog1*, *pbs2*, or *hog1 pbs2* double null is defective for activation of Mkc1p upon treatment with hydrogen peroxide (Arana, Nombela, Alonso-Monge, & Pla, 2005). These strains also show constitutive activation of Cek1p, which is expected given that the Hog1p pathway represses activation of the Cek1p pathway (Arana et al., 2005; Eisman et al., 2006). The hyperfilamentous phenotype displayed by the *hog1* null under various conditions is not, however, dependent upon Cek1p. Additionally the response of Hog1p to osmotic and oxidative stress is independent of Cek1p (Eisman *et al*., 2006).

Aberrant crosstalk can lead to adverse outcomes, as cells could suffer if incorrect responses to the environment were made due to muddled signaling. In *S. cerevisiae*, where ScKss1p and ScHog1p both respond to osmotic stress and even share some pathway components, there have evolved methods of maintaining pathway specificity. One is phosphorylation of the shared component ScSte50p, which prevents prolonged ScKss1p activation during osmotic stress, preventing the switch to filamentous growth under those conditions (Nan Hao *et al*., 2008). There is also a scaffold protein, ScAhk1, which prevents ScHkr1p activation from leading to

ScKss1p activation, keeping ScHkr1p specific to ScHog1p, despite sharing downstream signaling components (Nishimura *et al.*, 2016).

In summary, the Hog1p, Mkc1p, and Cek1p MAP kinase pathways in *Candida albicans* promote filamentation and other cellular responses under various conditions. This work will focus primarily on the role of the Cek1p pathway in filamentation. The Cek1p pathway is activated by several small GTPases, reviewed in the next section.

1.3 Small GTPases

Small GTP-binding proteins, also called p21s or small GTPases, control a wide array of cellular processes. These proteins are defined by their size and their ability to hydrolyze GTP to GDP. GTPases are molecular switches that cycle between a GDP-bound state, which is considered inactive, and a GTP-bound state, which can interact with effectors to activate downstream pathways. This cycling is facilitated in both directions by two kinds of regulatory proteins: GAPs and GEFs. GTPase activating proteins (GAPs) stimulate hydrolysis of GTP, promoting the transition from the GTP-bound state to the GDP-bound state. Guanine nucleotide exchange factors (GEFs) stimulate the exchange of GDP for GTP, facilitating the transition from the GDP-bound state to the GTP-bound state. Some small GTPases have lipid modifications, such as geranylgeranylation, which allow them to associate with membranes. Members of another class of regulatory proteins, guanine dissociation inhibitors

(GDIs), bind these lipids to keep inactive GTPases in the cytosol, coordinating GDP/GTP switching with cytosol/membrane localization (Cherfils & Zeghouf, 2013).

1.3.1 Overview of Ras superfamily

The first small GTPase to be discovered was the oncogene Ras, and most of the known GTPases belong to the Ras superfamily (Colicelli, 2004). The Ras superfamily of GTPases now includes 5 families, based upon their sequence homology: Ras, Rho, Rab, Ran, and Arf (Rojas, Fuentes, Rausell, & Valencia, 2012). Although these proteins share a structural framework and enzymatic activity, their biological functions are diverse.

Ras subfamily GTPases are regulators of growth, differentiation, and survival. Ras GTPases are farnesylated at their C-termini to achieve membrane localization. In *Candida albicans*, Ras1p promotes filamentation in response to many stimuli and acts upstream of the Cek1p MAPK pathway and the cAMP/PKA pathway (Rojas *et al*., 2012; Wennerberg, Rossman, & Der, 2005).

Rho subfamily members regulate the cytoskeleton and vesicular trafficking. They can be either farnesylated or geranylgeranylated at their C-terminus to target them to the membrane. They are well known for their ability to regulate actin dynamics. The Rho GTPase family can be further subdivided into three groups: Rac, Cdc42, Rho. The best-studied members of the Rho family are Rac1, Cdc42, and RhoA, each being the paragon of its Rho family subgroup. These GTPases are of particular interest for this work due to their role in polarized growth and cell-matrix

interactions. In higher eukaryotes, Rac1 promotes membrane ruffling and lamellopodium formation; Cdc42 activation leads to filopodium formation; RhoA promotes focal adhesion formation and actin stress fiber assembly (Wennerberg *et al*., 2005). In *Candida albicans*, two Rho family proteins play significant roles in promoting filamentation: Cdc42p and Rac1p (Bassilana & Arkowitz, 2006).

Rho GTPases are activated by two families of GEFs: those containing a Dbl-homology (DH) domain and those containing a Dock homology region (DHR) domain. Most Rho GEFs belong to the former category, which was the first described; DHR domain-containing GEFs are sometimes termed "atypical GEFs" (Premkumar *et al*., 2010).

The Rab subfamily is the largest small GTPase family. Rab proteins have geranylgeranyl domains at their C-termini, and are bound by RabGDIs to maintain them in the cytoplasm. Rab subfamily members are involved in membrane trafficking. Individual Rab proteins can be targeted to different membrane localizations via a targeting region of 35-40 amino acids near the C-terminus, or via interactions with substrates (Cherfils & Zeghouf, 2013; Hutagalung & Novick, 2011).

Like the Rab subfamily, Arf is a regulator of intracellular traffic, with a major role in membrane trafficking. Unlike other small GTPases, Arf family members are myristoylated at their N-terminus. This modification is required for the function of these GTPases, but its role is incompletely understood (Liu, Kahn, & Prestegard, 2009). Arf subfamily members undergo a much larger conformational change between the GDP-and GTP-bound states than other small GTPases (Wennerberg *et al*., 2005).

Ran subfamily proteins are involved in transport across the nuclear membrane and assembly of the mitotic spindle. This function is facilitated by GEF presence in the nucleus and GAP segregation in the cytoplasm, resulting in a Ran-GTP gradient. Most eukaryotic genomes encode only one Ran protein (Melchior, 2001)

In summary, it is well documented that the Ras superfamily of GTPases is necessary for many essential cellular functions including growth, changes in morphology, membrane trafficking, and mitosis. In *Candida albicans*, Ras1p, Cdc42p, and Rac1p play roles in promoting filamentation in response to different cues. This work focuses on the role of Rac1p signaling in promoting contact-dependent Cek1p activation.

1.3.2 Rac1p in *Candida albicans*

The importance of Rac homologs in pathogenic fungi, especially in fungal plant pathogens, has been observed for some time. In plant pathogenic fungi, including *Ustilago maydis* and *Magnaporthe grisea*, Rac homologs are required for virulence, growth, and the morphological transition to hyphal or conidial development (Jisheng Chen *et al*., 2008; Mahlert, Leveleki, Hlubek, Sandrock, & Bolker, 2006; Nesher, Minz, Kokkelink, Tudzynski, & Sharon, 2011). Until recently, it was thought that the nonpathogenic model strain *S. cerevisiae* had no *RAC1* homolog. However, based upon the discovery of homologs of accessory proteins that activate Rac1p in mammalian cells, it is hypothesized that the *Sc*Rho5p is a Rac1p homolog (Schmitz, Jendretzki, Wittland, Wiechert, & Heinisch, 2015). In *Candida*

albicans, Rac1p has a clear role in embedded filamentation, although, like Dfi1p, it is dispensable for filamentation in response to other cues (Hope 2008, 2010). Rac1p in *C. albicans* can be geranylgeranylated, allowing it to associate with membranes. Association with the plasma membrane is important for its function in promoting embedded filamentation (Vauchelles, Stalder, Botton, Arkowitz, & Bassilana, 2010).

Rac1p function in *Candida albicans* requires two accessory proteins: the GEF Dck1p and its binding partner Lmo1p. Dck1p is a homolog of human Dock180 and *Caenorhabditus elegans* Ced-5, and therefore a member of the DHR-containing GEF family. Lmo1p is a homolog of the human protein ELMO and the *C elegans* protein Ced-12. ELMO/Ced-12 and Dock180/Ced-5 work together upstream of Rac1p, causing the latter protein to be activated. ELMO is not one of the previously discussed GTPase accessory proteins, but its interaction with Dock180 is required for Dock180 activation of Rac1p. Recently, homologs of *DCK1* and *LMO1* have been identified in *S. cerevisiae* (Schmitz et al., 2015). In human cells another Rho GTPase, RhoG, interacts with ELMO to activate Rac1p (Katoh & Negishi, 2003). A RhoG homolog in *C. albicans* has not been identified.

In summary, the Rac1p, Dck1p, and Lmo1p proteins are homologous to mammalian proteins that control cell morphogenesis. In *C. albicans*, these three proteins are needed for wildtype levels of embedded filamentation, but are dispensable for other forms of filamentation. Ultimately, the signals transmitted by GTPases and MAPK cascades result in transcriptional changes. Transcriptional control of filamentation is reviewed in the next section.

1.4 Regulation of Transcription in Eukaryotes

The ability to synthesize new proteins is crucial for an organism's ability to respond to its environment. This requires synthesis of new RNA to code for the desired proteins. However, organisms must maintain control over which genes are transcribed at any given time to avoid wasting resources or generating proteins that are harmful in their present environment. This section will review transcription in eukaryotes, as well as control of transcription by transcription factors.

Transcription in eukaryotic organisms is a tightly regulated process and involves several components. Central to transcription is RNA polymerase. In contrast to bacteria that only have one RNA polymerase, eukaryotes have three RNA polymerase enzymes, each specializing in a particular gene product. RNA polymerase I transcribes rRNA, while RNA polymerase III transcribes small RNAs, such as tRNAs. RNA polymerase II transcribes protein-coding genes. Transcription in mitochondria is carried out by another polymerase, which is similar to bacterial RNA polymerases (Cooper, 2000; Wray et al., 2003). All three eukaryotic RNA polymerase enzymes comprise many subunits (8-14, depending upon the polymerase). Several components are the same in all three polymerases, including the two largest subunits, which are similar to the β and β' subunits of prokaryotic RNA polymerases (Cooper, 2000). This section will focus on RNA polymerase II, as transcription of protein-coding genes is of most interest to this work.

Unlike bacterial RNA polymerases, RNA polymerases in eukaryotic cells require a series of additional proteins to initiate transcription. These proteins are known as general transcription factors, not to be confused with specific

transcription factors, which bind sequences upstream of specific genes. RNA polymerase II requires 5 general transcription factors: TFIID, TFIIB, TFIIF, TFIIE, and TFIIH. To make the abbreviations a little more easily digestible, TF indicates transcription factor and II indicates RNA polymerase II. TFIID is the first complex to bind, and it does so at the TATA box. Some eukaryotic promoters of protein-coding genes include a feature known as a TATA box, approximately 25 nucleotides upstream of the transcriptional start site. The TATA box is similar to the -10 element of a bacterial promoter. This sequence is recognized and bound by a protein known as TATA binding protein (TBP), which is a component of TFIID. The rest of TFIID is made up of 10-12 other polypeptides, called TBP-associated factors (TAFs). These polypeptides can associate with initiation elements, which are another promoter motif that may be present alongside or instead of the TATA box. Initiation elements span the transcription initiation site. Some promoters have neither TATA box nor initiation element, demonstrating the variability of eukaryotic promoters (Cooper, 2000; Hantsche & Cramer, 2017).

Once TBP is bound, it can interact with TFIIB; this complex forms a bridge to RNA polymerase, which also interacts with a third factor, TFIIF. TFIIE and TFIIH join the complex after RNA polymerase arrives at the promoter. TFIIH plays several important roles in transcription initiation. Two subunits of TFIIH have both helicase activity and are needed for the proofreading ability of the polymerase. Thus, these subunits are believed to help open the DNA for transcription and to promote fidelity of the transcript. Additionally, another subunit of TFIIH has kinase activity and phosphorylates sites on the C-terminal domain of the largest subunit of RNA

polymerase II. This phosphorylation is believed to allow RNA polymerase to be released from the initiation complex and begin transcription elongation. Assembly of the initiation complex is thought to typically occur through sequential binding of the promoter, but complexes formed prior to DNA binding have been observed (Cooper, 2000; Hantsche & Cramer, 2017; Wray et al., 2003).

Transcription initiation is crucial, but controlling which genes are expressed is also of great importance. Coordinating transcription so that the appropriate genes are expressed when needed and expressed with any other genes whose products are needed relies upon specific transcription factors, which act alongside the elements described above. Like in bacteria, eukaryotic transcription factors act either as repressors or activators of transcription. The most straightforward action of a repressor is to block binding of an activating factor or other part of the transcription machinery, although it is also possible to interfere with transcriptional elongation. Activators typically bind upstream of the TATA box or other promoter element (such as initiation elements) and recruit early members of the initiation complex, such as TFIID. Activators can also stabilize the initiation complex at the promoter (Latchman, 1990).

One of the most well-studied transcription activators is Gal4p. Gal4p promotes transcription of genes needed for galactose uptake and utilization. Gal4p is a zinc finger transcription factor, with two zinc-containing domains connected via a linker. Gal4p also has two activating domains, which contain acidic residues, which exert their effect on transcription by inducing a conformation change in TFIID (Latchman, 1990; Traven, Jelicic, & Sopta, 2006). This altered conformation

facilitates binding of other members of the transcription initiation complex. During growth on glucose, a preferred carbon source, the activity of Gal4p is repressed by binding of Gal80p to the activating domains of Gal4p. This prevents the activating domains from interacting with TFIID. When galactose and not glucose is present Gal3p relieves repression by causing Gal80p to dissociate from Gal4p (Traven et al., 2006). Gal4p is a useful example for this work, as two other transcription factors important to this work belong to the same family as Gal4p and can be artificially activated by the addition of a Gal4p activating domain (Schillig & Morschhäuser, 2013a).

In summary, eukaryotic transcription is a complex process that is still incompletely understood. This process is coordinated by sequence-specific transcription factors that promote transcription of particular groups of genes. One of the most well-known transcriptional activators in eukaryotic biology is Gal4p.

1.5 Transcriptional control of Filamentation

Filamentation is a complex process that can be induced by numerous stimuli. While signaling molecule pathways are key to sensing each of these stimuli, ultimately, control of transcription, mediated by an array of transcription factors, is key to initiating hyphal morphogenesis. In this section, transcription factors playing a role in filamentation, especially embedded filamentation, are reviewed.

Despite the breadth of cues for hyphal morphogenesis, filamentation can be prevented under most conditions by deletion of genes encoding two transcription factors: *cph1* and *efg1* (Lo *et al*., 1997). Cph1, as previously discussed, is a Ste12p

homolog and acts downstream of the Cek1p MAPK pathway to promote filamentous growth. Efg1p, enhanced filamentous growth protein 1, is a homolog of *S. cerevisiae* Phd1p. Efg1p acts downstream of the Ras1/PKA pathway to promote filamentous growth in response to many liquid growth conditions, sometimes working with another transcription factor, Flo8p (Cao *et al*., 2006).

One stimulating condition in which this double deletion strain does not abrogate filamentous growth is embedded, or contact-dependent filamentation (Giusani, Vinces, & Kumamoto, 2002). Interestingly, deletion of *efg1* or *flo8* enhances embedded filamentation. This suggests that regulation of genes during embedded filamentation is different from regulation under other conditions that induce filamentation. Contact dependent filamentation is severely defective in a strain deleted for *cph1* and *czf1* (Brown Jr, Giusani, Chen, & Kumamoto, 1999).

Identified in the same screen that identified Cek1p, Czf1p is a zinc finger transcription factor belonging to the same family as Gal4p (Whiteway 1992, Macpherson 2006). Czf1p promotes filamentation in embedded conditions, but is dispensable during liquid growth. Czf1p promotes filamentation by removing repression by Efg1p (Giusani *et al*., 2002). This effect is dependent upon Efg1p, indicating that Efg1p is epistatic to Czf1p (Petrovska & Kumamoto, 2012).

Expression of *CZF1* is subject to complex regulation. Expression is regulated by a number of environmental cues, including carbon source, growth phase, physical environment, and temperature. Under all conditions tested to date, *CZF1* expression depends upon Efg1p. Negative autoregulation also plays a role in *CZF1* transcript

levels (Vinces, Haas, & Kumamoto, 2006; Zordan, Miller, Galgoczy, Tuch, & Johnson, 2007).

Czf1p is also a regulator of the white-opaque switch in *C. albicans*, another example of the yeast's morphological plasticity. The white-opaque switch is an epigenetic switch between two distinct morphological forms. These forms are the white form and the opaque form. The white form is the typical form of laboratory strains, while the opaque form is needed for successful mating (Lohse & Johnson, 2009). Switching occurs stochastically at a low frequency, but can be encouraged by environmental conditions, such as anaerobic growth (Ramírez-Zavala *et al*., 2013). Czf1p works with other transcription factors, especially Wor1, to promote the transition to an opaque cell form, while Efg1 promotes the white cell form (Noble *et al*., 2016). Like embedded filamentation, white-opaque switching is a process in which Czf1p and Efg1p have opposing effects.

It is unclear which pathway activates Czf1p during embedded growth, although some experimental results suggest a possible link to the MAPK Mkc1p. Ectopic *CZF1* expression restores embedded filamentation in an *mkc1* null. Ectopic expression of *CZF1* does not, however, complement the *mkc1* null strains sensitivity to Nikkomycin Z, suggesting that if Czf1p is downstream of Mkc1p, it does not mediate all the transcriptional outputs of the Mkc1p MAPK pathway (Kumamoto, 2005). Additionally, ectopic expression of *CZF1* does not complement the embedded filamentation defect in a *rac1* null strain, suggesting that either Czf1p is not downstream of the Cek1p MAPK pathway or Czf1p requires input from the Cek1p pathway to be functional (Hope *et al.,* 2010).

Filamentation is also negatively regulated. The transcription factors Nrg1p and Tup1p are repressors that prevent filamentation until an inducing environment is encountered. Tup1p is a global regulator, and Nrg1p is a sequence-specific binding protein (Kadosh & Johnson, 2005). Strong evidence supports that deletion of *nrg1* greatly favors the filamentous form, while deletion of Tup1 results in constitutive filamentous growth (Braun, Kadosh, & Johnson, 2001). Deletion of *TUP1* in *S. cerevisiae* causes many different phenotypes, such as flocculation, loss of glucose repression, and inability to sporulate, suggesting that Tup1 controls several groups of genes in *S. cerevisiae* whose roles vary widely (Smith & Johnson, 2000). The variety of phenotypes of the *tup1* null is consistent with the role of Tup1p as a global regulator. Filamentous growth is also prevented by the quorum sensing molecule farnesol, which inhibits the Ras1/PKA pathway. *tup1* null and *nrg1* null strains produce an excess of farnesol, and farnesol can complement the haploinsufficiency of the *tup1/Tup1* strain (Kebaara *et al*., 2008).

Decreasing the amount of Nrg1p is an important step in hyphal initiation. This temporary decrease requires Efg1p and Flo8p. Levels of Nrg1p decrease in the first 30 minutes after hyphal induction at 37°C, but begin to rise again after an hour. Hyphal maintenance is achieved by blocking Nrg1p from rebinding to the promoter by chromatin remodeling. Remodeling chromatin to exclude Nrg1p involves recruitment of a histone deacetylase, Hda1p. Recruitment of Hda1p occurs downstream of the Tor pathway, which senses nutrient poor environments. Promotion of hyphal maintenance by input from the Tor pathway demonstrates the importance of environmental sensing in prolonged hyphal extension. In nutrient

replete conditions, Nrg1p is allowed to rebind, directing *C. albicans* back to yeast growth (Lu, Su, Wang, Liu, & Hazan, 2011).

In summary, transcriptional control of filamentation is a complex process that involves many potential players. Efg1 and Flo8 promote filamentation downstream of the Ras1/PKA pathway during liquid growth. Cph1 is an output of the Cek1p MAPK pathway and promotes filamentous growth in some liquid media and also on solid media; embedded filamentation is also promoted by Czf1p. Negative regulation of filamentation is orchestrated by Tup1p, Nrg1p, and the quorum-sensing molecule farnesol. These pathways work in concert to produce responses appropriate to the environmental conditions.

1.6 Iron homeostasis in *Candida albicans*

Most known organisms require transition metals, such as iron, copper, zinc, and manganese. These metals can be incorporated as essential parts of metalloprotiens, including metalloenzymes and transcription factors. The roles of transition metals can vary, especially between prokaryotes and eukaryotes. For example, around 80 percent of zinc-binding proteins in prokaryotes are enzymes, while 44 percent of eukaryotic zinc-binding proteins are transcription factors. Many of the transcription factors discussed in this work require zinc. In both prokaryotes and eukaryotes, however, around half of proteins with iron are enzymes, usually utilizing iron as a redox cofactor (Hood & Skaar, 2012).

Although iron is the fourth most common element in the earth's crust, it is a limited resource within the human body (Hsu, Yang, & Lan, 2011). Systems to maintain a low amount of free iron provides two benefits: protection from oxidative damage to cellular components via the Fenton reaction and protection from invading pathogens, called nutritional immunity. Storage and sequestration of iron in tissue and blood is, therefore, of great importance to the host. For its part, *C. albicans* has evolved methods of wresting iron from its host environment (C. Chen, Pande, French, Tuch, & Noble, 2011). The storage of iron in the human body and the ways in which it is acquired by *C. albicans* are reviewed below.

About two thirds of the iron within the human body, not including iron found in the digestive tract, is found in the bloodstream. This iron is usually bound to hemoglobin in erythrocytes, although some is bound by transferrin. Transferrin binding is important for iron distribution within the body (Andrews, 1999).

C. albicans utilizes several activities to obtain iron during growth within a host. *C. albicans* hemolysins release hemoglobin from red blood cells. The surface protein Rbt5p then binds to the liberated heme porphyrin rings; the complex is then endocytosed and the heme oxygenase Hmx1p releases the ferrous iron into the cytosol (Santos *et al*., 2003). *C. albicans* can also obtain iron from transferrin, although the mechanism is unclear. Direct contact between the yeast cell and transferrin is required, suggesting a membrane-bound receptor. The ferric iron freed from transferrin must then be reduced to ferrous iron by an iron reductase (Knight, Vilaire, Lesuisse, & Dancis, 2005). The iron is then imported via a

multicopper ferroxidase or iron permease; the *C albicans* genome encodes several copies of both methods of import (Noble, 2013).

In tissue, iron is stored as ferritin. Ferritin is a less common source of iron for pathogens, with only some bacterial species known to use it. *C. albicans* can use ferritin as an iron souce by accumulating ferritin at the cell surface with the hyphal specific protein Als3p. Like transferrin, ferritin binds ferric iron, which is imported by *C. albicans* using a mechanism similar to the mechanism used to acquire iron from transferrin. Interestingly, deletion of *ALS3* results in a defect in virulence in oral epithelial infection models, but not bloodstream infection models. Als3p has more than one function. For example, Als3p promotes adhesion to host surfaces, endocytosis by host cells, and contributes to development of interspecies biofilms (Phan et al., 2007; Silverman, Nobbs, Vickerman, Barbour, & Jenkinson, 2010). These results suggest that Als3p functions, incuding the use of ferritin, contribute to growth of *C. albicans* in tissue (Almeida *et al*., 2008).

Another method of iron acquisition is through siderophores, small molecule iron chelators that can scavenge iron from the host or environment (Behnsen & Raffatellu, 2016). Although it is unclear whether *Candida* produces siderophores, the yeast can utilize them for iron acquisition. The Sit1p sideophore importer can utilize ferrichrome-type siderophores. A *sit1* null strain has the same virulence phenotype as an *als3* null, consistent with siderophore acquisition being dispensable in the bloodstream, but not for growth in tissue (Heymann *et al*., 2002).

There are, clearly, many contingencies for acquiring iron, but, as previously mentioned, in some cases too much of a good thing can be toxic. Organisms must

have ways to shut down iron uptake if the environment is particularly iron rich, as taking up iron indiscriminately can have negative consequences such as cellular damage. Transcription of iron homeostasis genes is therefore tightly regulated.

The nonpathogenic yeast *S. cerevisiae* has a transcriptional circuit whose role is to tailor iron utilization and uptake to the iron availability in the environment. Transcriptional activators ScAft1p and ScAft2p respond to low iron availability by accumulating in the nucleus and increasing transcription of genes involved in iron uptake while repressing transcription of genes involve in iron utilization. When iron levels are high, the transcription factors are removed from the nucleus (Hsu *et al*., 2011). ScYap5 is a transcription factor that is active in iron rich conditions and promotes transcription of *CCC1*, a protein that imports iron into the vacuole (Baek, Li, & Davis, 2008). ScYap5 is a basic leucine zipper (bZip) transcription factor (Hsu *et al*., 2011).

Candida albicans has an iron responsive transcriptional circuit that differs from that in *Saccharomyces cerevisiae*. *C. albicans* has an *AFT2* homolog, which has recently been shown to function in iron homeostasis, as well as other processes (Xu *et al*., 2013). Aft2p is not, however, part of the main iron regulation transcriptional circuit in C. albicans. Instead three transcription factor units: Sfu1p, Sef1p, and the CCAAT-binding protein (CBP) complex form the main iron regulation circuit (C. Chen *et al*., 2011). This circuit, and those found in *S. cerevisiae* and *Schizosaccharomyces pombe* (*S. pombe*) are shown in figure 1.4.

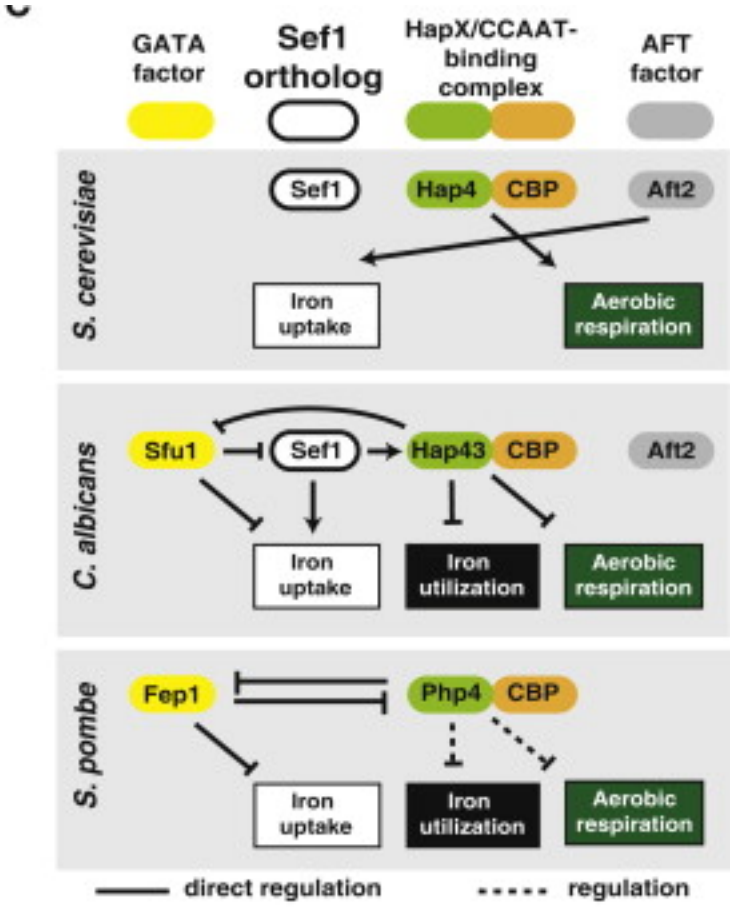

Figure 1.4 Regulatory circuits responding to iron in *S. cerevisiae*, *C. albicans*, and *S pombe.* Modified from Chen *et al* 2011. Changes include cropping of a multi-panel figure.

Sfu1p is a GATA family transcription factor. GATA transcription factors are classified based on a conserved amino acid sequence and their possession of Cys_2/Cys_2 zinc finger domains separated by a cysteine rich domain. In Sfu1p this cysteine rich region senses iron. Sfu1p homologs in several fungal species have been identified for their ability to coordinate responses to high iron, for example repression of siderophore production (Pelletier *et al*., 2007; Voisard, Wang, Mcevoy, Xu, & Leong, 1993). In *S. pombe* this protein is Fep1p (C. Chen *et al*., 2011). Although GATA transcription factors that respond to high iron availability are found in many

species in the Ascomycota and Basidomycota phyla, there is no homologue of Sfu1p in *S. cerevisiae*.

Sef1p is a Zn2Cys6 zinc finger transcription factor. It belongs to the same family of zinc finger transcription factors as the well-studied *S. cerevisiae* Gal4 transcription factor (MacPherson, Larochelle, & Turcotte, 2006). Sef1p upregulates iron uptake genes, as well as some virulence factors (C. Chen *et al.*, 2011). Sef1p also upregulates transcription of the third player in the iron regulatory circuit, the CBP complex.

The CBP complex is defined by the DNA motif to which it binds. The component proteins are homologous to the members of the mammalian NF-Y complex, which is comprised of three proteins: NF-Y A, B, and C. These proteins work together to bind DNA at a 5'-CCAAT-3' sequence. Several fungal species have known homologs of this complex, and some of them regulate genes in response to iron starvation. In *Schizosaccharomyces pombe*, the CCAAT binding protein complex is made up of the proteins Php2, 3, 4, and 5. Php2, 3, and 5 are homologs of NF-YA, B, and C; these proteins are responsible for binding to DNA. Php4 is a fourth member of the complex that occurs in yeast and acts as a regulatory subunit. In *Aspergillus nidulans*, the DNA binding protein complex is composed of HapB, C, and E with HapX acting as the regulatory subunit. In C. albicans, the NF-Y homolog proteins are Hap2, 3, and 5 and Hap43 is one of three homologs of HapX/Php4. Hap43p interacts with the DNA-binding members of the complex via a domain that interacts with Hap5p. Hap43 has a conserved N terminal region with a domain homologous to HapX in *A nidulans,* and a bZip domain, like Yap5 *in S. cerevisiae*, although the sequence and

function of the protein is more similar to that of HapX than Yap5. Interestingly, Hap43 is the only *C. albicans* HapX homolog with a bZip motif, and is the only one for which iron regulatory activity has been shown, suggesting that the bZip domain may be important for this function (Hsu *et al*., 2011; Singh, Prasad, Sinha, Agarwal, & Natarajan, 2011).

In *C. albicans*, Sef1p, Sfu1p, and the CBP complex act in a circuit to maintain iron homeostasis. When in an iron rich environment, Sfu1p is active and iron uptake genes are repressed, protecting the cell from excess iron. Sfu1p down-regulates Sef1p in two ways. First, Sfu1p represses *SEF1* transcription. Second, Sfu1p also binds Sef1p, keeping it in the cytosol and targeting it for degradation (C. Chen *et al*., 2011). When iron becomes limited, repression of gene expression by Sfu1p is alleviated. Sfu1p levels decrease due to CBP complex repression of *SFU1* transcription. Inhibition of Sef1p by Sfu1p is prevented by Ssn3p, which phosphorylates Sef1p, preventing Sfu1p from binding. Unbound Sef1p enters the nucleus, where it can promote transcription of iron uptake genes (C. Chen, Noble, Bitincka, Shamir, & O'Shea, 2012). The genes encoding two of the members of the CBP complex are known to be upregulated by Sef1p: HAP3 and HAP43 (C. Chen *et al*., 2011).

This transcriptional circuit is important for *C. albicans* adjustment to its various host niches. In the bloodstream and tissue, where all but a vanishingly small amount of iron is bound by host proteins, Sef1p and the CBP complex are critical for survival. These proteins are also important for causing disease; in an intravenous infection model in mice, deletion of either *SEF1* or *HAP43* results in significant

attenuation of virulence (C. Chen *et al*., 2011; Hsu *et al*., 2011). However, a comparatively large amount of iron is available in the digestive tract. An individual following a typical Western diet consumes 15mg of iron daily, of which only about 10 percent is absorbed by the host. Additionally, low pH, such as that found in the stomach, and anaerobic conditions, such as those found in the colon, increase bioavailability of iron. In the gut, Sfu1p is required for wildtype levels of colonization. Interestingly, Sef1p is also required for wildtype colonization of the gut, suggesting that there are iron poor regions of the gut. It is also possible that the virulence factors upregulated by Sef1p are needed for growth in the gut (C. Chen *et al*., 2011).

In summary, iron regulation is vital for organisms that utilize the element. The host aggressively sequesters iron in the bloodstream and tissues to protect itself from the Fenton reaction and invading pathogens, but *Candida albicans* has evolved multiple methods of extracting iron in these iron poor niches. In order to maintain appropriate levels of iron in its own cells, *Candida* has also evolved a transcriptional circuit whose role is to control iron uptake and utilization, depending upon how iron rich or poor the environment is.

Sef1p and Hap43p are of particular interest to this work, as Chapter 3 presents evidence that Dfi1p-dependent Cek1p activation results in upregulation of some of the genes previously observed to be regulated by these transcription factors.

1.7 Rationale for Study

The overall goal of my thesis work was to improve our understanding of Dfi1p-dependent signaling and its targets.

Dfi1p was previously shown to signal via the MAP kinase Cek1p during growth in contact with an agar matrix (Zucchi *et al*., 2010). Subsequent work showed that Dfi1p-dependent Cek1p activation could be obtained by exposing exponential phase cells to a calcium ionophore (A23187, referred to in this work as "ionophore") because Dfi1p binds calcium-bound calmodulin (Davis, Zucchi, & Kumamoto, 2013).

The first aim of my thesis was to identify signaling intermediates between the transient complex formed by the C-terminal tail of Dfi1p and calcium-bound calmodulin and Cek1p. This work will show the involvement of the classical Cek1p MAPK cascade, as well as the GTPase Rac1p, its GEF Dck1p, and the structural protein Lmo1p. Constitutive activation of Rac1p bypassed the defect in Dfi1p-dependent Cek1p activation, suggesting that Rac1p functions downstream of Dfi1p, perhaps in the same pathway.

The second aim of my thesis was to identify and characterize transcriptional targets of Dfi1p-dependent Cek1p activation. This work will show that many of the targets of the Dfi1p-dependent pathway are members of the low iron-responsive regulon controlled by Sef1p. Expression of some of these target genes responds to activation of another transcription factor, Czf1p, suggesting that Dfi1p-dependent Cek1p activation targets a subset of both regulons to achieve situation-specific regulation.

Sensing and responding to environmental cues is critical for any organism, but is especially important for opportunistic pathogens, like *Candida albicans*. Identifying signaling pathway members and transcriptional targets that respond to a particular stimulus can help us to understand how that signal fits into the biology and host interactions of *C. albicans*. Previous work has established that Dfi1p–dependent signaling is important for virulence. One hypothesis is that this is due to a defect in filamentation, but other stimuli leading to filamentation should also be present in any mammalian infection model. The results presented below give insight into why Dfi1p is required for wildtype virulence. Based upon the function of several of the identified transcriptional targets, I propose a model where Dfi1p signaling promotes virulence in ways other than promoting filamentation.

Chapter 2

Dfi1p-Dependent Cek1p Activation during growth in contact with an Agar Matrix Requires the Classical MAPK cascade and Rac1p in *Candida albicans* [1]

[1] Weeks, AE., Bassilana, M, and Kumamoto CA To be submitted to FEMS Yeast Research

Abstract

The opportunistic pathogen *Candida albicans* causes a significant disease burden in humans. The ability to transition between the yeast growth form and the filamentous hyphal growth form contributes to virulence. One pathway that promotes the transition from yeast to hyphae is the Cek1p mitogen activated protein kinase (MAPK) pathway. During growth in contact with an agar matrix, activation of this pathway is increased by the plasma membrane protein Dfi1p. In this work we demonstrate that Cek1p signaling during growth in contact with agar medium requires members of the classical Cek1p MAPK cascade, as well as the small GTPase Rac1p. Constitutive activation of Rac1p restored Cek1p activation in a *dfi1* null mutant.

Introduction

The diploid fungus *Candida albicans* is a common human commensal colonizer and can be isolated from mucosal surfaces in many adults (Odds 1987). The fungus is also capable of causing a variety of disease states ranging in severity from superficial infections, such as oral thrush, to disseminated spread, which is fatal in 30-60 percent of cases (Hirano, Sakamoto, Kudo, & Ohnishi, 2015). Treatment outcomes for disseminated candidiasis and candidemia have not improved in the last 40 years despite the introduction of new therapeutic agents. Understanding virulence-related processes and factors is, therefore, an important line of inquiry.

One such process in *Candida albicans* is filamentation, the transition between the yeast growth form and the hyphal growth form. *Candida albicans* has the ability to grow in many different forms in order to adapt to its many niches within the host (Noble *et al*., 2016). The ability to transition between ovoid yeast and elongated hyphae is believed to contribute to the fungus's ability to cause disease in its human host (Lo *et al*., 1997). Hyphal morphogenesis can occur in response to many stimuli *in vitro.* These stimuli likely mimic signals the yeast would receive during growth in various environments in its host (Brown Jr *et al.*, 1999). One such stimulus is growth in contact with a semi-solid material such as agar medium. The membrane protein, Dfi1p is required for filamentation during growth in contact with agar medium. Dfi1p promotes filamentation by promoting activation of the Sterile Vegetative Growth (SVG) Mitogen-activated protein kinase (MAPK), Cek1p (Elvira Román *et al.*, 2009; Zucchi *et al.,* 2010). Identified for its ability to interrupt the *Saccharomyces cerevisiae* mating pathway, Cek1p in *C. albicans* is part of a pathway that senses some of the environmental cues leading to hyphal morphogenesis (Csank *et al*., 1998a; Elvira Román *et al*., 2009).

MAPK signaling pathways are common eukaryotic signaling modules comprised of three or more protein kinases that transmit signals received upstream through stepwise activation of the pathway. The MAPK kinase kinase (MAPKKK) receives a signal, sometimes from a MAPK kinase kinase kinase (MAPKKKK), or p21 activated kinase (PAK), and phosphorylates the MAPK kinase (MAPKK), which then phosphorylates the MAPK. The MAPK typically then activates a transcription factor (Monge *et al*., 2006; Widmann *et al.*, 1999). The proteins that make up the conserved

portion of the SVG MAPK pathway in *C. albicans* are the p21-activated kinase (PAK) Cst20p, the MAPKKK Ste11p, and the MAPKK Hst7p. These proteins are homologues of the *Saccharomyces cerevisiae* MAPKKKK Ste20p, MAPKKK Ste11p, and MAPKK Ste7p, respectively (R. E. Chen & Thorner, 2007; Csilla Csank et al., 1998)Strains lacking these proteins exhibit defects in hyphal morphogenesis under conditions during which Cek1p is a driver of filamentation, such as growth on solid medium supplemented with serum and growth on low-ammonia-dextrose medium (SLAD) (Csilla Csank et al., 1998). Hst7p and Cst20p are also required for wildtype levels of Cek1p phosphorylation under other studied conditions, such as constitutive activation of the small G protein Cdc42p (Elvira Román *et al*., 2009). These results support a role for these proteins in the Cek1p signaling cascade, but the role of these proteins in signaling during growth in contact with an agar matrix had not yet been determined.

Another protein shown to promote contact dependent filamentation is the GTPase Rac1p (Bassilana & Arkowitz, 2006; Vauchelles *et al.*, 2010). *RAC1* is conserved in higher eukaryotes and, interestingly, plays a role in regulating morphogenesis in mammalian cells (Bassilana & Arkowitz, 2006; Wennerberg *et al*., 2005). Rac1p is activated by a guanine nucleotide exchange factor (GEF) Dck1p, which is part of the Dock180 family of GEFs (Hope *et al*., 2008). The function of Rac1p also depends upon Lmo1p, a regulatory protein (Hope *et al*., 2010). *C. albicans* cells deleted for *RAC1*, *LMO1*, or *DCK1*, are defective for hyphal formation when grown in contact with an agar matrix (Hope *et al.*, 2008, 2010). These results

suggest that Rac1p regulates Cek1p activation during growth in contact with an agar matrix.

In this study, we show that Rac1p, Dck1p and Lmo1p are required for wildtype levels of Dfi1p-dependent Cek1p activation during growth in contact with agar medium. Rac1p localization to the membrane is key to its function in this pathway. A constitutively active form of Rac1p bypasses the MAPK activation defect in the *dfi1* null strain, consistent with the model that Rac1p acts downstream of Dfi1p. We also demonstrate the importance of the classical MAP kinase pathway, with a severe defect in Cek1p activation in an *hst7* or *ste11* null mutant and a partial defect in a *cst20* null mutant.

Results

Normal levels of contact-dependent Cek1p activation require Rac1p and its associated proteins

The GTPase Rac1p is required for wildtype levels of filamentation during growth in contact with an agar matrix (Hope 2008). We previously demonstrated that Cek1p activation increases in cells grown in contact with an agar matrix; this activation promotes filamentation (Zucchi 2010). We therefore hypothesized that Rac1p is required for Cek1p activation during growth in contact with an agar matrix. To test this hypothesis, we grew cells deleted for *RAC1*, *DCK1*, or *LMO1* on YPS agar with 1% agar at 25°C for four days. On the fourth day, cells were washed off plates with ice cold PBS, collected by centrifugation, and total protein was extracted with

RIPA lysis buffer supplemented with protease and phosphatase inhibitors, as described in the materials and methods section. Levels of activated Cek1p in each extract were determined by Western blotting. Tubulin was used as a loading control. Blots were quantified using GeneTools (GeneSys). Each lane shown in Figure 2.1 shows the amount of Cek1p~Pi_2 and tubulin detected in 60μg of total protein extracted from each strain. The level of Cek1p-Pi_2 was higher in the wildtype protein extract (Figure 2.1, lane 1) compared to the *rac1*, *dck1*, and *lmo1* null mutants (Figure 2.1, lanes 2-4). This finding indicates that activation of Cek1p was defective in strains deleted for *RAC1*, *DCK1*, or *LMO1* when compared to a wildtype control. Interestingly, this defect is similar in magnitude to that conferred by deleting *DFI1* (Zucchi 2010). We concluded that Rac1p, Dck1p, and Lmo1p do indeed contribute to Cek1p activation during growth in contact with an agar matrix.

Membrane association of Rac1p is required to achieve wildtype levels of Dfi1p-dependent Cek1p activation

Wildtype Rac1p protein localizes to both the plasma membrane and the nucleus, cycling between them depending upon activation state. Localization of Rac1p to the plasma membrane is required for wildtype levels of embedded filamentation (Vauchelles *et al*., 2010). The goal of our experiment was to determine if localization to the plasma membrane was required for wildtype levels of Cek1p activation. Mutations in the C-terminal tail of Rac1p that prevent the protein from localizing to the membrane confer defects in embedded filamentation similar to the

rac1 null. In the *RAC1-5Q* allele 5 basic residues in the polybasic region of the C-terminus of Rac1p are substituted with Q's, altering the Nuclear Localization Sequence (NLS) of the protein. This protein is largely cytoplasmic. *RAC1-C233S* encodes a form of Rac1p that cannot be geranylgeranylated. The geranylgeranyl modification of the C-terminus of Rac1p is thought to override or block the NLS, and, as expected, Rac1-C233S is largely present in the nucleus, especially when the strain is grown in the absence of shaking (Vauchelles *et al*., 2010). *RAC1*Δ *181-221* is an allele with the non-conserved region of the *RAC1* C-terminus deleted. The localization of this allele has yet to be determined, but is predicted to be cytoplasmic because of the importance of the C-terminus of Rac1p in membrane localization. We hypothesized that these mutants would be defective for Cek1p activation during growth in contact with an agar matrix.

To address the role of the C-terminal portion of Rac1p, cells of each mutant strain (*RAC1-5Q*, *RAC1-C233S*, and *RAC1*Δ *181-221*) and wildtype cells were grown separately on YPS with 1% agar for 4 days at 25°C. As in the previous experiment, cells were washed off the agar with ice cold PBS and protein was extracted in RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Protein extracts were probed for phosphorylated Cek1p and tubulin via Western blot. The results showed that all three mutations in the C-terminal tail of Rac1p conferred a defect in Cek1p activation compared to activation in a wildtype strain (Figure 2.2). This finding further supports the importance of Rac1p for Cek1p signaling during growth in contact with an agar matrix, and additionally supports the hypothesis that

membrane localization of Rac1p is important for embedded filamentation because it is required for adequate levels of Cek1p activation.

Constitutively active Rac1p bypasses the defect in Cek1p phosphorylation due to the absence of Dfi1p

Both *DFI1* and *RAC1* are required for wildtype levels of filamentation during growth in contact with an agar matrix (Bassilana & Arkowitz, 2006; Hope *et al*., 2008; Zucchi *et al*., 2010) and we proposed that Rac1p functions downstream of Dfi1p. We therefore asked whether constitutive activation of Rac1p would rescue the defect in Cek1p activation conferred by deletion of *DFI1*. GTPases cycle between an active GTP-bound state and an inactive GDP-bound state, facilitated and regulated by accessory proteins and the GTPases native GTPase activity. Mutation of a particular residue, however, can mimic the active state, leading to constitutive activation of the GTPase. For Rac1p the constitutively active allele is *RAC1 Q61L* (Hope *et al*., 2008). The *RAC1 Q61L* allele was introduced into the strain pcz5 (dfi1 -/- RAC1+/+). The *RAC1 Q61L dfi1* strain was used to test whether constitutive activation of Rac1p could rescue the defect in Cek1p activation conferred by deletion of *dfi1*. Wildtype cells, *dfi1* null mutant cells, and *RAC1 Q61L dfi1* cells were separately grown for four days on YPS with 1% agar on 25°C. Cells were washed off the plates with ice cold PBS and total protein was extracted in RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Cell extracts were probed via Western blot for phosphorylated Cek1p and Tubulin. As shown in figure 2.3, the

wildtype cell extract showed a strong band of activated Cek1p (lane 1), while the band of activated Cek1p was fainter in the cell extract from *dfi1* null cells (lane 3). The level of activated Cek1p in the *dfi1* null *RAC1 Q61L* double mutant cells (lane 2) resembled that shown by wildtype cells. Therefore, one allele of *RAC1Q61L* expressed in a *dfi1* null strain resulted in at least wildtype levels of Cek1p activation (Figure 2.3). This finding is consistent with the model that Rac1p acts downstream of Dfi1p in the same pathway.

Contact-dependent Cek1p activation requires Hst7p and Ste11p, while deletion of *CST20* confers a partial defect.

The MAPKK Hst7p and the PAK Cst20p are required for Cek1p activation in some growth conditions (Román *et al*., 2009) and we hypothesized that Hst7p, Cst20p, and the MAPKKK Ste11p would be required for wildtype levels of Cek1p activation during growth in contact with agar. To test this hypothesis, cells deleted for *cst20*, *ste11*, or *hst7* were grown for four days at 25°C on YPS with 1% agar. Cells were washed off plates with ice cold PBS and total protein was extracted in the presence of RIPA buffer with protease and phosphatase inhibitors. Protein extracts were probed for phosphorylated Cek1 and Tubulin via Western blot. Western blot analysis showed that deletion of either of the genes encoding Hst7p (the MAPKK) or Ste11p (the MAPKKK) conferred a severe defect in Cek1p activation. Deletion of the gene encoding Cst20p, however, conferred a partial defect in Cek1p activation, similar to the defect seen in the *rac1* null and the *dfi1* null strains (Figure 2.4).

These findings were consistent in experiments conducted with two sets of independently isolated MAPK cascade mutants (Csank *et al.*, 1997; Csank *et al.*, 1998b; Leberer *et al.*, 1996; Noble, French, Kohn, Chen, & Johnson, 2010a). This demonstrates that the proteins of the MAPK cascade are important for phosphorylation of Cek1p during growth in contact with an agar matrix.

Discussion

Integrating and responding to environmental signals is a crucial task for *Candida albicans*, and the proteins responsible for doing so participate in often-complex and inter-related pathways. We have shown that normal levels of Cek1p activation during growth in contact with an agar matrix require the MAPKK Hst7p, the MAPKKK Ste11p, and the PAK Cst20p. We also demonstrated that the GTPase Rac1p is required for wildtype levels of Cek1p activation under the same conditions, as are the Rac1p GEF Dck1p and the regulatory protein Lmo1p. The portions of the Rac1p protein that allow localization to the plasma membrane are also required for wildtype levels of Cek1p activation. Interestingly, our data suggest that Rac1p functions downstream of Dfi1p and the two proteins may be part of the same pathway. In addition, overexpression of *CEK1*, but not the other members of the MAPK cascade, rescued defective embedded filamentation, in a *rac1* null mutant (Hope 2010). In the absence of Rac1p, overproduction of Cek1p and inefficient activation may result in sufficient activated Cek1p to restore embedded filamentation. A model for Dfi1p-dependent Cek1p activation is shown in figure 2.5.

Crosstalk between MAPK pathways is well documented; in *C. albicans*, the MAPKKK of the SVG pathway can contribute to the osmosensing pathway that includes the MAPK Hog1p, and the cell wall integrity MAP kinase Mkc1p and its MAPKK, Mkk2p, contribute to Cek1p activation during growth resumption from stationary phase (Monge 2006, Roman 2015). Results presented here show that during growth in contact with an agar matrix there are no significant inputs to the SVG MAPK cascade between the MAPKKK Ste11p and the MAPK Cek1p because Cek1p activation is completely lost in the absence of either Ste11p or Hst7p. Therefore, growth in contact with an agar matrix is not one of the growth conditions under which Mkc1p and its MAPKK Mkk2p contribute to Cek1p activation (Elvira Román *et al.*, 2015). However, crosstalk above the level of the MAPKKK Ste11p may be occurring since the defect in Cek1p activation observed in the absence of Dfi1p, Rac1p, and Cst20p was only partial.

The partial defect in Cek1p activation observed in the *cst20* null contrasts with the more complete defect observed in the *ste11* and *hst7* null strains. A partial defect in *cst20* null has been shown under other conditions, demonstrating that this phenomenon is not restricted to contact-dependent Cek1p activation (Roman 2009). The partial defect displayed by the *cst20* null strain suggests that there is an additional input to the Cph1p-dependent MAPK cascade at the level of the MAPKKKK. One protein known to act at the MAPKKKK level is the Ste20-like protein Cla4p (Leberer 1997). Cla4p is a septin-associated protein kinase and is synthetically lethal with Ste20p in *S. cerevisiae,* suggesting that they share some functions (Leberer 1997, Huang 2014). However, cells deleted for *cla4* did not show

a defect in Cek1p activation when grown in contact with an agar matrix (data not shown). This indicates that Cla4p is not required for Cek1p activation under these conditions.

The importance of Rac1p to contact-dependent filamentation highlights a similarity between *C. albicans* and higher eukaryotes. In mammalian cells, Rac1p contributes to formation of lamellipodia by interacting with the actin cytoskeleton promote reorganization (Bosco 2009). Additionally, deregulation of Rac1p can increase invasiveness of cancer cells, not unlike promoting filamentation in yeast cells (Michiels 1995). Interactions between Rac1p and the cytoskeleton in yeast would be an interesting new direction for research.

In mammalian cells, Rac1p is activated by another GTPase, RhoG (Katoh 2003). There is no known RhoG homolog in *Candida albicans*, but another GTPase, Rsr1p, performs a similar function for the GTPase Cdc42p (Pulver 2012). Given that there are fewer G-proteins in yeast cells compared to mammalian cells, it is possible that elements of regulation that are specific to one protein or pathway in higher eukaryotes are shared in yeast cells. However, cells deleted for *rsr1* showed no defect in Cek1p activation, suggesting that it does not serve as the activating GTPase for Rac1p (data not shown). It would be interesting to know whether *Ca*Rac1p is able to act independently of an upstream GTPase.

In conclusion, this study demonstrates that Cek1p signaling in *Candida albicans* is complex and involves homologs of protein components from both *Saccharomyces* and higher eukaryotes. While crosstalk between MAPK modules is common, during growth in contact with an agar matrix, the SVG MAPK pathway

from the MAPKKK to the MAPK appears to function linearly. An interesting area for further study is the multiple inputs upstream of the core MAPK cascade, at the levels of Cst20p, Rac1p, or Dfi1p.

Materials and Methods

Growth Conditions and Strains

All strains used are detailed in table 1. *Candida albicans* cells were routinely cultured using YPD (1% Yeast Extract, 2% Peptone, 2% glucose) at 30°C. Several mutants were purchased as part of a deletion collection (Noble, French, Kohn, Chen, & Johnson, 2010b). Deletions in strains from the collection were confirmed by PCR. Additional *cst20* and *hst7* null mutant strains were kindly provided by the Whiteway lab (C Csank *et al*., 1997). The *rac1*, *dck1*, and *lmo1* deletion mutants and the *RAC1* C-terminal tail mutants were previously described (Hope *et al*., 2010; Vauchelles et al., 2010). The *dfi1* null RACQ61L mutant strain was constructed by transforming pcz5 with pExp-*PADHrac[Q61L]* (Hope *et al*., 2008).

Growth on agar medium

Growth in contact with an agar matrix was carried out as previously described (Brown Jr *et al*., 1999; Zucchi *et al*., 2010). Briefly, overnight cultures of *C. albicans*, grown at 30°C, were diluted 1:2500 and grown for 4 hours at 30°C on a culture wheel. Cells were plated for single colonies on yeast peptone sucrose (YPS) with 1%

agar. Plates were incubated at 25°C. After 4 days, colonies were collected by washing the plates with PBS. The PBS and cell mixture was combined with RNAlater and stored at -80°C.

Protein Extraction

Cells were poured over ice in conical tubes and collected via centrifugation. Cells were then resuspended in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (50 mM tris pH 8, 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% Na deoxycholate, 20 mM NaF, 10 mM Na orthovanadate, 50 mM β-glycerol phosphate, 50 mM Na pyrophosphate, 2 mM PMSF, 10 µl/mL fungal specific protease inhibitor (Sigma P8215), 0.1 Complete tablet (Roche 04693116001)/mL). Protein was extracted using 0.5mm zirconia silica beads and a Turbomix vortex attachment (Fisher) with six rounds of 30 seconds of vortexing followed by 1 minute on ice. Extracts were centrifuged for 10 minutes at maximum speed in a chilled microcentrifuge. Extracts were further clarified by an additional 1 mintute of centrifugation in a new tube. The final supernatants were stored in aliquots at -80°C. Concentration of protein in each sample was determined using the Pierce micro BCA kit (Pierce 23235).

Western blotting

60µg of protein was applied to each lane of an SDS-PAGE gel. Analysis was done using either 7.5 or 8.5 percent polyacrylamide gels, which were run for approximately 7 hours. Proteins were then transferred to a 0.2um PVDF membrane, which was blocked with 5% nonfat milk reconstituted in TBS 0.05% Tween-20 and probed with anti-p42/44 (Cek1p-Pi_2, Cell signaling 4370, 1:1000) or anti tubulin (BioRad MCA78G) incubated overnight at 4°C. HRP-conjugated goat anti-rabbit (phospho-MAPK, Invitrogen 656120) or HRP-conjugated goat anti-rat (Tubulin, Invitrogen 629520) was used as secondary antibody. Pierce ECL Western blotting substrate (32106) was used to produce the signal, which was detected on a Syngene G:Box Chemi-XT4 GENE*sys* imager. Blots were quantified using GeneTools (Syngene, version 1.5.7.0) using a standard curve run on each gel.

Table 2.1 Strains used in this study

Strain	Description	Genotype	Source
pcz1	Wildtype	SC5314ura3Δ::imm434/ura3Δ::imm434 ura3Δ::imm434/URA3	(Zucchi *et al*., 2010)
pcz5	*dfi1* null	pcz1, *dfi1Δ*::FRT/*dfi1Δ*::FRT	(Zucchi *et al*., 2010)
SN250	Wildtype (Noble)	*his1Δ/his1Δ, leu1Δ::C.dubliniensis HIS1/leu2Δ::C.maltose LEU2, arg4Δ/arg4Δ URA3/ura3Δ::imm^{434} IRO1/iro1Δ::imm^{434}*	(Noble *et al*., 2010b)
cek1	*cek1* null	SN250 *cek1Δ::ARG4/cek1Δ::URA3*	(Noble *et al*., 2010b)

cpp1	*cpp1* null	SN250 *cpp1Δ::ARG4/cpp1Δ::URA3*	(Noble *et al.*, 2010b)
CK43B-16	*cek1* null	*ura3/ura3 cek1Δ::hisG-URA3-hisG/cek1Δ::hisG*	(Csilla Csank et al., 1998)
29-1-7+	*cpp1* null	*cpp1Δ::hisG/cpp1Δ::hisG-URA3-hisG*	(C Csank *et al.*, 1997)
BWP17	Wildtype (Hope, Vauchelles)	*ura3Δ::λimm434/ ura3Δ::λimm434 his1Δ::hisG/ his1Δ::hisG arg4Δ::hisG/arg4ΔhisG*	(Wilson, Davis, & Mitchell, 1999)
PY189	*rac1* null, Arg-	Same as BWP17 with *rac1Δ::URA3/rac1Δ::HIS1 arg4Δ::hisG/arg4Δ::hisG*	(Bassilana & Arkowitz, 2006)
PY191	*rac1* null, Arg+	*Same as BWP17 with rac1Δ::URA3/rac1Δ::HIS1 RP10::ARG4*	(Bassilana & Arkowitz, 2006)
PY706	*dck1* null	*Same as BWP17 with dck1Δ::HIS1/dck1Δ::URA3 RP10::ARG4*	(Hope *et al.*, 2008)
PY1009	*lmo1* null	*Same as BWP17 with lmo1Δ-2::HIS1/lmo1Δ-2::URA3 RP10::ARG4*	(Hope *et al.*, 2010)
PY275	*RAC1* complemented strain	PY189 with *RP10::ARG4-PRAC1RAC1*	(Bassilana & Arkowitz, 2006)
PY406	Strain encoding Rac1p defective for geranylgeranylation	PY189 with *RP10::ARG4-PRAC1rac1[C233S]*	(Vauchelles *et al., 2010)*
PY534	Strain encoding Rac1p with a mutated polybasic region	PY189 with *RP10::ARG4-PRAC1rac1-5Q*	(Vauchelles *et al.*, 2010)
PY2156	Strain encoding Rac1p with a C-terminal deletion	PY189 with *RP10::ARG4-PRAC1rac1Δ181-221*	This Study
RAC1Q61L	Strain encoding constitutively active Rac1p	pcz5 RP10::ARG4-PADHrac1Q61L	This study
cst20	*cst20* null	SN250 *cst20Δ::ARG4/cst20Δ::URA3*	(Noble *et al.*, 2010b)
ste11	*ste11* null	SN250 *ste11Δ::ARG4/ste11Δ::URA3*	(Noble *et al.*, 2010b)

hst7	*hst7* null	SN250 *hst7Δ::ARG4/hst7Δ::URA3*	(Noble *et al.*, 2010b)
CDH22	*cst20* null	*ura3/ura3 cst20Δ::hisG-URA3-hisG/cst20Δ::hisG*	(Leberer *et al.*, 1996)
CDH9	*hst7* null	*ura3/ura3 hst7Δ::hisG-URA3-hisG/hst7Δ::hisG*	(Leberer *et al.*, 1996)

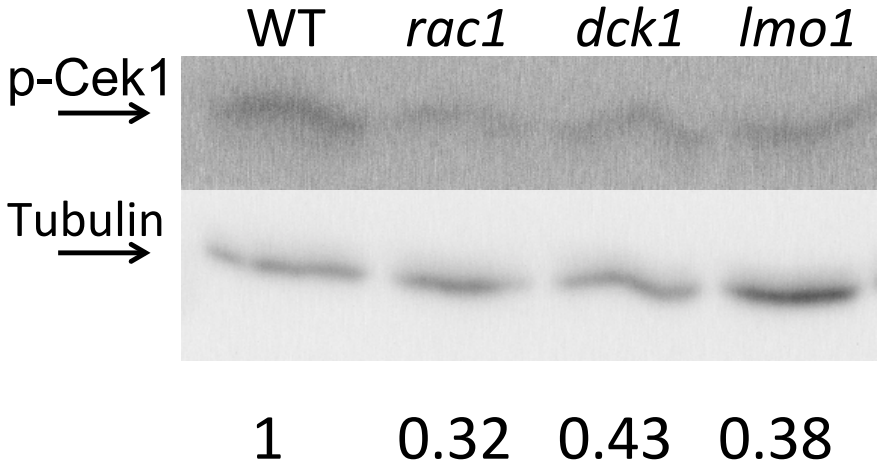

Figure 2.1 Rac1p is required for wildtype levels of Cek1p activation in *Candida albicans* during growth in contact with an agar matrix. Cells of the relevant genotypes were plated on YPS 1 percent agar and grown for 4 days at 25°C. On the fourth day cells were washed from the surface of the plates with cold PBS, harvested, and extracted by bead beating. The extracts were probed for phosphorylated MAPK (upper blot) and tubulin. Values below the blot indicate normalization to tubulin and then wildtype. Experiments were performed 3 times and a representative blot is shown.

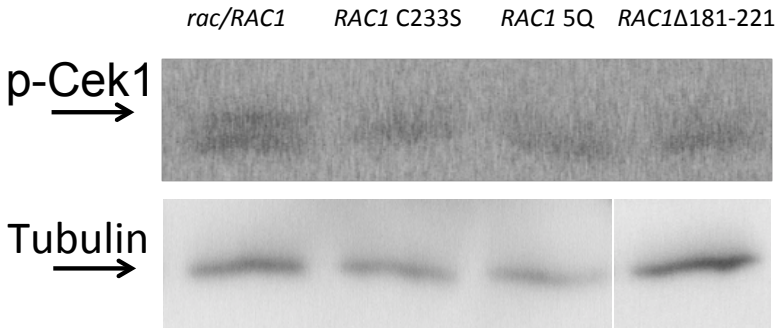

Figure 2.2 Mutation of the C-terminus of Rac1p results in decreased Cek1p activation as compared to wildtype when grown in contact with an agar matrix. Strains of the relevant genotypes were grown for 4 days at 25°C on YPS with 1 percent agar. On the fourth day, cells were washed from the agar with cold PBS, harvested, and extracted with bead beating. 60µg total protein were applied to an SDS PAGE gel and probed for activated MAPK (top blot) and tubulin (lower blot). All lanes were run on the same gel. Representative image shown.

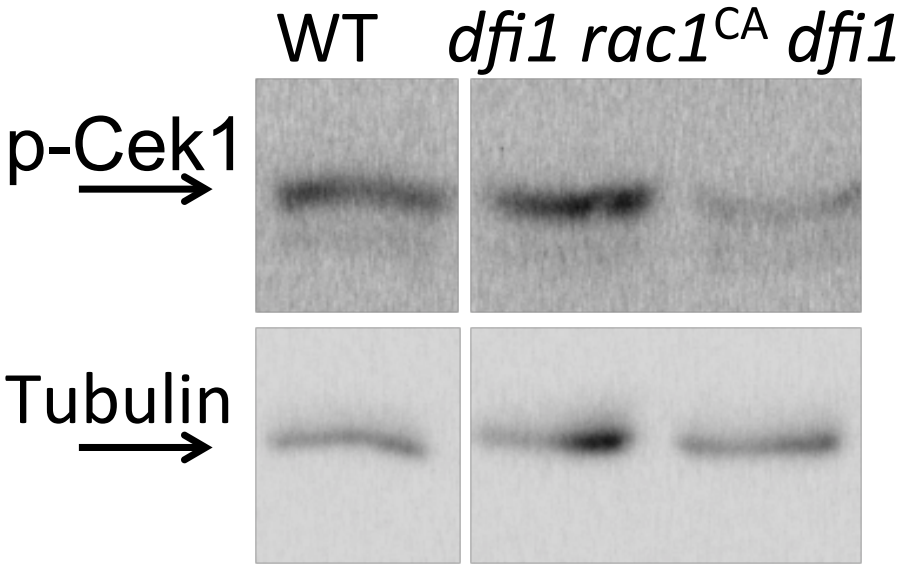

Figure 2.3. Mutants of Rac1p can alter Cek1p activation with respect to Dfi1p in plated cells. Cells were plated on 1 percent agar and grown for 4 days at 25°C. On the fourth day cells were washed from the surface of the plates with cold PBS, harvested, and extracted by bead beating. The extracts were probed for phosphorylated MAPK (MAPK-P) and Tubulin. All lanes were run on the same day with irrelevant lanes removed. Representative result of 3 repeats.

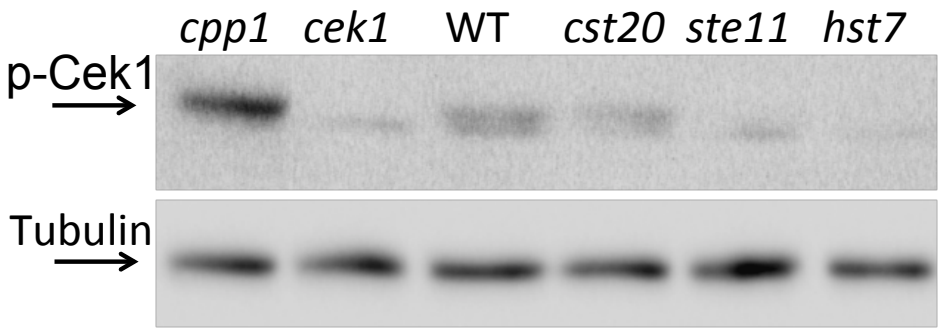

Figure 2.4 Members of the Cek1p MAPK cascade are required for wildtype levels of Cek1p activation in *Candida albicans* during growth in contact with an agar matrix. Cells were plated on 1 percent agar and grown for 4 days at 25°C. On the fourth day cells were washed from the surface of the plates with cold PBS, harvested, and extracted with bead beating. The extracts were probed for phosphorylated MAPK (upper blot) and tubulin, with extracts from the *cpp1* null and *cek1* null as positive and negative controls, respectively, for Cek1p activation. Representative result of 3 repeats is shown.

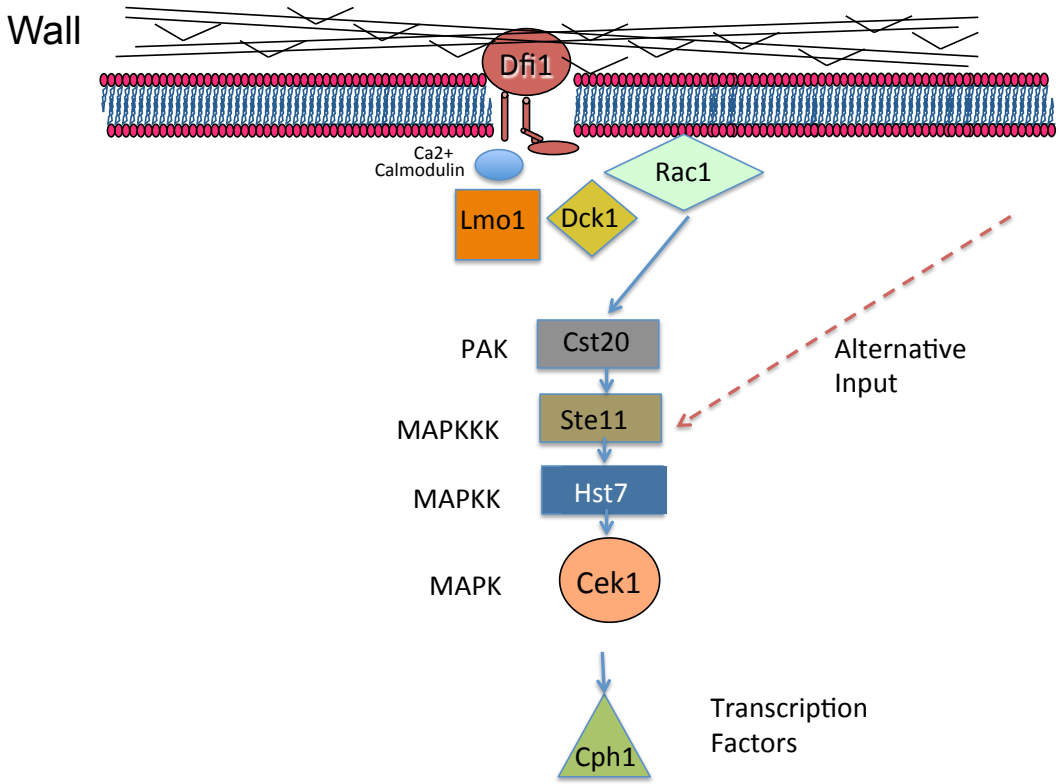

Figure 2.5 Model of Dfi1p-dependent signaling to Cek1p. Calcium-bound calmodulin interacts with the C-terminal tail of Dfi1p upon Dfi1p activation. This leads to activation of Dck1p, which, with Lmo1p, activates Rac1p. Rac1p activates Cst20p, which activates Ste11p. Ste11p activates Hst7p, which activates Cek1p. One potential transcriptional output of this pathway is Cph1p. There is another input above the level of MAPKKK (dotted line), as Ste11p and Hst7p are required for Cek1p activation, but Cst20p is only partly required.

Chapter 3

Contact-dependent Signaling Activates a Subset of the Sef1p Regulon in *Candida albicans* [+]

[+] Weeks, AE and Kumamoto CA. To be submitted to mSphere

Abstract

Candida albicans is the leading cause of fungal bloodstream infections. This important opportunistic pathogen benefits from its ability to change its morphological form; the transition between two such forms, yeast and hyphae, is linked to virulence. This transition can occur in response to many environmental cues, including growth in contact with agar medium. The protein Dfi1p is required for filamentation during growth in contact with agar and contributes to virulence of *C. albicans*. Dfi1p signaling is transmitted by the MAP kinase Cek1p. In this study we describe the identification of several genes regulated downstream of Dfi1p. Some of these genes belong to the Sef1p regulon, which responds to low iron availability. We report here a connection between contact-dependent signaling and signaling typically associated with the response to iron deprivation. This subset of genes could promote virulence of *C. albicans* in response to signals sensed by Dfi1p.

Importance

Candida albicans causes a variety of diseases in humans, ranging from superficial infections of the skin or mouth to bloodstream infections that can be fatal. The protein Dfi1p is needed to cause expected levels of disease in a mouse model. Dfi1p signaling appears to be important for causing disease, so we sought to identify gene targets of Dfi1p signaling. Understanding how Dfi1p contributes to *C. albicans* virulence could allow for new ways of preventing disease caused by *C. albicans*. This study demonstrates a link between Dfi1p signaling and signaling that is usually seen

in a low iron environment, like most of the human body. Several genes identified in this study could contribute to the ability of *C. albicans* to cause disease.

Introduction

Sensing and responding to the environment is critical to the survival of any organism. Free-living organisms must adapt to various environmental conditions and stresses, while organisms associated with a host may be additionally exposed to host-specific stresses, and must modify themselves accordingly. For opportunistic pathogens, sensing the environment can provide crucial information about when to shift from commensal growth to pathogenicity. One such opportunistic pathogen is the diploid yeast *Candida albicans*.

Candida albicans is a common colonizer of human mucosal surfaces and the fourth leading cause of hospital acquired bloodstream infections (J. Kim & Sudbery, 2011). One of the yeast's important virulence factors is its ability to change its morphology from ovoid budding yeast to elongated hyphae (P. E. Sudbery, 2011). This transition can occur in response to many stimuli, one of which is growth in contact with an agar matrix. In the context of agar invasion, the protein Dfi1p is required for wildtype levels of filamentation, although Dfi1p is not required for responding to other types of cues, such as nutritional cues (Zucchi *et al*., 2010).

Dfi1p is an integral membrane protein whose C-terminal tail interacts with calcium bound calmodulin. Dfi1p signals through the mitogen activated protein kinase Cek1p when cells are growing in contact with agar medium (Davis *et al*., 2013; Zucch*i et al.*, 2010). An alternative way to activate Cek1p in a Dfi1p-

dependent manner is to increase cytosolic calcium by treating cells with calcium ionophore. In the presence of ionophore, Dfi1p-dependent activation of Cek1p can be observed in cells growing in liquid medium (Davis *et a*l., 2013).

Deletion of *DFI1* attenuates *C. albicans* virulence in an intravenous infection model (Zucchi *et al*., 2010). Therefore, the Dfi1p signaling pathway contributes to the ability of *C. albicans* to cause a lethal infection. We hypothesized that identifying genes whose transcription responds to Dfi1p-dependent Cek1p activation would give insight into virulence-promoting processes that are activated via Dfi1p signaling.

In this study, we performed an RNA-seq analysis to gain an unbiased view of transcriptional changes that occur in cells in which Cek1p has been activated in a Dfi1p-dependent manner. This analysis led to the identification of several genes whose up-regulation depended upon Cek1p activation and the presence of Dfi1p. Some of these genes were also dependent upon Dfi1p in cells grown on soft agar plates. Genes that were up-regulated in a Dfi1p-dependent manner were members of the Sef1p regulon. Sef1p is known for regulating the transcriptional response to an iron deficient environment. Sef1p is also required for virulence in the intravenously inoculated mouse model and its target genes likely promote the virulence of *C. albicans* in this niche (C. Chen *et al.*, 2011). The results of this study highlight an intersection between the response to signaling during embedded growth and response to growth in low iron.

Materials and Methods

Strains and Growth Conditions

The strains used in this study are detailed in Table 1. *C. albicans* cells were cultured in Yeast Peptone Dextrose (YPD) medium (1% Yeast Extract, 2% Peptone, 2% glucose), either as liquid medium or with 2 percent agar. In some studies, cells were grown in complete minimal medium minus uridine (CM-U) (Treco & Lundblad, 1993) or Yeast Peptone Sucrose (YPS) medium (1% Yeast Extract, 2% Peptone, 2% sucrose). Cells were routinely cultured at 30°C or 25°C.

Ionophore Treatment

Ionophore treatment was performed as previously described (Davis *et al.*, 2013). Briefly, cells were grown for 8 hours at 30°C in YPD over the day, diluted 1:1000 or 1:2000 and grown overnight. When cultures had reached an OD600 of 0.75-1.5, the calcium ionophore A23187 (Sigma) was added to a final concentration of 4μM, or a vehicle control of ethanol. Ionophore exposure was allowed to proceed for 30 minutes or 1 hour before cells were collected.

Growth on agar medium

Growth in contact with an agar matrix was carried out as previously described (Brown Jr *et al.*, 1999; Zucchi *et al.*, 2010). Briefly, overnight cultures of *C. albicans*,

grown at 30°C, were diluted 1:2500 and grown for 4 hours at 30°C on a culture wheel. Cells were plated for single colonies on yeast peptone sucrose (YPS) with 1% agar. Plates were incubated at 25°C. After 4 days, colonies were collected by washing the plates with PBS. The PBS and cell mixture was combined with RNAlater and stored at -80°C.

Growth in Low Iron Medium

Growth of cells in low iron medium was done as previously described (C. Chen *et al.*, 2011). Briefly, log phase cells, grown in YPD at 30°C, were diluted 1:10,000 into either YPD (high iron medium) or YPD with 500μM bathophenanthroline disulfonic acid (BPS) an iron chelator. Cells were incubated in this medium for 6 hours at 30°C and were collected via centrifugation. Pellets were resuspended in RNAlater and stored at -80°C.

Protein Extraction

Cells were poured over ice in conical tubes and collected via centrifugation. Cells were then resuspended in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (50 mM tris pH 8, 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% Na deoxycholate, 20 mM NaF, 10 mM Na orthovanadate, 50 mM β-glycerol phosphate, 50 mM Na pyrophosphate, 2 mM PMSF, 10 μl/mL fungal specific protease inhibitor (Sigma P8215), 0.1 Complete tablet (Roche 04693116001)/mL).

Protein was extracted using 0.5mm zirconia silica beads and a Turbomix vortex attachment (Fisher) with six rounds of 30 seconds of vortexing, each followed by 1 minute on ice. Extracts were centrifuged for 10 minutes at maximum speed in a chilled microcentrifuge. Extracts were further clarified by an additional 1 minute of centrifugation in a new tube. The final supernatants were stored in aliquots at -80°C. Concentration of protein in each sample was determined using the Pierce micro BCA kit (Pierce 23235).

Western blotting

60µg of protein was applied to each lane of an SDS-PAGE gel. Analysis was done using either 7.5 or 8.5 percent polyacrylamide gels, which were run for approximately 7 hours. Proteins were then transferred to a 0.2um PVDF membrane, which was blocked with 5% nonfat milk reconstituted in TBS 0.05% Tween-20 and probed with anti-p42/44 (Cek1p-Pi_2, Cell signaling 4370, 1:1000) or anti tubulin (BioRad MCA78G) incubated overnight at 4°C. HRP-conjugated goat anti-rabbit (phospho-MAPK, Invitrogen 656120) or HRP-conjugated goat anti-rat (Tubulin, Invitrogen 629520) was used as secondary antibody. Pierce ECL Western blotting substrate (32106) was used to produce the signal, which was detected on a Syngene G:Box Chemi-XT4 GENE*sys* imager. Blots were quantified using GeneTools (Syngene, version 1.5.7.0) using a standard curve run on each gel.

RNA extraction

RNA was extracted using beadbeating (Minibeadbeater 24, Biospec), and the Qiagen RNeasy kit. Briefly, cells stored at -80°C were thawed on ice, collected by centrifugation, and resuspended in buffer RLT with added beta mercaptoethanol (BME). This suspension was added to screw cap tubes with baked 0.5mm silica zirconia beads; RLT-BME was added until the tubes were full. Three rounds of bead beating for 1 minute and cooling on ice for at least one minute were conducted. Lysates were collected and treated with the Qiagen RNeasy kit, including RNeasy DNAse treatment. RNA was stored at -80°C until converting into cDNA using Superscript III Reverse Transcriptase with oligo dT priming. cDNA was stored at -20°C.

RNA Sequencing

RNA sequencing of ionophore-treated and vehicle control treated wildtype and *dfi1* null mutant strains was done as follows. Three biological replicates were generated per sample. Libraries were constructed using 1 µg of RNA and the Illumina TruSeq RNA library preparation kit by the Tufts University Genomics core facility. Sequencing was performed with the HiSeq 2500 instrument. Prepared libraries were mixed prior to sequencing and distributed evenly between two sequencing lanes. Single-end 50bp reads were sequenced. The sequencing data was analyzed as

previously described using the Tuxedo Suite (Trapnell *et al.*, 2012). Reads were aligned to the *Candida albicans* genome, modified from the Candida Genome Database (CDG), with Bowtie, using standard parameters. Differential gene expression was examined using CuffDiff. Cuffdiff was run using aligned reads from Bowtie and an annotation file of C. albicans genes. This annotation file was modified from CDG, as well. Genes with a fold change over 2 were considered.

RT-qPCR

cDNA was diluted 1:20 prior to use. q-PCR reactions were set up using Sybr green mastermix. A LightCycler480 machine was used for q-PCR. Primers used for q-PCR are listed in table 2.

Transcription Factor Binding Site Analysis

in silico searches for transcription factor binding motifs were done in two ways. The program FIMO was used to search for Czf1p binding sites (Grant, Bailey, & Noble, 2011). Promoter regions were derived from the Upstream Regions: Fungal option in FIMO, further selecting *Candida albicans*. Searches for the conserved Ste12p binding site was done by scanning individual promoter sequences for the binding motif. Promoter sequences for analyses were obtained from the Candida genome database (CDG) (Skrzypek *et al*., 2017).

Results

RNAseq analysis of gene expression in response to Dfi1p-dependent Cek1p activation

In order to identify genes whose expression responds when Cek1p is activated in a Dfi1p-dependent manner, an RNAseq procedure was conducted. The premise of this experiment was to take advantage of the fact that treatment with the ionophore A23187 resulted in Dfi1p-dependent Cek1p activation in a liquid culture. Wildtype and *dfi1* null mutant cells were treated with A23187 or vehicle control. After thirty minutes of treatment, a portion of cells were collected, preserved in RNAlater, and stored at -80°C. Protein was also extracted from another portion of the culture and extracts were analyzed for the presence of activated Cek1p by Western blot using anti-p42/44 antibody (Cell Signaling). Figure 3.1 lane 1 shows activation of Cek1p in a *cpp1* null mutant grown in contact with an agar matrix; this is a positive control for Cek1p activation. Cpp1p is a phosphatase that removes the phosphate from Cek1p, so that activated Cek1p accumulates in the cell in the absence of Cpp1p (C Csank *et al*., 1997). Figure 3.1 lane 2 shows the absence of phosphorylated Cek1p in a *cek1* null. This is a negative control for Cek1p activation. Lane 3 shows activation of Cek1p in wildtype cells treated with ionophore. Lane 4 shows the level of activation of Cek1p in wildtype cells treated with a vehicle control. Lanes 5 and 6 show the level of Cek1p activation in *dfi1* null mutant cells treated with ionophore and treated with a sham induction, respectively. All

experiments used for the RNA-seq study (3 biological replicates for each condition) showed this pattern of Cek1p activation.

RNA was extracted using the RNeasy kit (Qiagen) and libraries were prepared using the Illumina TruSeq RNA library preparation kit. Sequencing was performed with the HiSeq 2500 instrument. The sequencing data was analyzed using the Tuxedo Suite as described in materials and methods (Trapnell *et al*., 2012). Briefly, reads were mapped with Bowtie and differential gene expression was examined using CuffDiff.

We focused on genes whose expression was higher in wildtype cells treated with ionophore as compared to both wildtype cells treated with a sham induction and *dfi1* null mutant cells treated with ionophore. Ionophore treatment had a large effect on cells and 117 genes were more highly expressed in ionophore-treated wildtype cells compared to vehicle-treated wildtype cells. A smaller group of genes (35) were more highly expressed in the ionophore-treated wildtype cells compared to ionophore-treated *dfi1* null mutant cells. The overlap between these groups, 12 genes, were candidates for targets of the Dfi1p-dependent signaling pathway (Table 3).

To verify that these genes showed differential expression, cultures of wildtype or *dfi1* null cells were treated with ionophore or vehicle, RNA was extracted and quantitative reverse transcription PCR (RT-qPCR) was used to measure expression of the candidate genes. 6 of the 12 genes showed a Dfi1p-dependent response to ionophore. We decided to focus on three genes: *SOD4*, *BMT9*, and *OPT1* (Figure 3.2) for further study.

SOD4 and *BMT9* encode activities that affect host-pathogen interactions. *SOD4* encodes a Cu/Zn superoxide dismutase in the Sod1, 4, 5, and 6 family (Frohner, Bourgeois, Yatsyk, Majer, & Kuchler, 2009). Sod4p is a GPI-linked protein localized to the cell surface (de Groot *et al*., 2004). Sod4p works with Sod5p to protect *C. albicans* from reactive oxygen species (ROS) generated by macrophages *in vitro* (Frohner *et al*., 2009). Protection from ROS is crucial for survival in the host. *BMT9* encodes a beta mannosyltransferase. This class of proteins adds β-1-2-mannans to cell wall proteins and lipids. β-1-2-mannans modulate cytokine responses to *C. albicans* and promote adherence to epithelial cells (Dalle *et al*., 2003; Ueno *et al*., 2013). Little study of *BMT9* has been done to date, but *BMT9* is highly similar to *BMT1* (C. Mille *et al.*, 2012). Bmt1p adds B-1,2-mannosides to the acid stable fraction of cell wall proteins (Céline Mille *et al.*, 2008). *OPT1* encodes an oligopeptide transporter that transports peptides 3-5 amino acids in length, and is capable of transporting peptides up to 8 amino acids in length (Lubkowitz, Hauser, Breslav, Naider, & Becker, 1997; Reuss & Morschhauser, 2006). *OPT1* does not appear to directly influence virulence (Reuss & Morschhauser, 2006). Up-regulation of these target genes as a result of Dfi1p-dependent signaling may contribute to *C. albicans* pathogenicity.

Dfi1p-dependent gene expression during growth in contact with agar medium

The use of calcium ionophore to activate Cek1p in a Dfi1p-dependent manner was devised as a way of mimicking Dfi1p signaling during growth in contact with

agar. We hypothesized that expression of the genes of interest would depend upon Dfi1p when cells were grown in contact with an agar matrix. To determine whether the regulation of the genes of interest was dependent upon Dfi1p in plated conditions, wildtype and *dfi1* null cells were grown on YPS with 1% agar for 4 days at 25°C. Cells were harvested with PBS and RNA was extracted using the Qiagen RNeasy kit, including DNAse treatment, as described in materials and methods.

RT-qPCR analysis of the genes of interest showed that *BMT9* and *OPT1* required Dfi1p for wildtype levels of expression during growth in contact with an agar matrix. *SOD4* did not require Dfi1p for expression under the same conditions (Figure 3.3). These results support the model that at least *BMT9* and *OPT1* represent targets of the Dfi1p-dependent signaling pathway.

Genes of Interest Belong to the Sef1p regulon

The three genes identified as potential targets of the Dfi1p-dependent signaling pathway were previously shown to be members of the Sef1p regulon (C. Chen *et al.*, 2011). Sef1p is a zinc finger transcription factor whose role is to stimulate transcription of iron uptake and other genes during growth in low iron environments. *SOD4* and *OPT1* were identified as members of the Sef1p regulon, and their promoters are bound by Sef1p. *BMT9* is not directly regulated by Sef1p, but is up-regulated by the CCAAT binding protein (CBP) complex, which is active during low iron growth. Sef1p up-regulates the expression of two members of the CCAAT binding complex, Hap43 and Hap3, which form a complex with the other two

members, Hap2 and Hap5. The CBP complex upregulates *BMT9* and other genes (C. Chen *et al*., 2011; Hsu *et al*., 2011). These findings suggest an overlap between iron regulation and contact-dependent signaling.

Sef1p is required for wildtype expression of genes of interest

The genes of interest are regulated by Sef1p during growth in iron-poor conditions. Therefore, the role of Sef1p in transcription during ionophore treatment was assessed by growing a *sef1* null strain and its congenic wildtype strain and treating with ionophore, as described in the materials and methods section. Cells were incubated at 25°C and samples were taken after 30 minutes and 1 hour.

RNA was extracted and RT-qPCR analysis of the samples showed that wildtype up-regulation due to ionophore treatment for *SOD4* and *BMT9* required Sef1p. *OPT1*, by contrast, did not require Sef1p for expression (Figure 3.4).

We hypothesized that Sef1p would also be needed for expression of the genes of interest in cells grown on agar plates. Cells were grown at 25°C for 4 days, cells were harvested, and RNA was extracted. RT-qPCR analysis showed that *SOD4*, but not *BMT9* or *OPT1* required Sef1p for wildtype levels of expression (Figure 3.5). Thus, in cells grown on agar, *SOD4* expression required Sef1p, and *BMT9* and *OPT1* expression required the Dfi1p-signaling pathway.

Dfi1p is not required for transcriptional response to low iron

Since both Sef1p and Dfi1p are required for up-regulation of the genes of interest in cells growing with ionophore, we hypothesized that Dfi1p might also participate in regulating the expression of the genes of interest in low iron. To address this hypothesis, we grew *sef1* null cells and a congenic wildtype strain in low iron medium as described in (C. Chen *et al*., 2011). Cells were grown in YPD medium with or without 500μM of the anionic iron chelator, bathophenanthroline disulfonic acid (BPS) for 6 hours. Cells were harvested and RNA was extracted. RT-qPCR analysis of the genes of interest showed that transcription of *BMT9* and *SOD4* increased in the low iron condition. This increase in transcription occurred in both the wildtype and the *dfi1* null strain ($p<0.001$ for all), indicating that Dfi1p is not required for the response to low iron (Figure 3.6). *OPT1* expression showed a different pattern. *OPT1* transcription levels decreased upon iron starvation, and transcription levels were not significantly different in *dfi1* null cells compared to wildtype cells in either low iron or high iron conditions (data not shown). Therefore, Sef1p-dependent gene expression in low iron does not require Dfi1p.

Activation of Czf1p1 results in upregulation of *SOD4* and *OPT1*

The transcription factors Czf1p and Cph1p promote filamentation in cells growing in contact with an agar matrix. The Ste12p-homolog Cph1p is the predicted output of the Cek1p-MAPK pathway. Czf1p is a transcription factor known for its

role in embedded filamentation and white opaque switching (Brown Jr *et al*., 1999; Hernday *et al*., 2013; Petrovska & Kumamoto, 2012). It is a zinc finger protein in the same family as Gal4p and Sef1p (Schillig & Morschhäuser, 2013a). *in silico* analysis of promoter regions of genes of interest did not show the conserved Ste12p binding motif (TGAAACA) (Wong Sak Hoi & Dumas, 2010). Instead, analysis of promoter sequences via FIMO, part of the MEME suite of analysis tools, showed a binding site for Czf1p (TTWRSCGCCG) upstream of *SOD4* and *OPT1* (Grant et al., 2011; Hernday et al., 2013).

To further explore the role of Czf1p, we took advantage of a construct in which Czf1p was artificially activated by fusion to the Gal4 activating domain (Schillig & Morschhäuser, 2013a). This hybrid protein is expressed under control of the *ADH1* promoter, allowing for expression that is not subject to the complex timing of native *CZF1* expression (Vinces *et al.*, 2006).

To measure transcript levels in this strain and its congenic wildtype, cells were grown as described for the ionophore treatment, but ionophore was not added. Cells were collected by centrifugation and prepared for subsequent RNA extraction. RT-qPCR analysis of expression of genes of interest showed that *SOD4* and *OPT1* were up-regulated in cells carrying the CZF1-GAL4AD fusion (figure 3.7, $p<0.05$). *BMT9* was not up-regulated by activated Czf1p (data not shown). Therefore, as a regulator of embedded filamentation, Czf1p, is able to up-regulate the expression of two members of the Sef1p regulon.

Sef1p does not regulate *CZF1* expression

Sef1p up-regulates the expression of the transcription factor Hap43p, and therefore we tested for regulation of *CZF1* by Sef1p. Expression of *CZF1* in wildtype and *sef1* null cells grown in contact with agar was measured. RT-qPCR analysis showed no change in *CZF1* expression in the *sef1* null strain as compared to the wildtype (Figure 3.8). Therefore *CZF1* does not require Sef1p for expression under these conditions.

Discussion

The goal of this study was to understand how the Dfi1p signaling pathway contributes to the virulence of *C. albicans*. We show that Dfi1p-dependent signaling influences the expression of a subset of the Sef1p regulon. Dfi1p is not required, however, for up-regulation of genes in response to low iron, which is the primary function of the Sef1p regulon. Further, other genes that are up-regulated by low iron (C. Chen *et al.*, 2011) were not up-regulated by ionophore treatment. Therefore, ionophore exposure did not merely produce or mimic a low iron environment.

The identity of targets of *dfi1* signaling may shed light on why Dfi1p is required for full virulence in a model where you would expect other cues for filamentation to be more than sufficient. The three targets described in this work could all contribute to fitness in an animal host.

The role Sod4p would play in virulence is straightforward. Superoxide dismutases are known to contribute to virulence (Broxton & Culotta, 2016). The superoxide dismutase would be predicted to help *C. albicans* survive reactive oxygen species (ROS) it would encounter in the host. The related superoxide dismutase Sod5p also promotes GI colonization (Pierce, Dignard, Whiteway, & Kumamoto, 2013). Sod4p and Sod5p work together in vitro to protect *C. albicans* from macrophage-derived ROS (Frohner *et al*., 2009). The role of Bmt9p as a beta mannosyltransferase could contribute to host-pathogen interactions because B-1,2-mannosides increase adherence of the fungal cell, which would be useful for sticking to a surface that *C. albicans* had encountered and was about to invade (Dalle *et al*., 2003). B-1,2-mannosides expression also interferes with the host immune response; *Candida c*ells lacking B-1,2-mannosides elicit an increased inflammatory cytokine response (Ueno *et al.*, 2013). However, more work needs to be done to understand the role of BMTs in infection, as cells deleted for *bmt1* show an increase in virulence in intravenous infection in mice (Courjol *et al*., 2015). Peptide transport via Opt1p could help meet the nutritional needs of a cell during tissue invasion. However, a strain deleted for several *OPT* genes is not defective in a mouse intravenous model of infection (Reuss 2006).

A range of transcriptional patterns amongst the three genes of interest was observed, suggesting that regulation during ionophore treatment or growth in contact with an agar matrix is complex. *SOD4* expression was strongly dependent on Sef1p, under most conditions but showed Dfi1p-dependent up-regulation during ionophore exposure. The *SOD4* promoter also contains a binding site for Czf1p.

Expression of *BMT9* is regulated by the Sef1p-controlled factor Hap43p, and also by Dfi1p signaling. *BMT9* was not very responsive to activated Czf1p, suggesting that like Sef1p, the Dfi1p signaling pathway regulates *BMT9* indirectly through another factor. *OPT1*, interestingly, responds strongly to Czf1p activation. Expression of this gene requires Dfi1p during both ionophore treatment and growth in contact with agar. *OPT1* expression was down-regulated during growth in low iron (data not shown). Therefore, the Dfi1p pathway played a larger role in up-regulation of the gene than Sef1p.

Expression of genes of interest during plated growth at low temperature was of great interest, as this is the original condition for which Dfi1p's importance was noted. The complementary observations reported here are intriguing: *BMT9* and *OPT1* require Dfi1p for wildtype expression levels, but not Sef1p, and *SOD4* requires Sef1p, but not Dfi1p.

The embedded growth signaling pathway includes both Czf1p and Dfi1p. Czf1p may act downstream of Dfi1p, although a direct link between the two has yet to be shown. It is possible that Dfi1p is required for activation of Czf1p.

The proposed model for upregulation of these genes is as follows. When commensally growing *C. albicans* grows in contact with a soft surface, like the intestinal epithelium, Dfi1p is activated. Dfi1p activation leads to activation of Cek1p and possibly other signaling pathways. This activation results in activated Czf1p. Activated Czf1p and Sef1p, which is also active under these conditions, upregulate expression of *SOD4* and *OPT1*. Sef1p promotes transcription of *HAP43*, and Hap43p

then upregulates *BMT9.* The candidate gene products are then able to promote virulence.

The combined regulation of these genes by Sef1p and the embedded growth signaling pathway could be a way for *C. albicans* to make sure important virulence factors are produced during growth in a hostile host environment. Sef1p regulation is expected to occur primarily in the bloodstream due to the low amount of iron available, so expression of these genes would occur in that niche. Dfi1p could contribute to up-regulation when the cells are invading tissue, prior to reaching the bloodstream. This dual regulation would ensure expression of these genes when *C. albicans* encounters a hostile host environment.

Table 3.1 Strains used in this study

Strain	Description	Genotype	Source
pcz1	Congenic wildtype of pcz5	SC5314ura3Δ::imm434/ura3Δ::imm434 ura3Δ::imm434/URA3	(Zucchi *et al.*, 2010)
pcz5	*dfi1* null	Pcz1 dfi1Δ::FRT/dfi1Δ:: FR BWP17, *dfi1Δ*::FRT/*dfi1Δ*::FRT, *ura3Δ::imm434/URA3*	(Zucchi *et al.*, 2010)
SN152	Congenic wildtype of *sef1* null	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434	(Noble & Johnson, 2005)
sef1	*sef1* null	SN152 *sef1Δ*::HIS1/*sef1Δ*::LEU2	(Homann et al., 2009)
SC5314	Congenic wildtype of *CZF1*-GAD	Clinical isolate	(Gillum, Tsay, & Kirsch, 1984)
CZF1-GAD	Strain expressing allele encoding artificially activated Czf1p	SC5314 pADH1-*CZF1*-GAD	(Schillig & Morschhäuser, 2013b)

Table 3.2 Primers used in this study.

Primer name	Sequence (5'-3')	Purpose
Bmt9_1726F	TCAGAAACCATGTATGCACCA	qPCR of *BMT9*

Bmt9_1906R	CCACATTTTTCCCCTCACAT	qPCR of *BMT9*
Orf_1225F	GGGGCTTCATTTGAAGGTTT	qPCR of *orf19.7445*
ORF_1301R	CCAAAACGGCCATTGTTATT	qPCR of *orf19.7445*
CSA1_1955F	CAGCTAACGTGCAAACGAGT	qPCR of *CSA1*
CSA1_2042R	GATTCGGAAGCAGAAGCAAC	qPCR of *CSA1*
CFL5_1216F	CCAACAGTTGCTGTTTGGTG	qPCR of *CFL5*
CFL5_1289R	GCCTTAGGGAATCCGAAAAC	qPCR of *CFL5*
AMO1_935F	GTGCTCCAGATTTCCCACAT	qPCR of *AMO1*
AMO_1021R	CCAATGCCAATGGGTTAGTC	qPCR of *AMO1*
GAP2_1452F	TGCTATTTGGGGTGCTTTTT	qPCR of *GAP2*
GAP2_1536R	ACTTGGGGAAGAATGCAATG	qPCR of GAP2
NUP_915F	ATTGGCTAAACATGGCTTGG	qPCR of NUP
NUP_1002R	TGACATTGATGGAGGTGGTC	qPCR of NUP
OPT1_1724F	CGCAGTTTGCTGCTACCATA	qPCR of *OPT1*
OPT_1804R	ACAAATTGTCGATTGCACCA	qPCR of *OPT1*
ORF19.6017_1328F	ATCATCATGGAGGGGTTCTG	qPCR of *orf19.6017*
ORF19.6017_1411R	CTTCATCGTCGTCATCCTCA	qPCR of *orf19.6017*
SOD4_236F	AGCCAGTTCCAGAATCCAAA	qPCR of *SOD4*
SOD4_322R	CAGCTGGAGTTTTGGCAGTA	qPCR of *SOD4*
ACT1_163F	GTTGGTGATGAAGCCCAATC	qPCR of *ACT1*
ACT1_241R	CCCAGTTGGAAACAATACCG	qPCR of *ACT1*
CZF1_735F	TCCATCAGAGCAGCAACAAG	qPCR of *CZF1*

CZF1_864R	TGGAACCTGTGGTGTTTCAG	qPCR of *CZF1*

Table 3.3 Genes of interest and results of RT-qPCR follow up. Genes listed were more highly expressed in ionophore-treated wildtype cells compared to both ionophore treated *dfi1* null mutant cells and sham treated wildtype cells based on the RNAseq analysis. Genes are listed in alphabetical order according to common name. An asterisk indicates that RT-qPCR analysis of that gene of interest reached statistical significance when comparing expression in ionophore-treated wildtype and expression in ionophore-treated *dfi1* null mutant ($p<0.05$ as determined by T-test).

Gene of Interest	Common name	Upregulated by ionophore in a Dfi1p-dependent manner
orf19.5784	*AMO1*	Yes*
orf19.4673	*BMT9*	Yes*
orf19.7114	*CSA1*	No
orf19.1930	*CFL5*	Yes*
orf19.6993	*GAP2*	No
orf19.4647	*HAP3*	ND
orf19.6570	*NUP*	No
orf19.2602	*OPT1*	Yes*
orf19.6017	*orf19.6017*	Yes *
orf19.6570	*orf19.6570*	ND
orf19.7445	*orf19.7445*	No
orf19.2062	*SOD4*	Yes*

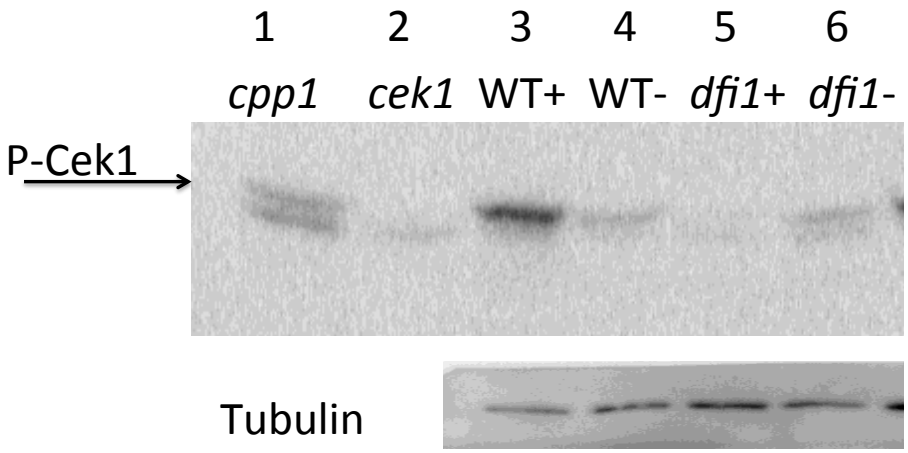

Figure 3.1 Ionophore treatment results in increased Cek1p activation in wildtype cells. Mid-log cultures of *Candida albicans* were treated with 4μM A23187 (denoted by a "+") or with EtOH (vehicle control denoted by a "-"). After 30 minutes of shaking at 25°C, the cells were collected by centrifugation, washed with cold PBS, and extracted by bead beating. 60μg of total protein was loaded on an 8.5% polyacrylamide gel. Blots were probed with p-Cek1p and tubulin antibodies, as described in materials and methods. Extracts from the *cpp1* null and *cek1* null were used as positive and negative controls, respectively, for Cek1p activation. The blot for P-Cek1p is shown at the top of the figure, with the tubulin blot below.

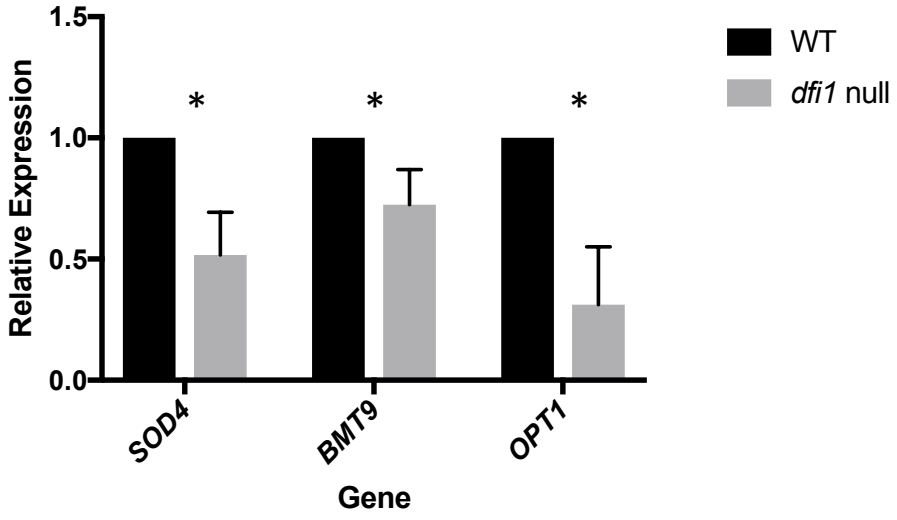

Figure 3.2 *SOD4*, *BMT9*, and *OPT1* are more highly expressed in wildtype cells treated with calcium ionophore than *dfi1* null cells treated the same way. Expression of the genes indicated below was normalized using *ACT1*. The expression in WT+ is set to one and the expression in the *dfi1* null is expressed as a fraction of WT+ expression. Statistical significance was determined by paired T-test.

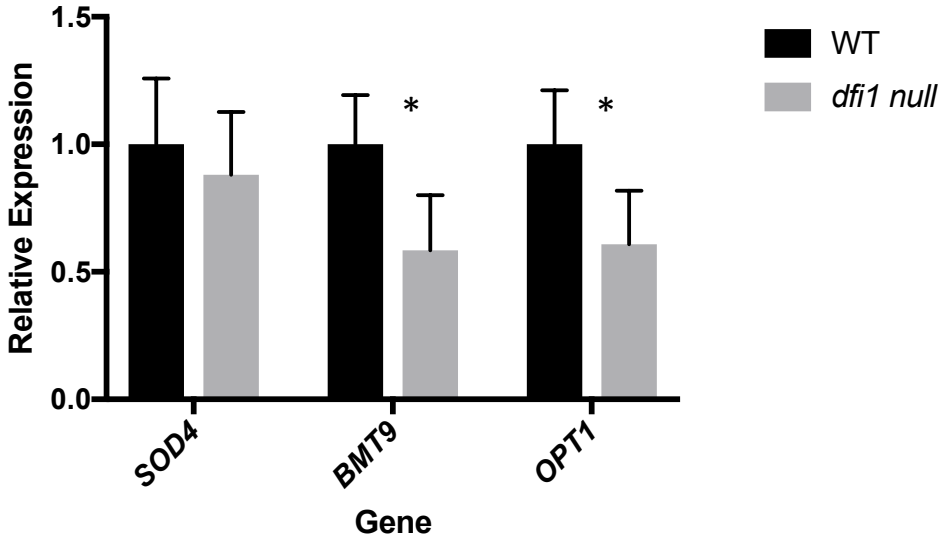

Figure 3.3 *BMT9* and *OPT1* expression is lower in plated cells lacking *DFI1*, while *SOD4* expression is unchanged. Cells were grown at 25°C on 1 percent agar for 4 days. Cells were then washed off using cold PBS and preserved with RNAlater. RNA was extracted with beadbeating and RT-qPCR analysis was performed with *ACT1* used for normalization. Expression was normalized to the average expression value of the wildtype strain within each experiment. Results from one representative experiment done in triplicate are shown; errors bars show one standard deviation. The asterisk indicates $p<0.05$ by T-Test.

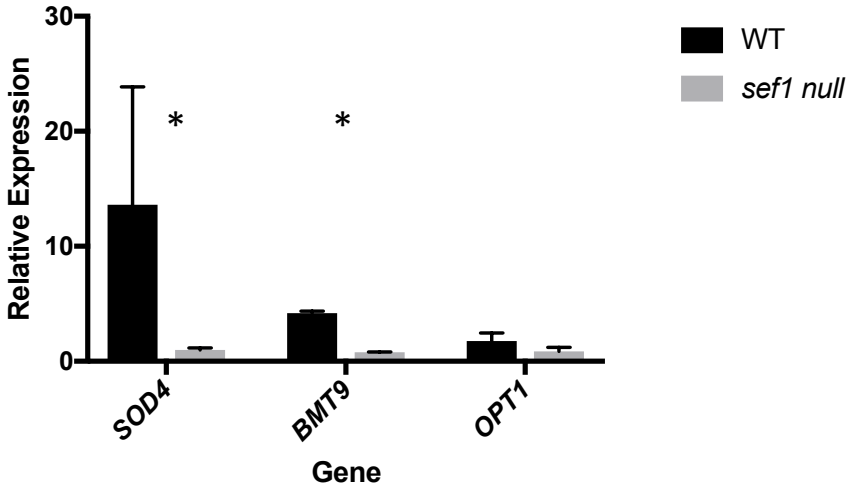

Figure 3.4 Wildtype expression of genes of interest requires *SEF1*. Cells were grown to log phase in minimal medium at 25°C. Samples were taken before addition of ionophore and at 30 minutes post-ionophore addition. Ionophore was added to a final concentration of 4uM. Cells were collected via centrifugation, resuspended in RNAlater, and stored at -80°C. RNA was extracted with beadbeating and RT-qPCR was performed. Expression of genes of interest was normalized to *ACT1*, and then normalized to the value at t=0. Significance was determined with a Student's T-test.

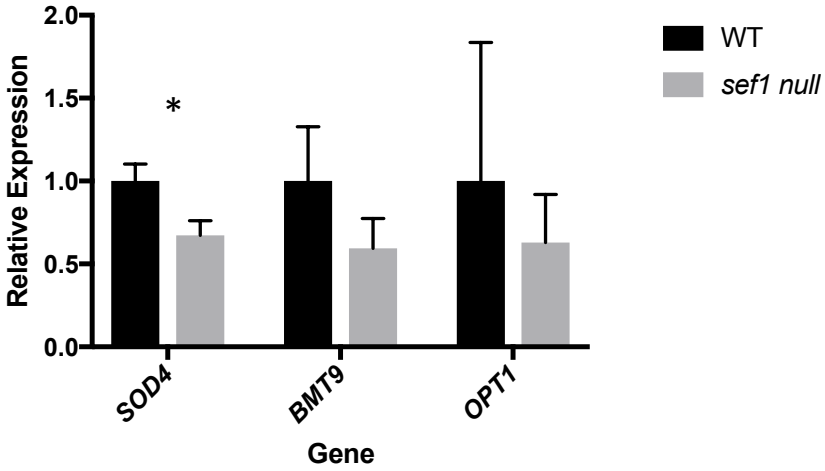

Figure 3.5 Deletion of *sef1* leads to a small decrease in expression of genes of interest in plated cells. Cells were plated on 1% agar and grown at 25°C for 4 days. Cells were scraped off the plates with PBS, resuspended in RNAlater, and stored at -80°C. RNA was extracted using beadbeating and RT-qPCR was performed. Expression values were normalized to *ACT1* and then to the average value of the corresponding wildtype condition. *=$p<0.05$, as determined by a Student's T-test.

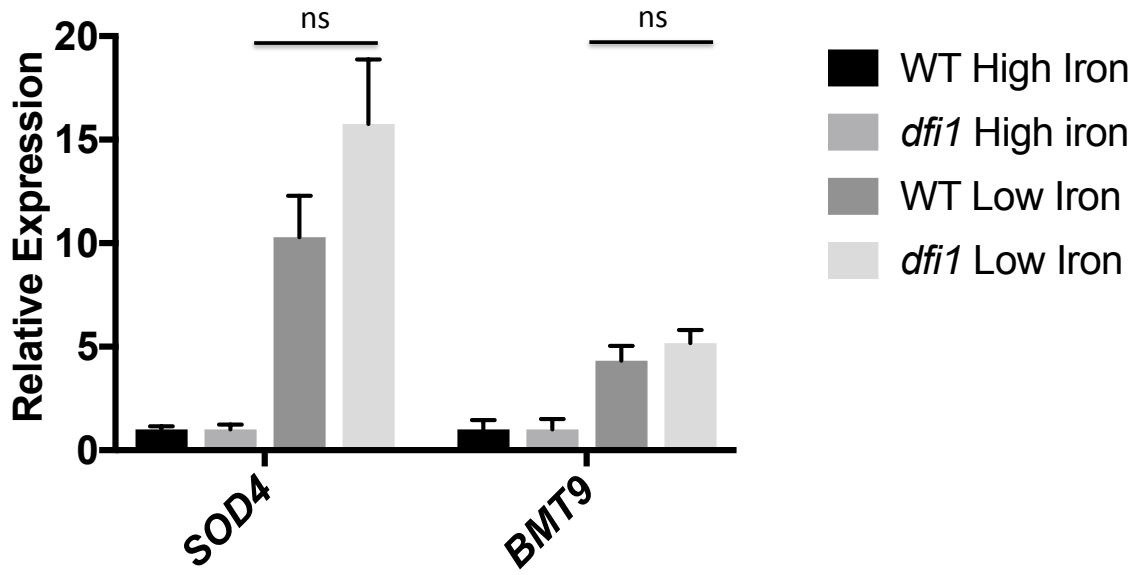

Figure 3.6 *DFI1* is not required for the transcriptional response of genes of interest to low iron. Cells were grown as in Chen 2011. Briefly, log phase cells were diluted in either YPD or YPD+500µg of BPS, an iron chelator. After 6 hours, cells were collected via centrifugation, resuspended in RNAlater, and stored at -80°C. RNA was extracted using beadbeating and RT-qPCR was performed. Expression values were normalized to *ACT1* and then normalized to the average value of the corresponding high iron condition. Significance was determined by T test.

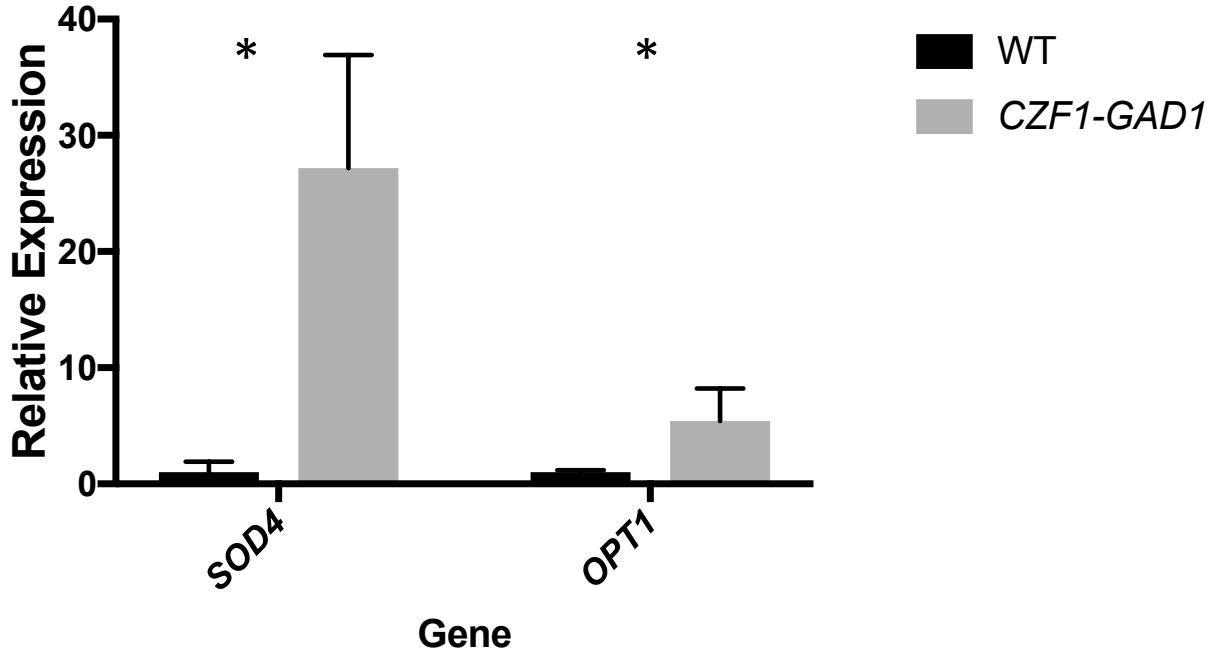

Figure 3.7 Expression of an activated allele of *CZF1* promotes expression of *SOD4* and *OPT1*. Cells were grown to log phase in minimal medium. Cells were collected via centrifugation, resuspended in RNAlater, and stored at -80°C. RNA was extracted using beadbeating and RT-qPCR was performed. Expression values were normalized to *ACT1* and then to the average expression value for the wildtype strain. These differences are significant based on a permutation test, $p<0.05$.

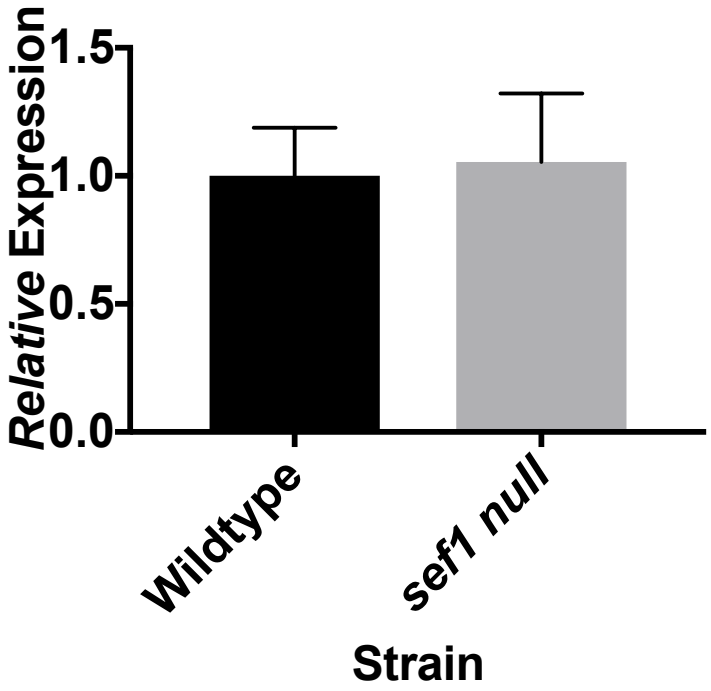

Figure 3.8 Expression of *CZF1* does not require Sef1p. Cells were grown to log phase in minimal medium. Cells were collected via centrifugation, resuspended in RNAlater, and stored at -80°C. RNA was extracted using beadbeating and RT-qPCR was performed. Expression values were normalized to *ACT1* and then to the average expression value for the wildtype strain.

Chapter 4: Discussion

Candida albicans is an opportunistic pathogen equipped with many virulence factors, including morphological plasticity (Noble 2017). The transition from ovoid yeast to elongated hyphae plays a considerable role in *C. albicans* pathogenesis and can be induced by a variety of environmental cues, including growth in contact with an agar matrix (Brown Jr *et al.*, 1999; P. E. Sudbery, 2011). The membrane-bound protein Dfi1p is required for wildtype levels of filamentation, and wildtype levels of Cek1p MAPK activation, during growth in contact with an agar matrix (Zucchi *et al*., 2010). The observations reported here demonstrate that the classical MAPK pathway proteins, as well as the GTPase Rac1p and its accessory proteins, Dck1p and Lmo1p, are needed for wildtype levels of Cek1p activation during growth in contact with an agar matrix.

Dfi1p signaling is required for wildtype virulence in a mouse intravenous model of infection (Zucchi *et al*., 2010). Dfi1p's contribution to filamentation is well documented, but other filamentation cues are present in the host. To learn more about how Dfi1p signaling affects the *Candida albicans* cell using an unbiased approach, we used RNA sequencing to identify genes whose expression was increased in response to Dfi1p signaling activation. We identified several hits and chose to follow up on three genes, *BMT9*, *OPT1*, and *SOD4*. These genes are known members of the Sef1p regulon that responds to low iron availability (C. Chen *et al.*, 2012, 2011). Observations reported here demonstrate that Sef1p is required for wildtype levels of expression during ionophore treatment. We also demonstrate that

Dfi1p is needed for the response to ionophore, but not low iron. The plated condition is of interest to us, as it is the original environment in which Dfi1p was shown to be important. Dfi1p is required for wildtype levels of expression of *BMT9* and *OPT1*, but not *SOD4* in plated cells. Interestingly, Sef1p is required for wildtype levels of expression of *SOD4*, but not *BMT9* or *OPT1* in plated cells.

All three genes of interest have large promoters, opening the possibility for multiple transcription factor binding sites. *in silico* analysis shows that *OPT1* and *SOD4* have binding sites for Czf1p, a transcription factor known for its role in promoting embedded filamentation (Brown Jr *et al.*, 1999). The expression of both genes is increased during expression of artificially activated Czf1p. We propose that Dfi1p pathway-dependent transcription is a complex response that requires activation of at least two transcription factors.

4.1 Model for Dfi1p signaling and transcriptional response

4.1.1 Requirement for the Classical Cek1p MAPK proteins

During growth in contact with an agar matrix, Dfi1p senses contact and activates Cek1p. Observations reported here demonstrate the importance of the classical MAPK cascade in activating Cek1p. The absolute requirement for Hst7p has been reported for Cek1p activation downstream of constitutively activated Cdc42p, and results presented in this work support the requirement for Hst7p (Elvira Román *et al.*, 2009). Preliminary evidence demonstrates the requirement for Hst7p

in Cek1p activation during growth resumption, showing that Hst7p is required for Cek1p activation in many, if not all conditions.

Likewise, the partial defect of a *cst20* null strain had been reported downstream of activated Cdc42p (Elvira Román *et al.*, 2009). Deletion of *cst20* in cells grown in contact with agar showed a similar effect, in the absence of artificial activation of any signaling elements. These observations suggest that there is a second input to the pathway that is partially redundant with Cst20p.

The exploration of the role of Ste11p in Cek1p activation in this work fills a gap in the literature. The MAPKKK Ste11p was assumed to function in the Cek1p MAPK cascade due to homology to ScSte11p. The role of Ste11p in activating Cek1p in *Candida albicans* had been little explored. The results reported here demonstrate that Ste11p is an essential part of signaling to Cek1p during embedded growth. Preliminary evidence also suggests that this is true during growth resumption. The severe defect of the *ste11* and *hst7* null strains, compared to the partial defect of the *cst20* null strain suggests that a Cst20p-independent input to the core Cek1p MAPK cascade exists.

4.1.2 Requirement for Rac1p

Hope *et al* (2008, 2010) established that Rac1p and its accessory proteins, Dck1p and Lmo1p, are required for normal embedded filamentation, and this effect can be rescued by overexpressing *CEK1*. Observations presented in this work demonstrated Rac1p, Dck1p, and Lmo1p participate in promoting activation of the

Cek1p MAPK cascade during growth on an agar matrix. Deletion of *rac1*, *dck1*, or *lmo1* leads to a defect in Cek1p activation that is reminiscent in magnitude of the defect of a *dfi1* null strain.

Artificial activation of Rac1p can bypass the Cek1p activation defect of the *dfi1* null strain. This shows that Rac1p is epistatic to Dfi1p, and that they may function in the same pathway. A connection between Dfi1p and Rac1p, Dck1p, and Lmo1p is intriguing as they are all proteins without homologs in *S. cerevisiae*, which deos not respond to growth on agar and does not form true hyphae. These proteins appear to represent a pathway evolved by *C. albicans* to sense contact in order to better sense its host and enhance virulence.

4.1.3 Transcriptional Response to Dfi1p-dependent Cek1p Activation

Previous work established Dfi1p-dependent Cek1p activation as a signaling pathway that promotes filamentation and MAPK activation. The targets of MAP kinases are often transcription factors, so we hypothesized that Dfi1p-dependent Cek1p activation would result in transcriptional changes. We also hypothesized that these transcriptional changes would tell us more about the role that Dfi1p plays in *Candida* cell biology. I used RNA sequencing to find genes whose expression was increased in conditions that promote Dfi1p-dependent Cek1p activation.

Plated cells are the original context in which Dfi1p was found to be important, but plated cells are heterogeneous and that risked adding too much noise to the transcriptional landscape to find candidate genes. To avoid this, I used a

system for Dfi1p-dependent Cek1p activation in liquid culture devised by Talya Davis (2013). We hypothesized that Dfi1p-dependent Cek1p activation would activate the same targets whether induced by growth in contact with an agar matrix or ionophore. We hypothesized that using a liquid culture would provide a less variable environment, compared to growth in a colony, and that this would allow us to identify targets of Dfi1p-dependent signaling. We could then use RT-qPCR to examine the expression of those genes in cells grown in contact with an agar matrix. To do this, we used RNA sequencing to measure expression, comparing ionophore treated cells to cells treated with a sham induction, and comparing wildtype cells to *dfi1* null cells.

After analyzing the RNA sequencing data, candidate genes were validated with RT-qPCR. I then focused on measuring expression of those genes, *SOD4, OPT1*, and *BMT9* in several contexts. Interestingly, these three genes are regulated, directly or indirectly, by Sef1p (C. Chen *et al*., 2011). Observations reported here support the involvement of Sef1p in regulating the genes of interest, especially *SOD4*. *SOD4* and *OPT1* also responded strongly to activation of Czf1p, which is known for its role in promoting embedded filamentation (Brown Jr *et al*., 1999; Petrovska & Kumamoto, 2012).

These transcription factors may work independently or together. Cooperation between transcription factors is a known phenomenon in yeast, with the paradigm based on ScSte12p. Ste12 is activated downstream of ScKss1p or ScFus3p to promote invasive growth or mating, respectively. Complexes formed with ScSte12p and other transcription factors, most famously ScTec1p, promote

expression of different subsets of the ScSte12p regulon (Chou, Lane, & Liu, 2006). A complex formed by ScSte12p and ScTec1p upregulates genes involved in biofilm and filamentous growth, whereas ScSte12p in a complex with another transcription factor, ScMcm1, promotes expression of genes related to mating (van der Felden, Weisser, Brückner, Lenz, & Mösch, 2014). Something similar could occur with Czf1p and Sef1p, where each regulates its own set of genes, but together they regulate a subset of the total regulon.

The proposed model for up-regulation of these genes is shown in Figure 4.1, and is as follows. When commensally growing *C. albicans* grows in contact with a soft surface, like the intestinal epithelium, Dfi1p is activated. Dfi1p activation leads to activation of Cek1p and possibly other signaling pathways. This activation results in activated Czf1p. Activated Czf1p and Sef1p, which is also active under these conditions, up-regulate expression of *SOD4* and *OPT1*. Sef1p promotes transcription of *HAP43*, and Hap43p then up-regulates *BMT9.* The candidate gene products are then able to promote virulence.

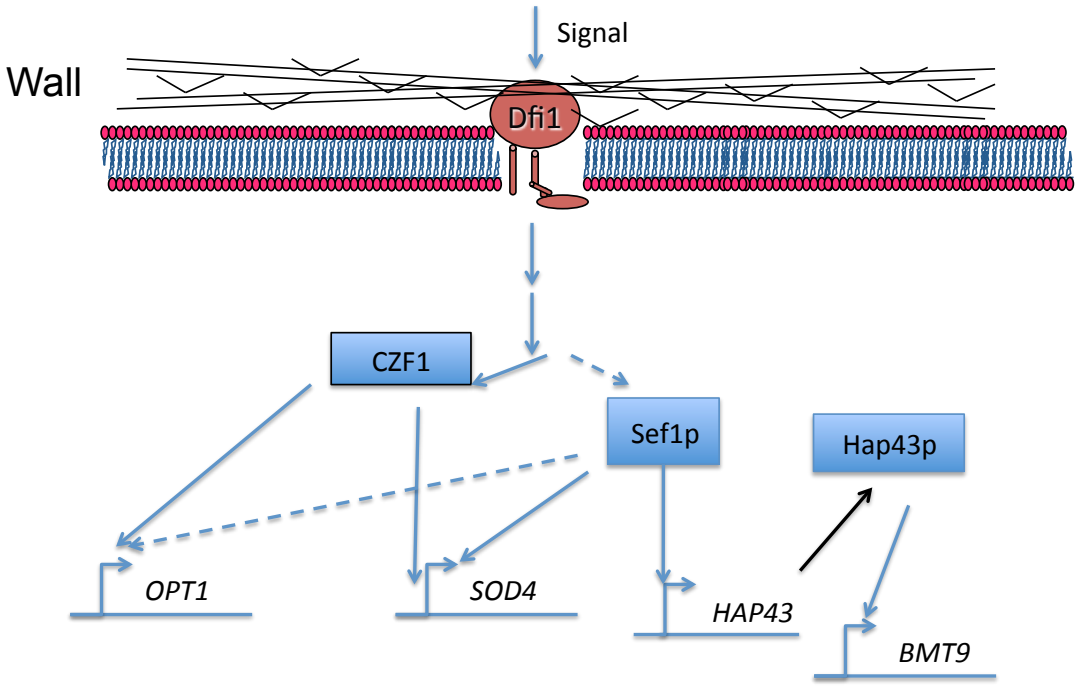

Figure 4.1. Model for gene expression regulation downstream of Dfi1p activation. Genes are upregulated by Czf1p and Sef1p, possibly promoting virulence. Dashed line indicates hypothesized positive regulation.

Based on animal experiments done in our lab, we think of Dfi1p as acting in *C. albicans* cells in the gut. Chen *et al* (2011) showed that both Sef1p and Sfu1p, which responds to high iron, are important for gut colonization, suggesting that there are iron-rich and iron-poor niches in the gut. Compared to the gut, however, the rest of the body has much lower amounts of free iron. We propose that the signaling we observed, which relies on both Dfi1p and Sef1p, could help *Candida albicans* anticipate an iron-poor niche to come. Previous research has suggested that microbes can associate one growth condition or stress with another and activate pathways needed to prepare for a condition they have yet to experience. This is called anticipation. For example, *E coli* can associate high temperature with low oxygen, given the appropriate experimental priming (Tagkopoulos 2008). Given *C.*

albicans's long history with warm-blooded animal hosts, it is possible that sensing contact has become associated with reaching an interface with the host and the yeast is preparing to meet a hostile environment.

4.2 Future Directions

4.2.1 When is Dfi1p activated?

Dfi1p is required for wildtype filamentation during growth in contact with an agar matrix. A physiologically important two-fold activation of Cek1p has been observed at days 3 and 4 of growth, when hyphal development is advanced enough for observation at 4X magnification. It remains to be determined when this signaling and the transcriptional response begin.

We hypothesize that Dfi1p is important in the transition to pathogenesis, due to its role in promoting switching to hyphal growth. It would be interesting to investigate when in the invasion of tissue Dfi1p is activated, or if it is activated. Using fluorescent proteins as transcriptional fusions downstream of promoters of genes of interest would allow us to visualize when Dfi1p-promoted transcription had been activated.

4.2.2 Where is Dfi1p activated?

Another area of research is to determine where within a colony Dfi1p is activated. Experimental observations have demonstrated that Dfi1p contributes to

Cek1p activation in colonies grown in contact with agar, but it remains to be determined how many cells have activated MAPK. One hypothesis is that only cells in direct contact with the agar surface would have Dfi1p-dependent Cek1p activation. It is also possible that activation extends further into the colony. Furthermore, the activation of Dfi1p in the hyphae that invade the agar remains to be determined. We hypothesize that Dfi1p signaling would contribute to continued growth as hyphae, especially given that *dfi1* null cells sometimes invade the agar but produce very short hyphae, suggesting a defect in hyphal elongation even if the defect in hyphal initiation is overcome.

This question also applies to *C. albicans* growth in the host. *Candida albicans* can occupy a number of niches within the host, including the skin, the mouth, the gastrointestinal tract, and the genitourinary tract. It would be interesting to examine the role of Dfi1p in different host niches.

This is another question that can be addressed with fluorescent protein reporter strains where GFP, for example, is fused to the promoter of a gene that is upregulated in a Dfi1p-dependent manner. Using fluorescent microscopy to visualize Dfi1p-dependent signaling would allow us to determine where Dfi1p signaling was occurring.

4.2.3 Is there a second Dfi1p signaling pathway?

Preliminary observations demonstrate that the filamentation defect of *rac1* and *dfi1* strains in embedded conditions is more severe than the defect of *ste11*,

hst7, or even *cek1* nulls (unpublished data). It is possible that this is because defects in MAPK signaling can result in complex feedback. There is also the possibility of Dfi1p signaling via another pathway along with the Cek1p MAPK cascade. This is especially interesting considering the hypothesis that Sef1p could be activated in a Dfi1p-dependent manner. Sef1p can be activated by phosphorylation by Ssn3p, but there is no known connection between the MAPK Cek1p and Ssn3p (C. Chen *et al*., 2012). Further exploration of signaling during embedded growth and ionophore treatment could determine whether Dfi1p is signaling via one pathway or multiple.

4.2.4 Connecting the signaling dots

Observations presented in this work identify proteins required for wildtype levels of Cek1p activation, but more work is needed to determine whether or not these proteins function in the same pathway. Protein-protein interaction studies would be needed to establish connections between members of the pathway. Of particular interest are the signaling steps directly downstream of Dfi1p.

One way to identify proteins that bind to Dfi1p is to use a system devised to identify proteins that come into close contact with membrane proteins. This system uses a modified version of the BirA protein from *E. coli* that can be fused to membrane proteins and will promiscuously biotinylate proteins that are physically close to the tagged protein. Tagged proteins can be purified using a streptavidin column and analyzed using mass spectrometry (D. I. Kim *et al.,* 2016). This would be an unbiased approach for identifying proteins that may interact with Dfi1p.

One question for which this approach is particularly apt is that of activation of Dck1p. In higher eukaryotes, Dck1p activates Rac1p after Dck1p is itself activated by another GTPase (Katoh & Negishi, 2003). This protein is called RhoG, and no homolog has yet been identified in *C. albicans*. Cdc42p, another GTPase activated in a similar way, is activated downstream of Rsr1p in *C. albicans* (Bassilana, Hopkins, & Arkowitz, 2005; Pulver *et al.*, 2013). Rsr1p, however, is not required for Rac1p activation (data not shown). An unbiased proximity-based screen would be a great way to find a *C. albicans* RhoG if one exists. It would be very interesting if Dck1p were not activated the same way it is in higher eukaryotes.

4.2.5 Which genes in the Sef1p regulon also respond to Czf1p?

We propose that the pattern of transcription we see is a result of gene regulation by at least two transcription factors. Here we present evidence that Czf1p regulates some of the genes of interest. Czf1p is a zinc finger transcription factor in the same family as Gal4p (Petrovska & Kumamoto, 2012). Czf1p can be ectopically expressed in its native form or artificially rendered constitutively active by expressing the protein with the activating domain of Gal4 fused to it (Schillig & Morschhäuser, 2013a). Overexpressing the native protein would be expected to push the equilibrium towards activated protein by sheer number of molecules present. This is likely the mechanism behind rescue of the *mkc1* null mutant's embedded filamentation defect by overexpression of Czf1p (Kumamoto, 2005). For some processes, this may not be enough, and use of the construct expressing constitutively active Czf1p could allow discovery of more subtle Czf1p roles.

It would be interesting to examine further how much of the Sef1p regulon also responds to Czf1p. One approach would be to compare expression levels of known members of the Sef1p regulon in the strain expressing artificially activated Czf1p to expression levels in a wildtype strain. Given the variability of the Czf1p binding motif, this would be a way to complement *in silico* data to get a fuller perspective of which genes in the Sef1p regulon are also regulated by Czf1p. Another option would be chromatin immunoprecipitation (ChIP) sequencing, which could be used under the conditions we used to show where Czf1p binds.

Overexpression of native Czf1p has been shown not to complement the filamentation of a *dfi1* null strain. It would be interesting to express the constitutively activated Czf1p in a *dfi1* null strain to determine whether bypass of Dfi1p to filamentation would occur if Czf1p were artificially activated.

4.2.6 How are Czf1p and Sef1p activated during embedded filamentation?

The transcription factor Czf1p is needed for embedded filamentation, but it remains unclear how it becomes activated. Speculation about a connection with the Cek1p and Mkc1p MAPK cascades, given their common functions promoting embedded filamentation, has always been intriguing (Brown Jr *et al.*, 1999; Zucchi *et al., 2010)*. Past experimental evidence has supported a connection to the Mkc1p pathway, but the observations presented in this work suggest that there may also be a Czf1p response downstream of the Cek1p MAPK pathway (Kumamoto, 2005). It would be interesting to determine if one or both MAP kinases interact with Czf1p.

One way to address this would be to examine binding of Cek1p or Mkc1p to Czf1p. Another possibility would be to use an *in vitro* kinase assay to determine whether Mkc1p or Cek1p is capable of phosphorylating Czf1p.

The same question applies to Sef1p, which responds to iron starvation. Sef1p is needed for wildtype *SOD4* expression in plated cells, suggesting that the protein is active under those conditions. It remains to be determined whether Sef1p is being activated by low iron availability or an embedded-specific signal. A relatively straightforward way to start addressing this question would be to measure the iron in the media and inside cells during plated growth. This can be done using a colorimetric assay, although the solid nature of the agar may complicate the procedure (Riemer, Hoepken, Czerwinska, Robinson, & Dringen, 2004). If iron is still abundant at day 4, that suggests an embedded pathway input, although the opposite result does not rule one out.

4.2.7 Is the Sef1p connection the key to the *dfi1* defect in virulence?

Another question that remains to be answered is how Dfi1p contributes to virulence. It is tempting to believe that it is Dfi1p's role in promoting transcription of a set of genes that makes the protein important for virulence, and the work presented here suggests that this is an interesting avenue of future research. Further research into the Dfi1p-dependent transcriptional response could shed light on the full extent of Dfi1p's role in yeast cell biology, as we refocus on Dfi1p, not just as a way of sensing contact and promoting filamentation, but as a potential barometer for changes in host niche that gives *C. albicans* a leg-up in virulence.

Chapter 5: Bibliography

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