Mating MAPK pathway in *Candida albicans*

Golnaz Rastghalam

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science (Biology) at
Concordia University
Montreal, Quebec, Canada

September 2017

CONCORDIA UNIVERSITY

School of Graduate Studies

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By: Golnaz Rastghalam

Entitled: **Mating MAPK pathway in *Candida albicans***

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Master of Science (Biology)

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_____Examiner

Dr. D. Walsh

_____Examiner

Dr. D.G. Stirling

_____Thesis Supervisor

Dr. M. Whiteway

Approved by _____

Dr. G. Brown (Graduate Program Director)

_____2018 _____

Dean of Faculty of Arts and Science

Abstract

Mating MAPK pathway in *Candida albicans*

Golnaz Rastghalam, M.Sc.

Concordia University, 2017

The Cek1 and Cek2 MAP kinases have been implicated in mating in *C. albicans*. I investigated the relationships of these MAP kinases and a putative MAP kinase phosphatase Cpp1 in the mating process of this fungal pathogen. Consistent with previous observations, mating type homozygous opaque cells that lack both kinases are sterile. However, several lines of evidence show that the two kinases do not simply provide redundant functions in the mating process. Loss of *CEK1* reduces mating significantly, to about 0.3% of wild type strains, and also reduces shmoo formation and pheromone-mediated gene expression. By contrast, loss of *CEK2* function has a relatively minor effect on mating, reducing it to approximately one third that of the wild type strain. Intriguingly, *CEK2* loss enhances pheromone responsiveness. It increases shmoo formation, pheromone-mediated cell cycle arrest halos, and pheromone-mediated gene expression. The behavior of the *cek2* mutants mimics that of *cpp1* mutants defective in the putative MAP kinase phosphatase; *cpp1* mutants are also hyper responsive to pheromone, generating large halos, high levels of shmoos, and increased pheromone responsive gene expression. Surprisingly, *cpp1* mutants are more mating defective than *cek2* mutants. The double *cek2 cpp1* mutant shows enhanced responsiveness over either single mutant. Analysis of protein phosphorylation shows that Cek1 undergoes pheromone-mediated phosphorylation of the activation loop, and this phosphorylation is enhanced in cells lacking either the Cpp1 phosphatase or the Cek2 kinase. Analysis of GFP localization of Cek1-GFP shows enhanced

nuclear localization in response to pheromone treatment, consistent with Cek1 playing a central role in pheromone response. Overall, these results show a complex interaction among the MAP kinases and MAP kinase phosphatase that function in the *C. albicans* mating pathway.

Acknowledgments

First and foremost I offer my heartfelt and sincere gratitude to my supervisor, Prof. Malcolm Whiteway, who has supported me throughout my thesis with his patience, deep knowledge and matter-of-fact academic brilliance whilst allowing me the room to develop my work in my own way. I owe my Masters to his encouragement and effort, without him this thesis, would neither have been attempted nor completed.

I would like to thank Dr. David Walsh and Dr. Donald Gray Stirling for agreeing to be on my thesis committee. Their assistance is gratefully acknowledged.

I would like to extend my special thanks to all the members of the Whiteway laboratory, including former group members Dr. Hannah Regan, Yoalin Chen and Dr. Yuan Sun, who were always willing to provide me help and guidance to move the research forward.

In particular, I would like to thank Dr. Jaideep Mallick for helping me with western blots, Raha Omran for RNA extraction and data analysis, and Tuana Mesquita and Amjad Islam for all their friendly supports and technical advices at all times.

Finally, I express my deep gratitude to Armoon Doughanian and my family for their love, support and constant encouragement during the course of my masters.

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List of Abbreviations

5-fluoroorotic acid	5FOA
Cyclic AMP- protein kinase A	cAMP-PKA
Clustered regularly interspaced short palindromic repeats	CRISPR
Endoplasmic reticulum	ER
Green fluorescent protein	GFP
Gene replacement and conditional expression	GRACE
N-Acetylglucosamine	GlcNAc
Loss of heterozygosity	LOH
Mitogen-activated protein kinase	MAPK
Mating type-like	MTL
Nourseothricin	NAT
Phosphate-buffered saline	PBS
Polymerase chain reaction	PCR
Room type	RT
Tetracycline	TET
Total internal reflection fluorescence	TIRF
Transcripts per million	TPM
Scanning electronic microscope	SEM
Wild type	WT
Yeast extract, peptone, dextrose	YPD

1. Introduction

1.1. What is *Candida albicans*?

Candida albicans is a fungus of the Eukaryota domain and Ascomycetes class first described in 1839 (McCullough, Ross et al. 1996). Phylogenetically it is located between the two primary model yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. These three species represent an estimated 200 million to 1.2 billion years of evolutionary divergence from a common ancestor. It has been suggested that *C. albicans* and *S. cerevisiae* diverged from a common ancestor about 600 million years ago (Hedges 2002). After this divergence many processes remain conserved between these species; however, there are also significant differences. Fundamental among these differences are the adaptations that have resulted in *C. albicans* becoming a commensal/opportunistic pathogen while *S. cerevisiae* is non-pathogenic and in fact has been exploited as a useful tool for thousands of years in the production of bread and alcohol (Steensels, Snoek et al. 2014), and more recently as a key workhorse in the biotechnology industry (Borodina, Nielsen 2014). An important distinction as well is that *C. albicans* is a member of the CUG clade of species; the genetic code used in *C. albicans* is non-canonical, encoding serine with CUG instead of the standard leucine (Santos, Tuite 1995).

C. albicans is a diploid fungus with eight chromosome pairs in its genome and about 6,500 pairs of allelic genes (Wilson, Mayer et al. 2014). In the laboratory *C. albicans* exhibits a parasexual cycle in which mating of diploid cells form tetraploids, followed by random chromosome loss to return to the diploid or near to the diploid state (Forche, Alby et al. 2008). This is in contrast to *S. cerevisiae*, where mating of haploid cells normally generates a stable diploid (**a**/α) cell that can

return to the haploid state through meiosis (Kassir, Adir et al. 2003). Despite the presence of many genes in the genome of *C. albicans* whose homologues function specifically in meiosis in other fungi to promote a reduction in cell ploidy (Forche, Alby et al. 2008), so far attempts to identify a meiotic process in *C. albicans* have failed. A genomic assessment of the potential has suggested that the parasexual pathway in *C. albicans* is an alternative pathway to meiosis.

C. albicans has diverse morphological forms. In particular, *Candida* cells have the ability to switch between a unicellular yeast form and a multicellular filamentous form (pseudohyphae, and true hyphae). Yeasts are oval shaped single cells that can exhibit both axial and bipolar budding patterns (Thompson, Carlisle et al. 2011). Pseudohyphae are unseparated chains of elongated cells, while true hyphae, which help the virulence of the *C. albicans,* are long tubes with parallel sides and the cells separated by septa (Sudbery, Gow et al. 2004). As well, *C. albicans* has the ability to reversibly switch between two different yeast form cell types, white and opaque (Fig.1). Opaque cells are elongated and pimpled-shell type cells that form flat colonies on solid agar whereas white cells are the normal, round, smooth surfaced yeast cells that form round, dome shaped colonies on solid agar (Anderson, Soll 1987).

C. albicans contains multiple regulatory pathways that recognize external cues, such as pH, temperature, and concentration of CO_2 and in response to any changes in these cues they can change their morphology and physiology between among these different cell types (yeast, hyphae, pseudohyphae, white, and opaque) (Miller, Johnson 2002).

1.1.1. Medical aspects of *Candida albicans*

C. albicans is the most prevalent opportunistic fungal pathogen colonizing humans (Nobile, Johnson 2015) and is a natural resident of diverse niches within a healthy host (Brand 2012). This yeast is commensal in healthy individuals, colonizing niches of a host's mucosal oral cavity, gastrointestinal tract and gastro-urinary tracts without causing any pathogenesis (Schulze, Sonnenborn 2009). However, *C. albicans* may cause both mucosal and systemic infection in immunocompetent and immunocompromised hosts (Sardi, Scorzoni et al. 2013) such as in HIV patients or patients undergoing organ transplantation or chemotherapy; bloodstream infection (candidaemia) can cause death (Denning 1991). Such systemic *Candida* infections are very dangerous, and *C. albicans* has several attributes that enable it to rapidly adapt to changing environments and that aid the pathogen to colonize every organ it settles in. Phenotypic switching and yeast-hyphal transition are the two examples of these attributes that affect the virulence of *C. albicans* in systemic disease, therefore such infections need intensive medical treatment (Rex, Walsh et al. 2000).

Overall, *C. albicans* represents a significant source of fungal infections in immunocompromised individuals which can infect the lungs, mouth, vagina, lymphatic system, brain or the urinary tract, generating up to as high as 49% mortality rates (Leroy, Gangneux et al. 2009). About 75% of women experience vulvovaginal candidiasis at least once in their lifetime (Mardh, Rodrigues et al. 2002). *C. albicans* species is detected in 40-65% of normal feces (Kumamoto 2011), and *Candida* oropharyngeal colonization found in almost 30-55% of young adults. Since *C. albicans* is a eukaryote that shares many central biological processes with humans they present challenges for the development of antifungal therapies (Berman, Sudbery 2002). Due to deleterious side

effects many antifungal drugs are used under conditions where they are fungistatic rather than fungicidal (Onyewu, Blankenship et al. 2003) so the ideal antifungal agent remains an elusive goal (Chapman, Sullivan et al. 2008).

The targets of current drugs are mainly limited to the cell wall and cell membrane. At present the treatments used to manage *Candida* infections vary substantially. The major classes of drugs used for treatment of candidiasis include echinocandins (e.g. Caspofungin) (Glockner, Steinbach et al. 2009), polyenes (e.g. Amphotericin B), and azoles (e.g. Fluconazole) (Ghannoum, Rice 1999). The echinocandin antifungal agents block the action of the glucan synthase and cause cell wall damage and cell death (Ghannoum, Rice 1999). Amphotericin B binds to ergosterol and creates pores in the cell membrane and cause cell death (Gruszecki, Gagos et al. 2003). The azole family inhibits the CYP-450 (cytochrome P) dependent enzyme C-14α-demethylase and blocks conversion of lanosterol to ergosterol and increases the membrane permeability and causes cell lysis (Yoshida 1988). However, drug resistance and toxicity has been observed for all the classes of drugs; therefore, the search must continue to identify new targets of antifungal drugs (Chapman, Sullivan et al. 2008).

1.2. Mating in *Candida albicans*

1.2.1. Overview of *C. albicans* Mating

Intriguingly, *C. albicans* was thought to be asexual for more than a century. This idea was supported by consistent observations of clonal populations of this organism with no apparent

sexual development and no detection of recombination (Pujol, Reynes et al. 1993). However, the sequencing of the *C. albicans* genome revealed genes with strong sequence similarity to the *S. cerevisiae* mating genes that function in the intracellular pheromone response mating pathway. Some of these genes are *CAG1* (Sadhu, Hoekstra et al. 1992)*, STE4, STE18 a*nd *STE2* (Tzung, Williams et al. 2001) , which represent components of a receptor G protein module, the orthologues of which function exclusively in mating in *S. cerevisiae.* However, there was a possibility that during evolution the products of these genes took on different roles other than mating-related signaling.

Things changed when in 1999 Hull and Johnson discovered the mating-type-like (*MTL*) locus related to the *MAT* locus of *S. cerevisiae* and this revolutionized our understanding of sexual reproduction in *C. albicans* (Lee, Ni et al. 2010). In *S. cerevisiae,* sexual mating is controlled by genes located at the *MAT* locus. The *S. cerevisiae MAT* locus consists of two hetero-alleles: *MAT***a** *and MATα*, where *MAT***a** codes for a transcriptional regulator, a1, and *MATα* codes for the transcriptional regulators α2 and α1 (Haber 2012). Similar to S. *cerevisiae, C. albicans* also possesses a *MTL* locus which resides on chromosome 5, and strains are typically heterozygous at this locus, containing the two hetero-alleles *MTL***a** and *MTLα* (Lee, Ni et al. 2010). However, in *C. albicans* a fourth transcriptional regulator, *MTL*a2, was identified at the *MTL***a** locus; the orthologue of this gene is missing in *S. cerevisiae* (Tsong, Annie E., et al. 2003).Therefore, allele *MTL***a** in *C. albicans* encodes the transcriptional regulators **a**1 and **a**2, and *MTLα* encodes the transcription regulators α*1* and α*2* Transcriptional regulator **a**2 activates the a-specific genes while α1 activates the α-specific genes. The **a**1/α2 complex controls mating in *C. albicans* by repressing the genes required for mating and for white-opaque switching (Soll, Lockhart et al.

2003). White-opaque switching is regulated by the Wor1 regulatory protein and opaque cells are the mating competent form of the *C. albicans,* so blocking the white-to-opaque switch by inhibiting Wor1 will also block mating (Lee, Ni et al. 2010). *WOR1* was found to be both necessary and sufficient for white-to-opaque switching based on gene deletion and enforced-expression studies (Huang, Wang et al. 2006) (Fig.2).

1.2.2. White to opaque switching: a strategy for *C. albicans* to mate efficiently

In *S. cerevisiae*, **a** and *α* cells are fully competent to mate but this is not the case in *C. albicans*. Intriguingly, although *C. albicans* has the structural elements of a classic mating pathway, mating in this organism is rare. Part of this is due to the fact that the majority of clinical and laboratory strains are **a**/*α* heterozygotes at their mating type-like locus (*MTL*), and thus express both the a1 and α2 transcription regulators. As explained in previous section, the heterodimeric a1/α2 complex controls mating in *C. albicans* by repressing the genes required for mating and by inhibiting *WOR1* which blocks white-to-opaque switch (Lee, Ni et al. 2010, Huang, Wang et al. 2006).

Wor1 controls its own expression via a direct positive-feedback loop, thus *WOR1* is part of a bistable expression loop with two states: white (infertile) and opaque (fertile) (Fig.2). The negative feedback loop inhibits the white-to-opaque transition and the positive feedback loop makes opaque cells stable through several cell divisions (Huang, Wang et al. 2006). Subsequent studies have revealed other regulatory elements that participate in the complex cellular circuitry that governs the white-to-opaque transition. These include Czf1 (required for contact induced

response), Efg1 (a negative regulator of white to opaque switch) and Wor2 (ensures the formation and stability of opaque phase) (Huang 2012). Wor1 in opaque cells stimulates the expression of *WOR2*, *CZF1*, and *WOR1* and represses *EFG1*, however, in white cells; Efg1 represses *WOR1* and inhibits the white to opaque switch (Langford, Hargarten et al. 2013).

C. albicans that are homozygous at *MTL* locus miss either a1 or α2, thus, Wor1 expression is permited and the bistable state can switch from white to opaque (Huang, Wang et al. 2006) (Fig.2).

Overall, *C. albicans* regulatory circuits demand very specific conditions for mating. First, diploid **a**/α cells have to become homozygous at the *MTL* locus by either gene conversion or chromosome loss followed by duplication of the retained copy of the chromosome (Soll 2014). And subsequently they have to undergo the epigenetic switch between the normal, rounds, smooth yeast cells (white) to an elongated, flat, pimpled-shell cell type (opaque) (Slutsky, Staebell et al. 1987). Opaque cells mate 10^6 times more efficiently than do white cells, and so they represent the necessary mating competent state (Miller, Johnson 2002). However, white-to-opaque switching occurs infrequently and requires specific environmental cues and conditions to trigger the cells to enter the opaque phase; but once it occurs it is heritable for many generations at the room temperature of 25°C (Soll, Lockhart et al. 2003). This mode of mating regulation is unique to *C. albicans* and is critical for their adaptation and survival in different host niches (Morschhauser 2010).

1.2.3. Mating signaling pathways: G protein and MAPK pathway

We still need to understand the details of the regulation of *C. albicans* mating at the molecular level. Once in the opaque state, how do *Candida* cells recognize mating partners and communicate that information to the internal machinery? It has been shown that *C. albicans* are able to respond to pheromones and are able to mate. The mating process of *C. albicans* is mediated by a pheromone signaling pathway, similar to the one in *S. cerevisiae* where a conserved MAPK pathway transduces the pheromone signal from outside of the cell into a transcriptional response in the nucleus (Gustin, Albertyn et al. 1998). In *S. cerevisiae*, pheromone binding to its receptor Ste2 or Ste3 activates a heterotrimeric G protein where the Gα subunit (Gpa1) dissociates from the Gβγ subunit (Ste4 and Ste18) and this Gβγ subunit binds to the Ste5 scaffold protein carrying Fus3, Ste7, and Ste11 and brings the complex to the plasma membrane where it interacts with the Cdc42-bound Ste20. Ste20 is brought to the cell membrane via its binding to Bem1 adaptor protein (Winters, Pryciak 2005). The C-terminal kinase domain of Ste20 is held in an inactive state by association with an auto-inhibitory sequence present in its N-terminal domain that overlaps with a Cdc42/Rac interactive binding (CRIB) motif; binding of active (GTP-bound) Cdc42 to the CRIB motif relieves this auto-inhibition (Lamson, Winters et al. 2002). Therefore, Cdc42-GTP and Bem1 binding to Ste20 activates the Ste20 which in turn triggers Ste11. The cascade involves activation of Ste20 (MAPKKKK), Ste11 (MAPKKK), Ste7 (MAPKK), by successive phosphorylation, and finally activation of the MAPKs, Fus3 and Kss1. The activated MAP kinases in turn release the inhibitory activity of Dig1 and Dig2 which activates the Ste12 transcription factor. Ste12 and Dig1/Dig2 form a complex that inhibits Ste12 and when Fus3 gets activated it interacts with Dig1/Dig2 and the inhibition on Ste12 is released (Cook, Bardwell et al. 1996, Tedford, Kim et al. 1997). The activated Ste12 transcription factor

then induces the transcription of pheromone-responsive genes (Pi, Chien et al. 1997). Dig1/Dig2 proteins regulate MAPK controlled signaling pathways involved in both mating and filamentous growth (Chou, Lane et al. 2006). Bar1 and Sst2 are the major components that negatively regulate pheromone signaling in the yeast *S. cerevisiae* (Dohlman, Song et al. 1996) and the Msg5 protein phosphatase plays a minor role in deactivation by suppressing Fus3 MAKP activity (Doi, Gartner et al. 1994). Several components of the mating pathway (Ste20, Ste11, Ste7, Kss1 and Ste12) are also important for filamentous growth and invasion in *S. cerevisiae* (Madhani, Fink 1998).

Orthologues of each of these signaling components including Cst20 (Ste20), Hst7 (Ste7) (Kohler, Fink 1996), Cek1 (Fus3 or Kss1) (Whiteway, Dignard et al. 1992a) and Cph1 (Ste12) (Maiti, Ghorai et al. 2015) have been identified in *C. albicans.* The existence of these orthologues was one of the lines of evidence suggesting that *C. albicans* has a similar mating system to that of *S. cerevisiae*. The mating signaling pathway of *C. albicans* also includes a receptor G protein module connected to a MAP (mitogen-activated-protein) kinase pathway. The pheromone binds to Ste2/Ste3 seven-transmembrane-segment G protein coupled receptors; Ste2 is the α pheromone receptor and Ste3 is the **a** pheromone receptor similar to that of *S. cerevisiae* (Alvaro, Thorner 2016) and the signal will be transduced to a heterotrimeric G protein. The G protein activation transfers the signal to the upstream MAP kinase module involving a p21- activated kinase homologue Cst20, which is proposed to initiate a series of phosphorylations of the MAP kinase components, Ste11, Hst7 and the downstream Cek1/Cek2. Cek1 and Cek2 activation then will activate the transcription factor Cph1 to regulate gene expression involved in pheromone response and mating (Monge, Roman et al. 2006). One way to negatively regulate this pathway

is through inhibition of the Cph1 transcription factor by Dig1, the single *C. albicans* orthologue of *S. cerevisiae* Dig1p and Dig2p. Dig 1 influences filamentation and mating through multiple signaling pathways including those controlled by MAPK and by cAMP-PKA pathways (Regan, Scaduto et al. 2017). Another negative regulator of this pathway is the Cpp1 protein phosphatase which is an orthologue of the Msg5 protein in *S. cerevisiae.* We found in this study that Cpp1 protein negatively regulates the mating pathway by deactivating the MAPK Cek1.

Although an efficient mating apparatus has now been identified in *C. albicans*, the mating cycle differs in several important respects from that of *S. cerevisiae* and other fungi. One of the differences is in the G protein-mediated signaling pathway which is composed of Gα (Cag1), Gβ (Ste4) and Gγ (Ste18) subunits (Lu, Sun et al. 2014, Kurjan 1993). In contrast to the well-studied *S. cerevisiae* where Gα and Gβ play opposing roles (Whiteway, Hougan et al. 1989) both the Cag1 and Ste4 subunits in *C. albicans* are required for activation of the mating pathway (Dignard, Andre et al. 2008). As in *S. cerevisiae, C. albicans* has 2 Gα subunits and a single Gβ and a Gγ subunit. The Gα subunit that encodes *GPA2* regulates cAMP signaling (Kubler, Mosch et al. 1997), and the one that encodes *CAG1* is implicated in the MAP kinase pathway (Dignard, Whiteway 2006, Kurjan 1993).

One other possible difference between *C. albicans* and *S. cerevisiae* may be the absence of the full-length scaffold protein Ste5 in *C. albicans*. One way that cells ensure a proper response to a wide variety of stimuli without any crosstalk is through scaffold proteins. Scaffold proteins improve signaling efficiency, ensure that signals are connected to the correct response and prevent crosstalk between signals. As we know the MAPK cascade is an extensively used pathway in response to variety of signals. Thus, the MAP kinase pathway involves scaffold

proteins to improve signal efficiency and prevent crosstalk between signals that utilize the same core components.

In *S. cerevisiae*, the Ste5 scaffold protein ensures the signal is transducing properly by associating with the kinases and the Gβ protein subunit, and acts enzymatically to ensure the Ste7 MAPKK phosphorylates and activates the MAP kinase Fus3 of the system (Good, Tang et al. 2009). In fact, Ste5 catalytically unlocks Fus3 for phosphorylation by Ste7 and that's a reason why Fus3 selectively gets activated by the mating pathway and not by other pathways that utilize Ste7 (Good, Tang et al. 2009). The Cst5 scaffold in *C. albicans,* which is an orthologue of Ste5 has been found to have some major differences from Ste5. Cst5 has the conserved RING and PH domains, however, has only one MAPK phosphorylation site compared with the four phosphorylation sites on Ste5 in *S. cerevisiae* and it lacks the vWA domain that is critical in *S. cerevisiae* to differentiate response to mating signals from invasive growth signals (Cote, Sulea et al. 2011). So the mechanism of how Cst5 in *C. albicans* connects to regulation of the MAPK kinase pathway is still unclear.

2. Materials and Methods

2.1. Strains, Media and Culture conditions

The *C. albicans* strains used for these experiments were all derivatives of CAI-4 (*ura3*::imm434/ *ura3*::imm434) (Fonzi, Irwin 1993). Construction of the *cek1*Δ/Δ a/α (CK43B-16L) is described in Csank *et al*., 1998 (Csank, Schroppel et al. 1998), and construction of the *cpp1*Δ/Δ a/α (CP29-1-7L4) is described in Csank *et al*., 1997 (Csank, Makris et al. 1997). Equivalent **a/a** strains were derived from the parent strains by sorbose selection (Magee, Magee 2000).

CEK2 was disrupted in the CAI-4 **a/a**, *cpp1*Δ/Δ **a/a** and *cek1*Δ/Δ **a/a** parent strains using *C. albicans* CRISPR-Cas9 system following the detailed protocol as described in Vyas VK, 2015 (Vyas, Barrasa et al. 2015) with minor modifications described in section 2.2 (Fig.3A) .

CEK1 was disrupted in the parental strain *cpp1*Δ/Δ **a/a** using the same method (Vyas, Barrasa et al. 2015). *Cek1* and *Cek2* were targeted with oligonucleotides listed in Table 1.

Cek1 was tagged with GFP in separate strains (CAI4, *cpp1*Δ/Δ). The Cek1-GFP fusion strains were created to investigate protein localization in response to pheromone by live tracking of the Cek1 fusion GFP signal.

Strains were plated on synthetic complete medium supplemented with 2% glucose or 1.25% GlcNAc and 100 µg/mL uridine at 25°C, and grown in SC- liquid media with glucose or GlcNAc at room temperature prior to assays. For opaque colony identification, Phloxin B (5µg) (Anderson, Soll 1987) was added to the SC with glucose (2%) or GlcNAc (1.25%) media.

Strains used in this study are listed in Table 2.

2.2. CRISPR-CAS9

I used the "solo system" strategy for *Candida albicans* CRISPR-Cas9 (Vyas, Barrasa et al. 2015). I designed three sets of primers for CRISPR mutant construction: sgRNA, repair DNA, and screening primers. To design sgRNA, Benchling was used based on the guidelines as follows; single guide; Guide length: 20; Genome: CA22 (CANDIDA ALBICANS SC5314 (DIPLOID)); PAM: NGG. A guide sequence was chosen based on the position nearer to the start codon (for gene inactivation) and nearer to stop codon (for GFP tagging) and higher on-target and lower off-target scores. The candidate sequence was also checked in the file named (Targ.NoTs.subs12nt.HitsGenesOnly.Hits1Gene2Alle.3letterName) from the supplemental material of Vyas et al., 2015 (Vyas, Barrasa et al. 2015). This file shows sequences that provide a target in both alleles for any given gene. For the solo system the pV1093 plasmid with *CAS9* and both Amp and Nat as markers is used. The sgRNA (20 nt) has extended sequences representing the complementary sequences on both 5' and 3' ends to allow cloning into the BsmBI site of plasmid PV1093, so the set of primers had this sequence: Forward – 5'-atttgX20g-3' and Reverse – 5'-aaaacX20c-3'. The repair DNA was also designed through Benchling and was derived from homologous sequences flanking the sgRNA target sequence. Each inactivation repair DNA was modified with 4 different changes including two in-frame stop codons (TGA used in this mutant), a disrupted PAM region (NGG) and introduction of a restriction enzyme site for confirmation of transformants. The repair DNA was amplified by PCR and was co-transformed with the plasmid that contains the sgRNA and Cas9. To inactivate *cek1* and *cek2*, stop codons were introduced in each gene (on 19^{th} and 25^{th} amino acid in *cek1* and 32^{nd} and 37^{th} amino acid in *cek2*). The screening primers were designed to amplify around 1kb allowed for sequencing. All the oligos used in this experiment are listed in Table 1.

2.3. Phenotype Switching and Immunofluorescence

I followed the published protocol of Sun, et al. (2016). White and opaque cells were picked from single colonies of CAI4, *cpp1*Δ/Δ, *cek1*Δ/Δ, *cek2*Δ/Δ, *cpp1*Δ/Δ *cek*1Δ/Δ, *cpp1*Δ/Δ *cek2*Δ/Δ and *cek1*Δ/Δ *cek2*Δ/Δ strains grown on SC-Glucose and SC-GlcNAc media for 5 days at 25°C using screening on plates containing phloxine B. The cell concentration was adjusted by suspending them in water and suitable concentrations were plated onto agar media containing 5 μg ml^{-1} phloxine B with either 2% glucose or 1.25% GlcNAc as the carbon source. Plates were incubated at 25°C and were scanned on the 7^{th} day. The frequency of switching colonies was calculated by dividing the number of opaque colonies including colonies with sectors over the total number of colonies for white to opaque switching; and the number of white colonies including sectors over the total for opaque colonies for opaque to white switching (Table 3).

2.4. Mating Assays

Opaque colonies were identified on SC-GlcNAc agar media with 5μg phloxine B after 5 days of growth at RT. Opaque cells of strain 3315 *α/α* were used as the tester strain for mating. Opaque colonies of *MTL***a/a** versions of CAI4, *cpp1*Δ/Δ, *cek1*Δ/Δ, *cek2*Δ/Δ, *cpp1*Δ/Δ *cek*1Δ/Δ, *cpp1*Δ/Δ *cek2*Δ/Δ and *cek1*Δ/Δ *cek2*Δ/Δ strains were re-streaked as straight lines on separate YPD, SC-GlcNAc and SC-Glucose agar plates as the experimental strains. Opaque cells of tester strains were streaked as straight lines on YPD plates. After 48 hours of incubation at room temperature, the two sets of tester and experimental streaks were replica plated together onto both SC-GlcNAc and SC-Glucose plates. After 48 hours incubation on SC-GlcNAc and SC-Glucose plates at RT, cells were replicated onto SC-Glucose selection medium lacking uridine, tryptophan and lysine for prototrophic selection. After 3 days of incubation at 30°C all plates were scanned and re-

streaked on the selection medium for further confirmation of stable prototrophic colonies (Dignard, Andre et al. 2008).

2.5. Quantitative Mating Assays

Quantitative mating assays were done in liquid SC-Glucose medium. Opaque cells of strains 3315*α/α* were used as the tester strains for mating; and CAI4, *cpp1Δ/Δ, cek1Δ/Δ, cek2Δ/Δ, cpp1Δ/Δ cek1Δ/Δ*, *cpp1Δ/Δ cek2Δ/Δ* and *cek1Δ/Δcek2Δ/Δ* all *MTL***a/a** were tested as experimental strains. Opaque cells were selected from SC-Glucose agar medium containing phloxine B after 5 days culture at RT. Cells were cultured separately in liquid SC-Glucose medium at RT shaking at 220 rpm for 24 hours. Equal amounts of cells from each strain were adjusted after quantification with a hemocytometer at 400x magnification using an optical microscope. After centrifuging the cells, tester and experimental strains were mixed in separate 5 ml fresh SC-Glucose liquid medium in 50 ml Falcon tubes. Cells were gently shaken for 48 hours at RT. Cells were then plated onto selection plates for 3 days at 25°C before counting the colonies (Guan, Xie et al. 2013).

To calculate the mating frequency the number of prototrophic mating product colonies on the trp-, lys-, ura- plates was divided by the input on ura- plates (the experimental cells). The experiments were done two times and the average value is presented in Table 4.

2.6. Pheromone Response Assay

Opaque cells of all strains were incubated in SC-Glucose liquid medium for 24 hours at RT. Cells were diluted for a final OD600=0.8 and treated with α pheromone (1µg/ml) for 3h, 6h, and 24 hours before being photographed and scored for shmoo-formation. Strains were visualized using a Nikon eclipse TS100 microscope with 400X magnification using DIC optics.

2.7. Halo Assay

For halo assays, 5µl of a synthetic 13-amino-acid version of the α-factor mating pheromone dissolved in 50% methanol (1µg/ml) was spotted directly onto a lawn of opaque cells to determine the growth inhibition response. Opaque cells were identified on SC-Glucose medium containing phloxin B. A single colony was diluted into 800 µl of sterile water, and serial dilutions were done (½ and ¼) to come up with a cell density that gives the best concentration of the lawn; 150µl of each dilution were spread onto separate SC-Glucose plates, 5µl α-pheromone was added, and cells were grown for 24 hours at RT. Pictures of whole plates were scanned at 300 dpi using an Epson 3700 dpi scanner.

2.8. RNA-Sequencing

RNA was extracted from CAI4, *cpp1Δ/Δ, cek1Δ/Δ, cek2Δ/Δ, cpp1Δ/Δ cek1Δ/Δ*, *cpp1Δ/Δ cek2Δ/Δ* and *cek1Δ/Δcek2Δ/Δ* strains with and without pheromone treatment following the QIAGEN RNA extraction kit protocol with the exception that the cells were disrupted completely with bead beater shaking for 25 times for 20 seconds with 1 min cooling on ice between treatments. Samples were tested for quality control using a bioanalyzer, and submitted to the Genome

Quebec innovation Centre for sequencing using an Illumina Miseq. A description of the RNA-Seq processing method that was employed is as follow; The C. albicans_SC5314_Assembly22 ORF/gene coding sequences (SC5314_V22) were downloaded from the CGD website (Skrzypek, Binkley et al. 2017). A PERL script was written to create a gene/ORF ID descriptions file. Each RNA-Seq data file was processed to correct the read sequences (Song, Florea 2015), trim adapters (Jiang, Lei et al. 2014) and remove rRNA reads (Kopylova, Noé et al. 2012). The pre-processed reads for each sample were then mapped to the SC5314_V22 sequences and quantified (Patro, Duggal et al. 2017) to produce raw counts and transcripts per millions (TPM) values. Each set of sample raw counts and TPM values were annotated with gene descriptions using a PERL script and imported into a spreadsheet for further processing. Thanks to Raha and Debra.

2.9. Phosphorylation analysis and Western blotting

White and opaque cells of all strains were grown in SC-Glucose liquid media for 24h at RT. Cells were then diluted to OD600 = 1.0 at 25°C both in the presence and absence of alpha pheromone. After 3hrs, cells were harvested by centrifugation, washed with IP150 buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM MgCl2, 0.1%NP40], and lysed with glass beads in IP150 buffer supplemented with a protease inhibitor cocktail tablet (Roche), anti-phosphatase inhibitor (Roche) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The protein extract was clarified by centrifugation at 13000 rpm at 4^0C and the protein concentration measured by Bradford's Method and equal amounts of proteins were boiled with SDS gel loading buffer and resolved in 4–20% gradient SDS polyacrylamide gels. The separated polypeptides were transferred electrophoretically onto a nitrocellulose membrane and analyzed by western blotting

using anti - P44/42 MAPK polyclonal antibody. The membrane was developed using the Li-Cor Odyssey system using their anti-rabbit-IR-Dye 680-conjugated secondary antibody (Mallick, Whiteway 2013).

2.10. Immunofluorescence Microscopy

Cells from single colonies were grown in SC-Glucose liquid media for 24h at RT, 220rpm shaking and 5x 10^6 Cells were washed in 1 mL of 1xPBS three times. Cells were fixed with 1 ml blocking buffer (2% BSA, and 1% goat serum in PBS) for at least 30 mins. The cells were pelleted and the pelleted cells were incubated with 100µl of primary antibody (F223-5H1-1 antibody) for another hour at RT. Cells were then washed with PBS containing 0.05%Tween 20 for three times, 5 mins each time. Washed cells were incubated with 100µl of a 1/100 dilution of secondary antibody - Texas red conjugated goat anti-mouse antibody for 1 hour in the dark at RT. Cells were then washed with PBS containing 0.05%Tween 20 three times and once with PBS , 5 mins each time. Cells were resuspended in 50µl of PBS and 4µl was applied under a coverslip. Sealed sample slides were placed in a slide box for protection from light before observation under the Nikon_Ti fluorescence microscope. All strains used in this study were examined with Immunofluorescence microscopy to confirm opaque cells through staining with the F223-5H1-1 antibody (Fig. 4).

2.11. Cek1 tagging with GFP

The GFP plus marker *URA3* was amplified by PCR from the plasmid pFA-GFP-CaURA3 using primers that provided homology to regions before the stop codon of *CEK1* and to flanking

regions downstream of *CEK1* for the construction of the Cek1-GFP by using standard one-step disruptions. The PCR product was transformed to the parent strain CAI4 and *cpp1Δ/Δ*, and the transformants were selected from SC-ura- agar plates. Successful transformants of Cek1-GFP were further confirmed by PCR. And in order to check if the Cek1 is still functional after being tagged with GFP, we used the Spider media and checked the morphology of the cells under the microscope. Our strains had only one functional copy of the *CEK1* gene, therefore, if tagging disrupted the other copy of this gene (the other allele) the phenotype of the *cpp1* null mutant which is hyphal growth and germ tube formation on Spider media would be suppressed (Csank, Makris et al. 1997). Cek1-GFP containing cells were plated on Spider media for 3 days, then the colonies were washed with water and the plates were observed under the microscope (Fig. 5). Also, Cek1-GFP fluorescence was observed under the microscope for further confirmation.

3. Results

The *CPP1* gene encodes a putative phosphatase that was found to regulate the Cek1 kinase of the human fungal pathogen *C. albicans* in the regulation of hyphal development (Csank, Schroppel et al. 1998, Eisman, Alonso-Monge et al. 2006). Recent work has shown that Cek1 and the redundant MAP kinase Cek2 are required for mating (Chen, Chen et al. 2002). I have therefore investigated the function and the relationship between Cek1, Cek2 and the phosphatase Cpp1 in the mating pathway.

3.1. Is Cpp1 a negative regulator of the mating pathway?

To investigate whether the Cpp1 phosphatase is a negative regulator of mating, we constructed a *cpp1* null mutant strain by identifying a *MTL* homozygous version of strain CP29-1-7L4 by sorbose selection (Rustchenko 2007, Magee, Magee 2000). We confirmed the *cpp1* mutation and the *MTL* phenotype by PCR. We tested this mutant for any modification in its response to pheromone and we found that *cpp1*Δ/Δ mutants are hyper responsive to pheromone treatment as they generate large halos when compared to the wild type strain which generates almost imperceptible halos in a standard halo assay test (Fig. 6). As well, direct microscopic assessment of shmoo formation showed that the *cpp1* mutant formed more frequent and extensive shmoos than the control strain (CAI4) (Fig. 7 & Table 5). However, even though the cells were more pheromone responsive, quantitative mating experiments showed that loss of *cpp1* actually reduces mating to about 2% that of the wild type strains (Table 4 & Fig. 8). We did RNAseq analysis of pheromone treated *cpp1* mutant strains. As seen in Table 6, the *cpp1* mutant was responsive to pheromone treatment, inducing the entire standard set of pheromone-induced genes with the exception of the missing *CPP1* gene. In fact, the level of expression generated for the

pheromone induced gene set by the standard pheromone addition was around 3-fold greater in the *cpp1* mutant compared to the wild type strain.

3.2. Does the hyper responsiveness of the *cpp1* null require MAP kinase function?

3.2.1. cek1Δ/Δ

We created a *cek1* mutant and a double mutant of *cek1* and *cpp1* to assess whether the Cek1 kinase is needed for the hyperactivity observed for the *cpp1* null mutant during pheromone response. As previously observed (Chen, Chen et al. 2002, Yi, Sahni et al. 2008), we found the Cek1 kinase to be important, but not absolutely essential, for *C. albicans* pheromone response and mating. Quantitative mating assays show the *cek1*Δ/Δ strain reduces mating to about 0.3% that of the wild type strain (Table 4). As shown in figure 7, this level of mating allows the *cek1* mutant strain to form detectable prototrophic mating products in cross print mating assays with the tester *MTL*α/α strain. However, the *cek1* mutant strain shows reduced shmoo formation in response to a pheromone treatment (Fig. 7), and they do not undergo efficient pheromone-mediated cell cycle arrest and thus do not make halos in a standard halo assay (Fig. 6). As well, when assessed for pheromone induced gene expression, they fail to induce any of the genes chosen from the literature to represent a pheromone-induced regulon (Table 6).

3.2.2. cek1Δ/Δ cpp1Δ/Δ

We created a *cpp1 cek1* mutant by inactivating the *CEK1* gene in the *cpp1*Δ/Δ strain using CRISPR as described in materials and methods. Loss of the Cek1 function eliminated the hyper

response of the *cpp1* mutant. The double mutant showed no halos (Fig. 6) and had reduced shmoo formation even relative to the wild type in response to pheromone (Fig. 7). The *cek1*Δ/Δ *cpp1*Δ/Δ strain reduces mating to about 0.4% that of the wild type strains (Table 3). As also shown in figure 8, they form infrequent prototrophic mating products in cross print mating assays with the tester *MTL*α/α strain. Finally, the double mutant showed poor induction of members of the pheromone-induced regulon, with most of the regulon genes showing weak or no induction in the presence of pheromone (Table 6). Thus the Cek1 kinase is essential for the hyper-pheromone-responsiveness shown by the *cpp1* mutant strain, while *cek1* mutants also lacking *cpp1* do not show better rate of mating than the *cek1* mutant alone.

3.2.3. cek2Δ/Δ

We also investigated the role of the Cek2 kinase in the pheromone hypersensitivity of the *cpp1* mutant. Previous work (Chen, Chen et al. 2002) had shown the Cek2 of strain WO-1 to be a minor player in pheromone response. Intriguingly, although loss of Cek2 generated only a minor effect on overall mating, reducing mating efficiency to approximately 36% that of the wild type strain (Table 4 & Fig. 8); in the SN148 background this loss enhanced pheromone responsiveness.

The c*ek2* mutant showed increased shmoo formation (Fig. 7) and greater pheromone-mediated cell cycle arrest resulting in the formation of large halos (Fig. 6). However, loss of Cek2 function had little effect on expression of the pheromone regulon; most of the regulon genes had expression levels similar to that observed in the wild type strain (Table 6).

3.2.4. cek2 Δ/Δ cpp1 Δ/Δ

Because loss of both *Cek2* and *Cpp1* enhanced aspects of pheromone response, we created a double mutant to assess the relationship between the MAP kinase and the MAP kinase phosphatase (Fig. 3A). *Cek2* was not needed for the enhanced responsiveness shown by the *cpp1* mutant. In fact, the *cpp1 cek2* double mutant showed further enhanced responsiveness over either single mutant. The double mutant generated large halos (Fig. 6), somewhat larger than those formed by either of the single mutants. The double mutant formed high levels of shmoos (Fig.7), and generated a very strong induction of the pheromone regulon in RNA sequencing analysis (Table 6). However, they mate at 2% of the wild type strain which is similar to *cpp1*Δ/Δ strain (Table 4 & Fig. 8). Thus loss of the Cek2 MAP kinase does not reduce the hyper pheromone response of the *cpp1* mutant strain, in fact it exacerbates it.

3.3. Does mating require both Cek1 and Cek2?

3.3.1. cek1Δ/Δ cek2Δ/Δ

Because we had found that loss of *cek2* in the *SN148* background enhanced aspects of pheromone response while reducing mating, we examined the *cek1 cek2* double mutant for mating and pheromone response. As found previously (Chen, Chen et al. 2002, Yi, Sahni et al. 2008) the *cek1 cek2* double mutant was completely non-responsive and non-mating. As shown in Figure 8, this strain fails to show formation of prototrophic mating products in cross print mating assays with the tester *MTL*α/α strain 3315, or to generate prototrophic cells in quantitative mating assays.

The cells also fail to make shmoos in response to a pheromone treatment (Fig. 7), and they do not undergo pheromone-mediated cell cycle arrest and make halos in a standard halo assay (Fig. 6). Finally, the double mutant was totally unable to induce the pheromone regulon (Table 6).

3.4. Does Cpp1 physically act on phosphorylated Cek1?

We investigated whether phosphorylation of the Cek1 activation loop was dependent on pheromone signaling and on Cpp1. First, I tagged Cek1 with GFP in CAI4 and *cpp1Δ/Δ* background strains and I treated the cells with pheromone and I performed western blotting using an antibody against GFP. Opaque *MTL* **a/a** *cpp1Δ/Δ* strain showed the accumulation of Cek1-GFP protein in response to pheromone compared to the CAI4 (Cek1-GFP) strain (Fig.9). Also, in separate experiments we treated opaque *MTL* **a/a** mutant strains (No GFP tag on Cek1) with pheromone, and we used the antibody against a phosphorylated form of the MAPKs (Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody), we again detected enhanced phospho-Cek1 in the *cpp1*Δ/Δ strain compared to wild-type strain. Therefore, consistent with previous observations (Li, Puri et al. 2015) the *CPP1* deletion resulted in Cek1 hyper-phosphorylation (Fig. 10), implicating Cpp1 as a regulator of the mating MAPK cascades.

3.5. Detection of Phosphorylation of MAPKs in mutant strains in response to pheromone.

In order to assess whether different mutations would alter cell susceptibility to mating pheromone and activate MAPKs differently, we exposed mutant strains to pheromone and measured the phosphorylation of MAPKs. I treated the cells with pheromone at 25°C for 3hrs and extracted the proteins. As shown in Figure 10, in the absence of the Cek1 cells show no activation and phosphorylation of MAPKs. Phosphatase Cpp1 removes the phosphate group

from activated Cek1. So in the absence of Cpp1 there is an accumulation of the phosphorylated form of MAPKs, even in the absence of pheromone treatment. Intriguingly, the absence of Cek2 enhances the phosphorylation of the MAPK Cek1 (darker band on gel) compared to the wild-type CAI4. Also, the *cpp1*Δ/Δ*cek2*Δ/Δ strain showed a hyper-phosphorylated form of the MAPKs compared to the WT CAI4 and either *cpp1*Δ/Δ or *cek2*Δ/Δ. A question that remains is why we do not detect phospho-Cek2 in the absence of Cek1 MAPK; what happens to Cek2 MAPK upon pheromone treatment, does it get phosphorylated after pheromone signal detection? It is possible that Cek2 gets phosphorylated either earlier than 3hrs or later, therefore, I treated the cells for 2hrs, 4hrs, and 6hrs. As shown in Figure 11, in the absence of Cek1 we do not get a phosphorylated Cek2 band, however, in lane 2 (*cpp1* Δ /Δ strain has GFP on Cek1 (75kd)) we get 2 bands - one at 49kd which is about the size of the Cek1 and Cek2 MAPK proteins, and one at 75 which corresponds to Cek1-GFP. If we call the 49kd band in the *cpp1* Δ /Δ (Cek1-GFP) strain phosphorylated Cek2 then it would likely be enhanced in the *cpp1* Δ /Δ *cek1* Δ /Δ strain which is not the case. Also, the band disappears in the *cek1*Δ/Δ stain. This suggested that the band in lane 2 could be the Cek1 protein lacking the GFP tag. (2 and 4 hours data not shown)

3.6. Deleting the components of the pheromone response pathway plays no role in switching

Each mutant strain (*cpp1* Δ /Δ, *cek1* Δ /Δ, *cek2* Δ /Δ, *cpp1* Δ /Δ *cek1* Δ /Δ, *cpp1* Δ /Δ *cek2*Δ and the wild-type CAI4) were individually tested for spontaneous white-opaque switching by plating cells from single white colonies at low density on SC-Glucose and SC-GlcNAc containing phloxine B, which differentially stained opaque cells red (Anderson, Soll 1987). The experiment was done twice and approximately five hundred derivative colonies were scored for each strain. In every case, red and sector colonies formed on Glucose containing plates were at

lower frequency than on GlcNAc plates after 7 days; which shows that GlcNAc efficiently triggers the cells to switch to the opaque state. Cells from every red colony of each mutant were found to exhibit the unique elongate opaque cell shape and were tested with F223-5H1-1 antibody which only stains opaque cells (Fig.4). The same experiment had been done starting with opaque cells and plating them on SC-Glucose and SC-GlcNAc and scores their switching rate (Table 3). (Data for SC-GlcNAc plates is not shown). All the strains showed the same rate of switching as that of the wild-type. As demonstrated previously (Yi, Sahni et al. 2008); switching is unimpaired with deletion of the genes in the pheromone response pathway.

3.7. Cek1-GFP

The Cek1-GFP fusion strain was created in both CAI4 and *cpp1*Δ/Δ background strains to investigate protein localization in response to pheromone. *C. albicans* Cek1-GFP was found at a low level in both the cytoplasm and the nucleus in non-stimulated cells, whereas there was a dynamic accumulation in the nucleus after response to pheromone stimulation (Fig.12). This is consistent with Cek1 playing a central role in pheromone response.

4. Discussion

It is only recently that the fungal commensal and opportunistic pathogen *Candida albicans* was identified as being mating competent; prior to this the organism was classified as asexual. This idea of asexuality was supported by consistent observations of *C. albicans* in clonal populations with little or no recombination and the fact that proliferation was through budding (Pujol, Reynes et al. 1993). Sex in general can be advantageous by purging deleterious mutations and by speeding up adaptation; even though it generates progeny more slowly than mitosis (Zhang, Magee et al. 2015).

The absence of sex in *C. albicans* was suggested to be advantageous because it protected rare allele combinations that allowed *C. albicans* to become one of the very few *Candida* species that colonize the human intestinal tract (Zhang, Magee et al. 2015). Intriguingly, sequencing of the *C. albicans* genome revealed genes with strong sequence similarity to *S. cerevisiae* mating and meiosis genes (Sadhu, Hoekstra et al. 1992, Tzung, Williams et al. 2001). However, there was the obvious possibility that these genes took on roles in *C. albicans* other than mating-related signaling. This view changed when in 1999 Hull and Johnson discovered the *C. albicans* mating-type-like (*MTL*) locus related to the *MAT* locus of *S. cerevisiae* and this revolutionized our understanding of sexual reproduction in *C. albicans.* In *S. cerevisiae,* sexual mating is controlled by genes located at the *MAT* locus. The *C. albicans MTL* locus resides on chromosome 5, and strains are typically heterozygous (*MTL***a**/α) at this locus, containing the two hetero-alleles *MTL***a** and *MTLα,* similar to S. *cerevisiae* (Lee, Ni et al. 2010). Allele *MTL***a** encodes the transcriptional regulators **a**1 and **a**2, and *MTLα* encodes the transcription regulators α*1* and α*2* (Soll, Lockhart et al. 2003).

The **a**1/α2 complex controls mating in *C. albicans* by repressing the genes required for mating and for white-to-opaque switching (Soll, Lockhart et al. 2003). White-opaque switching is regulated by the Wor1 regulatory protein and opaque cells are the mating competent form of the *C. albicans,* so blocking the white-to-opaque switch by inhibiting Wor1 will also block mating (Lee, Ni et al. 2010) (Fig.2). Therefore, white to opaque switch requires strains to be homozygous at the *MTL* (*MTL***a**/**a** or *MTL*α/α) locus and the opaque cells are many fold more effective at mating than the white forms. Thus *C. albicans* mating is regulated by a cryptic signaling pathway; the cells ability to mate is hidden under different levels of regulations, including the need to homozygose the mating type like locus, and to activate the epigenetic white opaque switching (Sun, Gadoury et al. 2016). There are two main reasons behind *C. albicans'* mating: recombination and adaptation. Recombination removes the undesired genetic insertions and allows the amplification of the good genes that increase the fitness of the organism. And, adaptation favors the positive fitness changes in the genome.

Our understanding of the mating signaling pathway of *C. albicans* has been strongly influenced by the extensive research on the pheromone response pathway of the model yeast *Saccharomyces cerevisiae*, as most of the pathway components in *S. cerevisiae* have been identified in *C. albicans*. Mating in *S. cerevisiae* is initiated by binding of the pheromone to Ste2/Ste3 seven-transmembrane-segment G protein coupled receptors which activates downstream MAPKs Fus3, Ste7, and Ste11 through activation of heterotrimeric G proteins and Ste5 Scaffold protein (Dietzel, Kurjan 1987, Whiteway, Hougan et al. 1989, Pryciak, Huntress 1998, Whiteway, Wu et al. 1995). Activated Fus3 MAPK then activates Ste12 transcription factor induces the transcription of pheromone-responsive genes (Pi, Chien et al. 1997). Similar to *S. cerevisiae,* in *C. albicans* G protein activation transfers the signal to the upstream elements of

the MAP kinase cascade initiating a series of phosphorylations of the components, Cst20, Ste11, Hst7 and the downstream Cek1/Cek2. The transcription factor Cph1 then gets activated to regulate gene expression involved in pheromone response and mating (Monge, Roman et al. 2006).

In this study I investigated the relationship between Cek1, Cek2 MAPKs and the phosphatase Cpp1 in human pathogen *C. albicans*. Although *S. cerevisiae* is used as a model organism for *C. albicans*, not all the processes are the same in the two species. Therefore, it is important to investigate the aspects of *Candida*'s pheromone signalling pathway directly. To better understand the mating pathway of *C. albicans* we created mutants of the components of this pathway and studied their influence on pheromone responses.

Our results confirmed that Cek1 and Cek2 are redundant MAP kinases in the pheromone-response mating pathway (Yi, Sahni et al. 2008, Chen, Chen et al. 2002). We have tested whether each kinase performs an equivalent function in the mating process; we investigated the mating behavior of the *cek1*Δ/Δ and *cek2*Δ/Δ mutant strains separately by creating *MTL***a**/**a** opaque cells and testing their ability to mate with an opaque *MTLα/α* tester strain. We have shown that in the absence of *CEK1* opaque cells are not able to make shmoos in response to pheromone, they do not make detectable halos on plates, and they do not induce pheromone-specific gene expression. They are, however, still able to mate in a very low frequency, unlike the sterile *cek1 cek2* double mutant. This indicates that the Cek1 MAPK is required for normal mating in *C. albicans,* but in the absence of Cek1 kinase, the Cek2 kinase can inefficiently substitute for its mating function.

Interestingly, deleting *Cek2* alone enhances the pheromone response of opaque cells; in response to pheromone treatment the cells show a clear cell cycle arrest and make visible halos, they show

extensive shmooing, and they induce the expression of mating-specific genes. The ability of *cek2*Δ/Δ cells to form halos is intriguing, because it is challenging to detect halos in wild-type strains. Moreover, the mating defect in *cek2* was not as severe as that in the *cek1* mutant.

These results show that although these two MAP kinases are redundant for mating they function quite differently in the pheromone response pathway. Based on our western-blotting results, in the absence of the Cek2 MAPK cells accumulate more of the phosphorylated form of the Cek1 kinase, which can be a reason why these cells respond more strongly to pheromone; it is possible that the more phosphorylated Cek1 protein in the cell, the longer the MAPK pathway stays active and the more intense a response we get. But a question that remains to be answered is why and how a MAP kinase (Cek2) appears to act both negatively (removal heightens responsiveness of the pathway) and positively (allows mating in a *cek1* deleted strain).

Both the *S. cerevisiae* and *C. albicans* mating pathways nominally contain a pair of MAP kinases; Fus3 and Kss1 in yeast, Cek1 and Cek2 in *C. albicans*. We have investigated whether there is a one to one mapping of the *C. albicans* kinases to their yeast orthologues, in other words does Cek1 correspond to Fus3 and Cek2 to Kss1 (or vice versa) or is the relationship among these kinases more complex? The earlier studies on evolution of the yeast genome and the emergence of new genes suggest that an ancestor of *Saccharomyces cerevisiae* underwent a whole-genome duplication (WGD) after it had diverged from the lineage that evolved into the CUG clade containing *C. albicans* and its close relatives. This indicates that the ancestral MAP kinase had been duplicated once before the whole genome duplication (generating Cek1 and Cek2) and the WGD would then have created two copies of each of the initially duplicated MAPKs; Cek1A, Cek1B, Cek2A and Cek2B. It is unclear from which sets of these potential MAPKs; the Cek2 group, the Cek1 group or one gene from each group, were the *S. cerevisiae*

MAPKs (Fus3 and Kss1) derived. Therefore, we have compared the structure and function of these MAPKs to predict their evolutionary trajectory.

Structurally, Cek2 shares 56% homology in amino acid sequence with Cek1 from *Candida albicans*, 53% homology with Fus3 and 49% homology with Kss1 from *S. cerevisiae*. Cek1 shares 59% homology with Fus3 and 57% homology with Kss1 (Chen, Wang et al. 2000). Thus simply based on overall structural similarity, a prediction would be that both Fus3 and Kss1 would have been derived from the ancestral Cek1 gene.

However, the most similar structural proteins are not the most functionally similar proteins. Previous studies showed that the *CEK2* gene can complement the yeast *fus3*/*kss1* mutant and allow mating, but the *CEK1* gene could not complement the *fus3*/*kss1* mutant (Chen, Wang et al. 2000). Cek2 and several components of the Cek1 pathway, including *STE2*, *GPA2*, *CST20*, *HST7*, *CEK1* and *CPH1*, are involved in mating responses. Cek2 has overlapping functions with Cek1 in mating pathway (Yi, Sahni et al. 2008) but is dispensable for filamentous growth on artificial media (Zhao, Mehrabi et al. 2007). Therefore, since *CEK2,* but not *CEK1,* complements the mating deficiency of the *fus3*/*kss1* mutant, and since it encodes a MAPK similar to that of *Fus3* MAPK*;* w*e* can propose that Cek2 is the Fus3 orthologue in *C. albicans*. However, our study shows that Cek1 plays a major role in mating of the *C. albicans* similar to that of Fus3 function in *S. cerevisiae* and Cek2 plays a minor role.

Although the *CEK1* gene expressed in *S. cerevisiae* cannot substitute for *KSS1* or for *FUS3* and re-establish mating in a *fus3 kss1* mutant strain, expression of Cek1 blocks pheromone arrest in the baker's yeast (Whiteway, Dignard et al. 1992b). A similar phenotype led to the initial identification of *KSS1,* as overproduction of the kinase blocked pheromone response in *S. cerevisiae* (Courchesne, Kunisawa et al. 1989).

The Cek1 MAPK is required for the filamentation MAPK cascade in *C. albicans* and probably functions analogous to the Kss1p MAPK of *S. cerevisiae* (Csank, Schroppel et al. 1998). Also, these two proteins Cek1 and Kss1 are structurally similar and the Cek1 and Kss1 MAPK cascade has been well characterized for their role in the yeast-hypha transition and virulence. Thus, we can still think that both Fus3 and Kss1 derived from the Cek1 lineage.

We confirmed that the Cpp1 protein phosphatase removes phosphate groups from Cek1 of the mating pathway upon activation with pheromone to negatively regulate this pathway. When the *CPP1* gene is deleted, we observed the accumulation of a phosphorylated form of the Cek1 kinase in a Western-blot and we observed that cells responded more efficiently to pheromone. The *cpp1*Δ/Δ strain generated large halos almost the same size as the halos observed for the *cek2*Δ/Δ strain. This halo formation is again intriguing because it is challenging to detect halos in wild-type strains. The *cpp1*Δ/Δ strain also shows extensive shmooing and induces expression of mating-specific genes (more than the wild-type). Therefore, Cpp1 is a negative regulator of the mating pathway that works directly on the Cek1 MAPK by removing phosphate group from this kinase. However, we have no evidence as to whether or not this phosphatase also deactivates the Cek2 MAPK by removing the activating phosphates.

To further investigate the function of these two MAPK and their phosphatase in the *C. albicans* mating pathway, we created double mutant strains *cek1*Δ/Δ*cek2*Δ/Δ, *cpp1*Δ/Δ*cek1*Δ/Δ, *and* *cpp1*Δ/Δ*cek2*Δ/Δ. The pheromone sensitivity and hyper responsiveness of *cpp1*Δ/Δ strains disappears upon deletion of the Cek1 MAPK. Thus, opaque versions of the *cpp1*Δ/Δ*cek1*Δ/Δ double mutant strain generated no detectable halos, they formed no shmoos, and they did not induce pheromone-specific genes in response to pheromone treatment. However, they are still

able to mate in a low frequency and that is presumably due to the presence of normal Cek2 which weakly complements the mating defect of the *cek1* mutant, because the *cek1cek2* double mutants are totally sterile. But the responses are not as efficient as when Cek1 gets activated (Zordan, Miller et al. 2007).

Pheromone response analysis of the *cek1*Δ/Δ*cek2*Δ/Δ strain confirmed the previous observations that in the absence of both Cek1 and Cek2*, C. albicans* cells fail to mate. They make no detectable halos, no shmoos, and exhibit no expression of pheromone specific genes in response to pheromone treatment. Thus, similar to *S. cerevisiae* where Kss1 complement mating when Fus3 is missing, and deletion of both genes make the cells sterile, in *C. albicans* also deletion of both MAPKs (Cek2 and Cek) make cells sterile.

Next, we were interested to see how the other MAPK (Cek2) would function when we deleted it along with *Cpp1*. Interestingly, when both *Cpp1* and *Cek2* are absent in the *C. albicans* cells, cells showed extreme sensitivity to pheromone. They generated very large halos; larger than those generated by either of the single mutant cells, and they formed extensive shmoos. They also induce pheromone-specific genes at a higher induction level than either of *cpp1*Δ/Δ or *cek2*Δ/Δ mutants, and to a much greater level than the wild-type strain. Consistent with this, Western-blotting showed a higher accumulation of the phosphorylated form of Cek1 MAPK compared to the wild-type strain and each of the single mutant strains (*cpp1*Δ/Δ and *cek2*Δ/Δ). We hypothesize that Cek2 can act as a kinase (a positive regulator) and also as a negative regulator of the mating pathway; therefore, in the absence of Cek2 the pathway stays active for longer time and cells respond more efficiently to pheromone, similarly to the situation when we delete the Cpp1 phosphatase.

Intriguingly we have not detected activated Cek2 MAPK with our P44/42 MAPK (Erk1/2)(Thr202/Tyr204) polyclonal antibody which identifies the endogenous levels of p44 and p42 MAP Kinase (Erk1 and Erk2) when phosphorylated at Thr202 and Tyr204 of Erk1 (Thr185 and Tyr187 of Erk2).

There are alternative scenarios that can explain this observation. One possibility is that the antibody does not recognize *C. albicans* Cek2 MAPK because it was developed against the residues surrounding Thr202/Tyr204 of human p44 MAPkinase by immunizing animals with a synthetic phosphopeptide containing these residues. Protein alignment of Cek1 and Cek2 shows conserved kinase motif after TEY residues (Thr163/Tyr165 on Cek1 and Thr187/ Tyr 189 on Cek2) but it is not conserved before TEY so that could be a possibility why Cek2 MAPK is unrecognizable with the antibody. Another possibility is maybe Cek2 does not get activated by phosphorylation in response to pheromone, maybe it has an alternative way of responding to pheromone other than phosphorylation or maybe it does not respond at all to pheromone.

To further study the function of the MAPK Cek2, we are tagging Cek2 with GFP in different strain backgrounds (CAI4, *cek1*Δ/Δ, *cpp1*Δ/Δ and *cpp1*Δ/Δ*cek1*Δ/Δ). As a future work and as a first step to unveil the function of this intriguing MAPK Cek2 of *C. albicans* we can use these Cek2-GFP tagged strains and treat the cells with pheromone and use the antibody against GFP. This experiment can answer our question that whether or not Cek2 gets activated upon pheromone detection by cells by detecting the accumulation of Cek2 in pheromone activated cells. Also, we can look at the cells under the microscope to see the movement of the Cek2-GFP MAPK in response to pheromone.

Our investigation, in revealing how the two MAPKs might control the signal transduction in this pathogen, raises several questions. How does Cek2 function both as the activator and negative

regulator of the pathway? Why upon deletion of both Cek2 and Cpp1 do cells become so sensitive and super-responsive to pheromone more than each of the single mutants? Why are we were unable to detect Cek2 phosphorylation with our P44/42 MAPK (Erk1/2)(Thr202/Tyr204) polyclonal antibody?

As a summary, in the absence of Cek1 MAPK, Cek2 is able to activate the Cph1 mating specific transcription factor poorly so that the cells are able to mate at low rate of 0.3%. Interestingly, when we have a normal Cek1 MAPK and deleting Cek2 MAPK cells get hyperactive and show more sensitivity to pheromone signals as if Cek2 MAPK was a negative regulator of the Cek1 MAPK upon activation. So based on the results, we can hypothesize that Cek2 MAPK has some basal activities independent of detecting a pheromone signal and when cells miss the other MAPK (Cek1) Cek2 complements the mating. We also hypothesize that in the normal cells after the Cek1 mating pathway gets activated by detecting pheromone, Cek2 MAPK can then signal to deactivate the Cek1 MAPK, because when we remove Cek2 MAPK cells show more phospho-Cek1 in western-blotting, and cells stays active for longer time so they form halos and nice shmoos.

Therefore, a comprehensive understanding of the mating pathway and its underlying mechanisms in *C. albicans* still require more investigation.

5. Figures and Tables

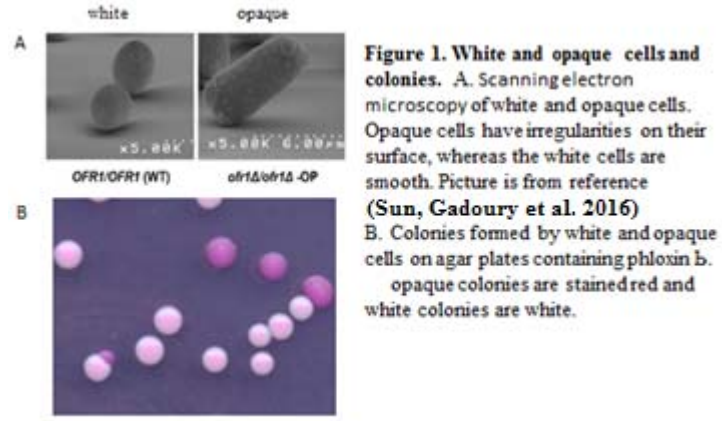


Figure 1. White and opaque cells and colonies. A. Scanning electron microscopy of white and opaque cells. Opaque cells have irregularities on their surface, whereas the white cells are smooth. Picture is from reference **(Sun, Gadoury et al. 2016)** B. Colonies formed by white and opaque cells on agar plates containing phloxin b. opaque colonies are stained red and white colonies are white.

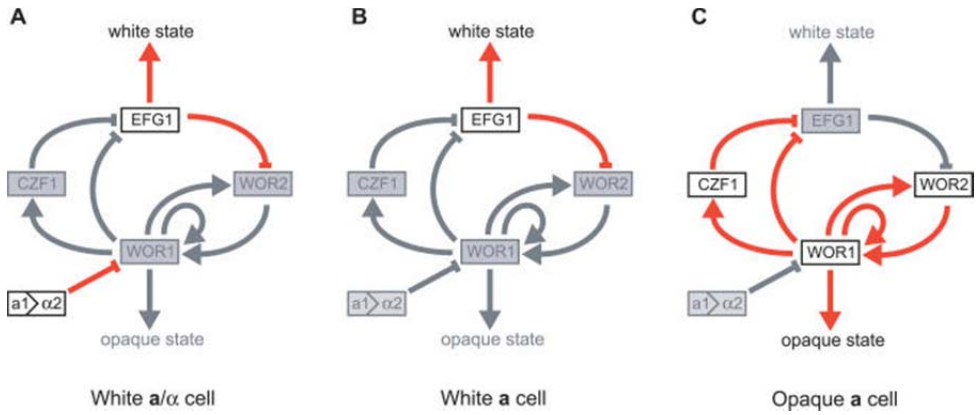


Figure 2. White-Opaque switching circuitry. Arrows indicate activation and bars indicate repression. Figure is taken from reference (Zordan, Miller et al. 2007).

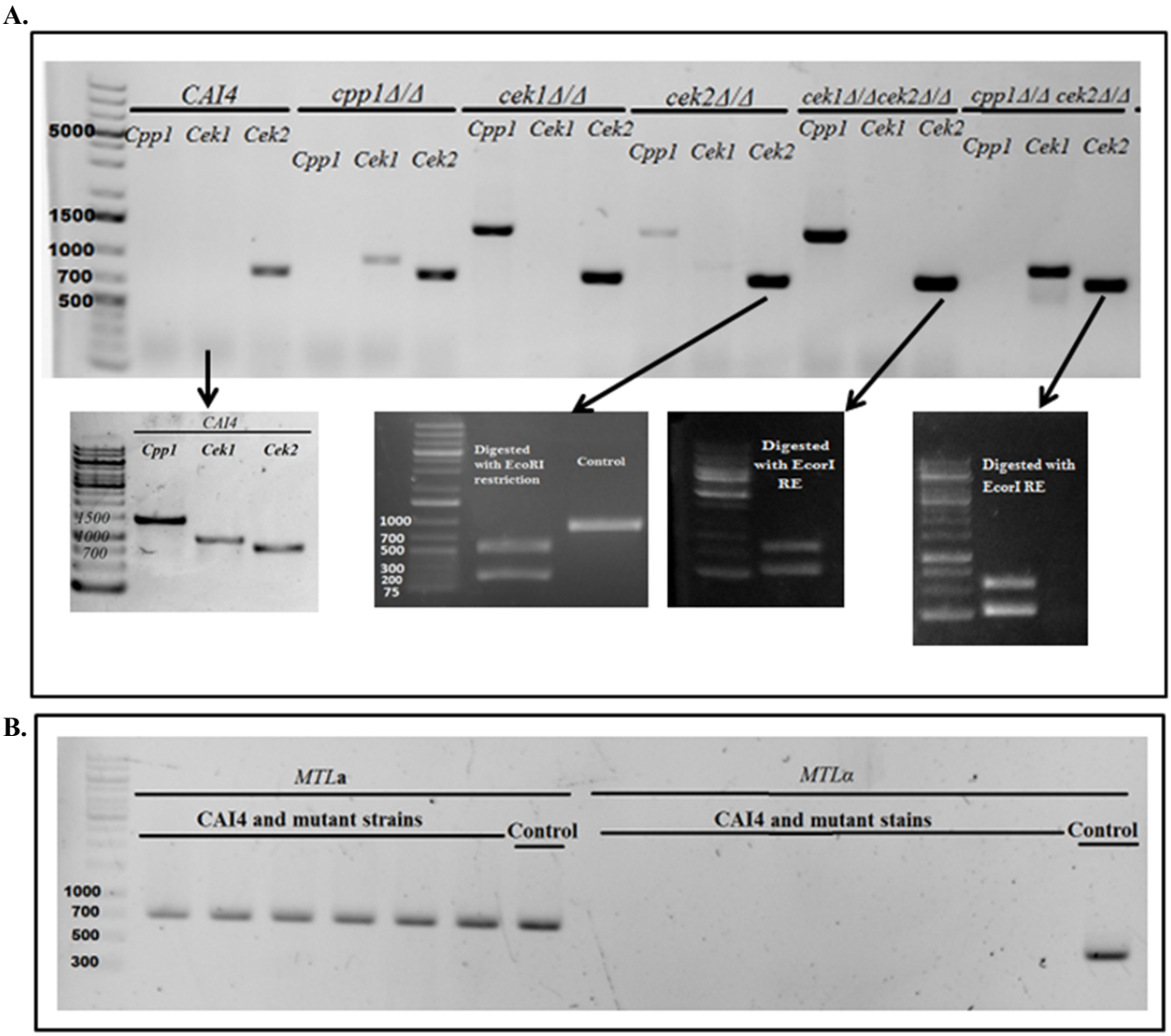


Figure 3. Genotype confirmation with PCR. A. *Cek2* gene was disrupted in *cpp1Δ/Δ cek1Δ/Δ*, *cek1Δ/Δ cek2Δ/Δ,* and *cek2Δ/Δ* strains by CRISPR method. Two consecutive stop codons and one EcoRI restriction enzyme were introduced in the beginning of the *Cek2* gene. Deletion of the gene was confirmed by amplifying the *Cek2* gene and digesting it with newly introduced EcoRI site. As you can see in the figure above two correct band size were shown after digestion. B. MTL locus of all strains were checked with *MTL* **a/a** and *MTL α/α* specific primer and as it is shown all the strains except the control are *MTL* **a/a.**

Table1. Oligonucleotides used to delete the C. albicans specific cell cycle genes

Name	Description	Sequence (5' to 3')	Source
CEK2 **deletion**	**SgRNA Forward**	atttgTGCTTATGGCATTGTTGCTTg	This study
	SgRNA Reverse	aaaacAAGCAACAATGCCATAAGCAc	This study
	Repair DNA Forward	atatctcaaaactttaaagtgctcaagattttgggagagggtgcttaAggAattCttgcttAgC	This study
	Repair DNA Reverse	ttttttgatggccaccttagtctctgtgggtaaatgtactgGcTaagcaaGaatTccTtaagc	This study
	Checking primers Forward	tacaaccaagttttacaatacctg	This study
	Checking primers Reverse	agataaacttcattgaagctgtc	This study
CEK1 **deletion**	**SgRNA Forward**	atttgacaacaacaagctcaggctcg	This study
	SgRNA Reverse	aaaacgagcctgagcttgttgttgtc	This study
	Repair DNA Forward	caa cat cat cag ctt caa cag caa cat caa caa caa atg tt aTaacaacGaATtcaggct Tag gc	This study
	Repair DNA Reverse	gttgttgttgttgttgttgtgcttgagcttgagcctgagcctAagcctgaATtCgttgttAt	This study
	Checking primers Forward	tgaatatttcgaccacgtca	This study
	Checking primers Reverse	agctacgtattctgtcatga	This study

***Cek1*-GFP**	**CEK1-S1-GFP**	TCGATTTTGATAAAATGAAAGATCAATTAACAATTGAAGATTTGA AAAAATTGTTATATGAAGAGATTATGAAGCCATTA ggtgctggcgcaggtgcttc	This study
	CEK1-S2-GFP	CCTATACAACAACAATTATGCTAAATCTACAACAACTACCAAGCC CAACCTATAGTTTTTAGTTTAGTTTAGTTTAGTTT tctgatatcatcgatgaattcgag	This study
	CEK1-CHK-F	TACCAACAACAATACTAGTAC	This study
	CEK1-CHK-R	CGGTGTTATTAAATCTCCTA	This study

Table 2. ***Strains used in this study.***

Strains	Parent	Mating type	Description	Source
CAI4 *MTLa*	**CAI-4**	**a/a**	*ura3 ::imm434/ ura3 ::imm434*	Doreen Harcus
CP29-1-7L4	**CAI-4**	**a**/α	*ura3/ura3 cpp1 ::hisG/cpp1 ::hisG*	Csilla Csank,1997
Cpp1Δ/Δ	**CP29-1-7L4**	**a/a**	*ura3/ura3 cpp1 ::hisG/cpp1 ::hisG*	This study
CK43B-16L		**a**/α	*ura3/ura3 cek1 ::hisG/cek1 ::hisG*	Csilla Csank,1998
Cek1Δ/Δ	**CK43B-16L**	**a/a**	*ura3/ura3 cek1 ::hisG/cek1 ::hisG*	This study
Cek2Δ/Δ	**CAI4 *MTLa***	**a/a**	*ura3/ura3 cek2::TAA(32tha.a&37tha.a)/cek2::TAA(32tha.a &37tha.a)*	This study
Cpp1Δ/ΔCek2Δ/Δ	**Cpp1Δ/Δ**	**a/a**	*ura3/ura3 cpp1/cpp1; cek2::TAA(32tha.a&37tha.a)/cek2::TAA(32tha.a &37tha.a)*	This study
Cek1Δ/ΔCek2Δ/Δ	**Cek1Δ/Δ**	**a/a**	*ura3/ura3 cek1/cek1; cek2::TAA(32tha.a&37tha.a)/cek2::TAA(32tha.a &37tha.a)*	This study
Cpp1Δ/ΔCek1Δ/Δ	**Cpp1Δ/Δ**	**a/a**	*ura3/ura3 cpp1/cpp1; cek1::TAA(19tha.a&25tha.a)/cek1::TAA(19tha.a &25tha.a)*	This study
Cpp1Δ/Δ;Cek1-GFP	**Cpp1Δ/Δ**			This study
Cai4, Cek1-GFP	**CAI-4**			This study
3315	**A505**	α/α	*trp1/trp1; lys2/lys2*	Magee

Table 3. Ratio of white-opaque switching on SC-Glucose media.

Row	Strains		Initial Cell Type	Carbon Source	Switching Ratio%
	Relevant genotype				
1	CAI4 (WT)	***MTLa/a***	WH	Glucose	0.45±0.05
2	Cpp1*Δ/Δ*	***MTLa/a***	WH	Glucose	1.3±0.3
3	Cek1*Δ/Δ*	***MTLa/a***	WH	Glucose	0.4±1
4	Cek2*Δ/Δ*	***MTLa/a***	WH	Glucose	1.1±0.1
5	Cek1*Δ/Δ* Cek2*Δ/Δ*	***MTLa/a***	WH	Glucose	0.16±0.3
6	Cpp1*Δ/Δ* Cek2*Δ/Δ*	***MTLa/a***	WH	Glucose	1.23±0.13
7	Cpp1*Δ/Δ* Cek1*Δ/Δ*	***MTLa/a***	WH	Glucose	0.7±0.1
9	CAI4 (WT)	***MTLa/a***	OP	Glucose	0.6±0.1
10	Cpp1*Δ/Δ*	***MTLa/a***	OP	Glucose	0.5±0.05
11	Cek1*Δ/Δ*	***MTLa/a***	OP	Glucose	3.3±1.5
12	Cek2*Δ/Δ*	***MTLa/a***	OP	Glucose	0.6±0.1
13	Cek1*Δ/Δ* Cek2*Δ/Δ*	***MTLa/a***	OP	Glucose	1.5±1.5
14	Cpp1*Δ/Δ* Cek2*Δ/Δ*	***MTLa/a***	OP	Glucose	0.4±1
15	Cpp1*Δ/Δ* Cek1*Δ/Δ*	***MTLa/a***	OP	Glucose	1.2±0.2

Strain	Mating partner	Mating percentage(%)	Percentage of mating relative to WT (CAI4)
CAI4	3315α	23.5±1.5	100%
*cpp1*Δ/Δ	3315α	0.33±0.03	1.4%
cek1Δ/Δ	3315α	0.07±0.02	0.3%
cek2Δ/Δ	3315α	8.5±0.5	36%
cpp1Δ/Δcek2Δ/Δ	3315α	0.37±0.02	1.6%
cek1Δ/Δcek2Δ/Δ	3315α	0	0%
cpp1Δ/Δcek1Δ/Δ	3315α	0.09±0.05	0.4%

Table 4. Quantitative mating assays. Tester strains and experimental strains were pre-cultured in SC-Glucose liquid medium for 24 hours and then mixed in fresh liquid SC-Glucose medium at a concentration of 1x 107 cells/ml for all strains. Mixed cells were incubated at RT for 48 hours and then plated onto selection media to detect auxotrophic mating products. The mating frequency is calculated as described in the materials and methods session. Opaque cells of the mutants' *cek1Δ*/Δ *cek2*Δ/Δ do not mate with opaque cells of opposite mating type 3315α strain; whereas, mutants *cek1*Δ/Δ and *cek2*Δ/Δ mate but at reduced frequency. Percentage of reduction in mating was computed by subtracting the percentage of mating of different mutant strains from the percentage of the mating of the wild-type (CAI4) strain divided by the percentage of the mating of the wild-type (CAI4) strain, and the fraction multiplied by 100.

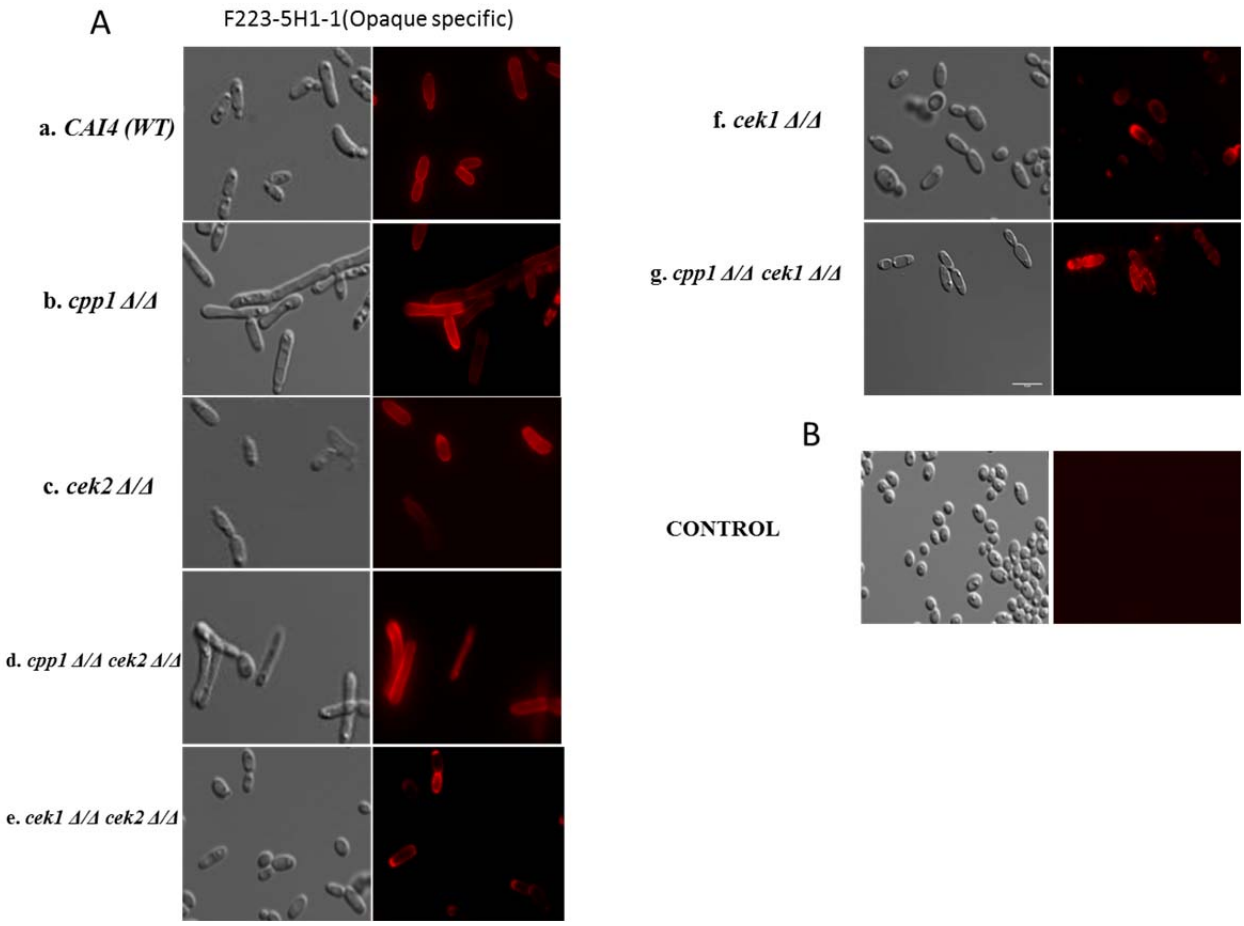


Figure 4. Immunofluorescence microscopy. (A) Immunofluorescence microscopy of opaque-like colonies of strains. (B) Control sample is the *cpp1* white cells treated only with the secondary antibody. Cells were washed with 1X PBS and blocked with 1ml blocking buffer (2% BSA, and 1% goat serum in PBS) for 30 minutes. F223-5H1-1 monoclonal antibody was used as a primary antibody and Texas Red-conjugated goat anti-mouse antibody was used as the secondary antibody to identify opaque cells. Cells were observed under LEICA_DM6000 fluorescent microscope at 40X magnification. Bar, 13.00 μm.

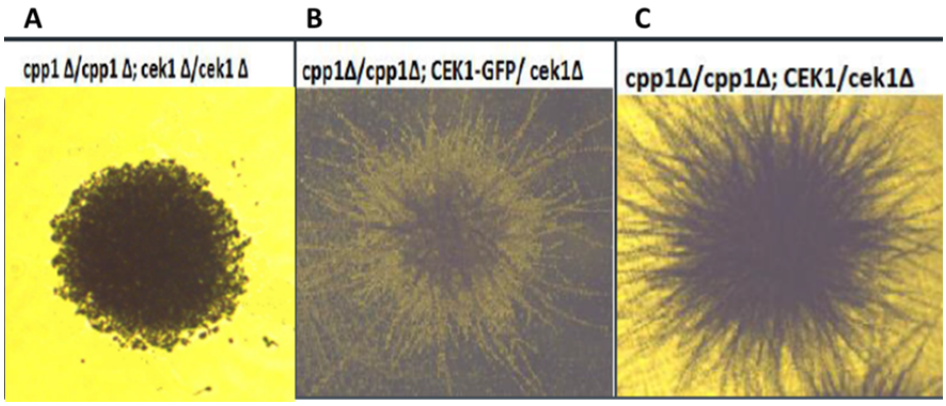


Figure 5. Spider media. Spider media were used to check if the Cek1 is still functional after being tagged with GFP. Our strains had only one functional copy of the Cek1 gene, therefore, if tagging disrupted the other copy of this gene (the other allele) the phenotype of the Cpp1 null mutant which is hyphal growth and germ tube formation on the spider media would be suppressed.

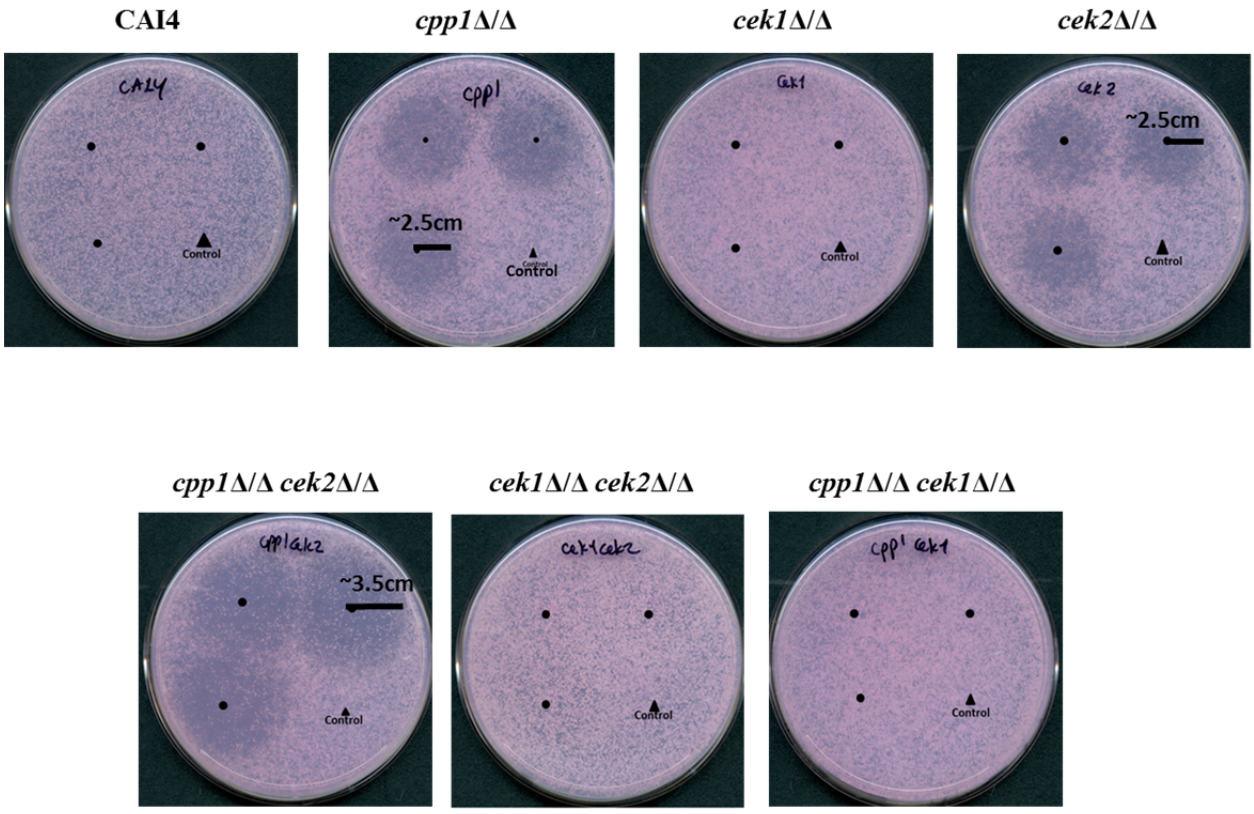
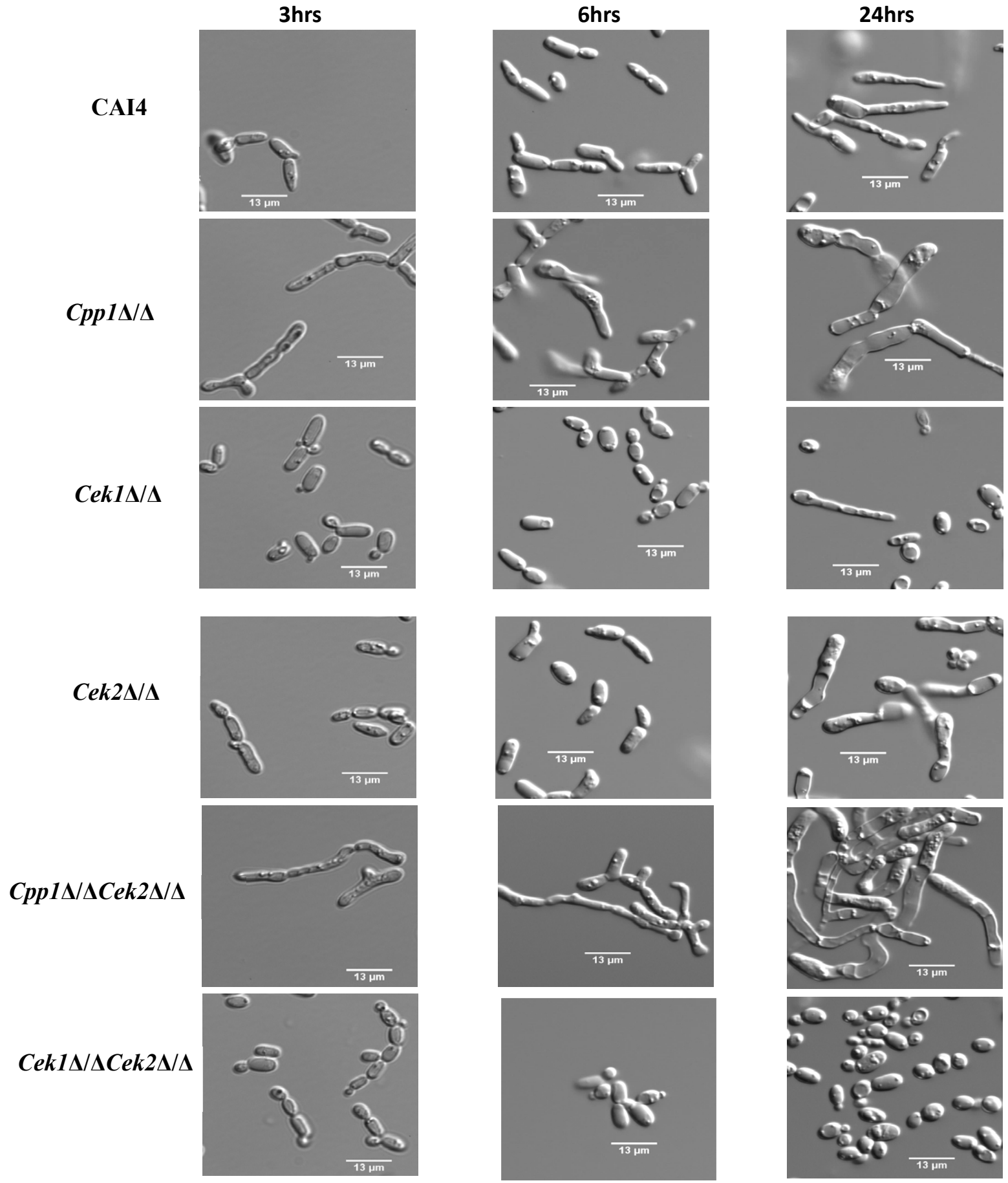


Figure 6. Halo assay with *C. albicans* mutant strains and wild-type strain. Halo assays were performed by plating a lawn of opaque **a** cells on SC-Glucose plates and spotting alpha pheromone (1 μg) or a control (50%methanol) onto each plate. Pheromone was spotted on the center of each three circle drawn on the plates, and the 50%methanol control was spotted on the fourth spot. Plates were incubated at room temperature for 2 days for the lawn of cells to grow up and then were photographed. Halo activities of opaque cells from mutants were compared to wild-type strains. *cpp1*Δ/Δ, *cek2*Δ/Δ and *cpp1*Δ/Δ *cek2*Δ/Δ are hyper responsive to pheromone treatment as they generate large halos compared to the wild type strain which generates almost imperceptible halos in a standard halo assay test. The size of the halos was measured over the plates.


3hrs
6hrs
24hrs
CAI4
Cpp1Δ/Δ
Cek1Δ/Δ
Cek2Δ/Δ
Cpp1Δ/ΔCek2Δ/Δ
Cek1Δ/ΔCek2Δ/Δ
13 µm

***Cpp1*Δ/Δ*Cek1*Δ/Δ**

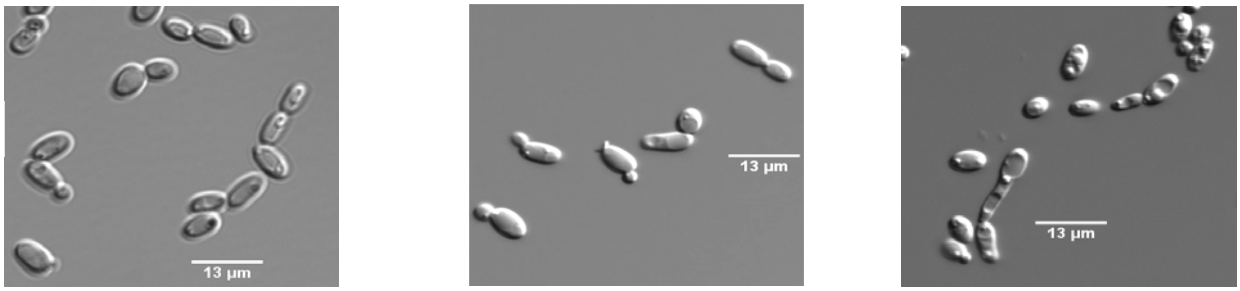


Figure 7. Pheromone response assay. Opaque-like cells were treated with α-pheromone for 3hours, 6 hours and 24 hours. Cells were observed under LEICA_DM6000 microscope. α-pheromone does not induce conjugation tube formation (shmoos) in opaque cells of the mutants *Cek1*Δ/Δ, *Cpp1*Δ/Δ*Cek1*Δ/Δ after 3hrs and 6 hrs of treatment and less than 0.1% of cells showed shmoo after 24 hrs of treatment. *Cek1*Δ/Δ*Cek2*Δ/Δ never induced conjugation tube formation. The "percentage of shmooing and reduction in induced shmoos" is computed in table 5. Bar 13µm.

Strain	Cell type	Hours of treatment with α-factor	Shmoo (%)	Reduction(R) or Increase (I) in induced shmoo (%)
CAI4	OP	3	57±3	_
		6	81±1	_
		24	100	_
***cpp1*Δ/Δ**	OP	3	91±1	60 (I)
		6	98.5±0.5	22 (I)
		24	100	_
cek1Δ/Δ	OP	3	2±0.25	97 (R)
		6	2.1±0.2	97 (R)
		24	7.5±0.5	92.5 (R)
cek2Δ/Δ	OP	3	21±1	63 (R)
		6	56.5±0.5	30 (R)
		24	90.5±0.5	9.5 (R)
cpp1Δ/Δcek2Δ/Δ	OP	3	95.5±0.5	68 (I)
		6	99	22 (I)
		24	100	_
cek1Δ/Δcek2Δ/Δ	OP	3	0.00	100 (R)
		6	0.00	100 (R)
		24	0.00	100 (R)
cpp1Δ/Δcek1Δ/Δ	OP	3	2.4±0.4	96 (R)
		6	4±1	95 (R)
		24	9.5±0.5	90.5 (R)

Table 5. Percentage of shmoo formation for different strains. The percentage of reduction or increase in induced shmoos was computed by dividing the difference in percentage of shmooing between CAI4 and mutant strain, by percentage of shmooing of CAI4, and multiplying by 100%.

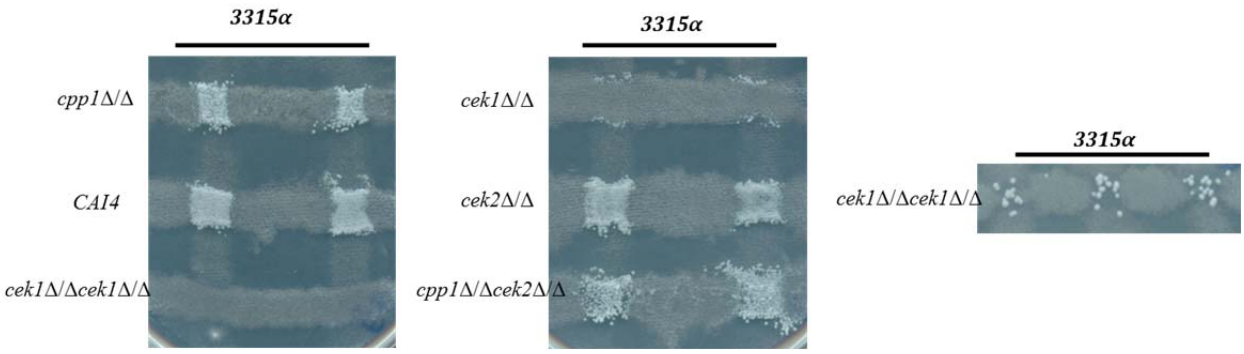


Figure 8. SC-GlcNAc media mating assay. Strain 3315α, in the opaque state, were used as mating type testers. It has the auxotrophic marker trp1/trp1 and lys2/lys2. This tester were crossed with mutant strains on both SC-Glucose and SC-GlcNAc agar medium at RT for 2 days and then replicated on selection medium SC-Glucose (Trp- Lys- Ura-) at 30°c for 3 days to detect auxotrophic mating products.

Gene	ORF	CAI4	cpp1Δ	cek1Δ	cek2Δ	cek1Δcek2Δ	cpp1Δcek2Δ	cpp1Δcek1Δ
PRM1	19.8286	13	25	0	10	0.8	182	0
FIG1	19.138	45	154	0	17	0	659	82
SST2	19.4222	7	19	0.6	6	0.5	116	0.9
RBT1	19.1327	13	72	0.7	7	0	93	5
HWP1	19.1321	8	40	0	9	0.1	37	0.2
CEK2	19.46	6	10	0.9	5.2	0.5	19	1.7
STE2	19.696	9	18	0.3	10	0.3	52	1.2
RBT4	19.6202	15	92	0.6	18	0.4	120	2.3
KAR4	19.3736	9	26	0.6	6	0.9	93	6
RAM1	19.5046	13	32	0.6	19	0.6	108	1.8
CEK1	19.2886	4	11	0.6	8	0.2	34	0
PCL1	19.2649	7	38	0.2	7	1.5	91	8
CPP1	19.4866	2.5	0.84	1.4	1.8	0.9	2	0.2
FAV1	19.3801	12	56	0.8	30	0.6	127	5
FAV2	19.112	25	73	0.7	14	0	65	4.5
FAV3	19.1914	3	12	0.9	1.4	0.6	60	2
ASG7	19.552	19	11	0	8	0	61	0.7
FGR23	19.1616	26	50	2.6	25	0.9	189	6
C2_07220	19.2278	1.4	25.6	1.3	1.8	0.8	59	16
DAG7	19.4688	4	12	0.7	4	0.5	24	1.7
POL	19.2219	18	20	1.2	10	0.9	54	2.9

Strains	CAI4	*Cpp1Δ/Δ*	Cek1Δ/Δ	Cek2Δ/Δ	Cek1Δ/ΔCek2Δ/Δ	Cpp1Δ/ΔCek2Δ/Δ	Cpp1Δ/ΔCek1Δ/Δ
# of upregulated genes out of 21 pheromone induced selected genes	18	19	0	17	0	20	2*

**FIG1* and C2_07220 genes are induced in Cpp1Δ/ΔCek1Δ/Δ

Table 6. RNA-Sequence analysis: Twenty-one pheromone-induced genes from the literature (Zhao, Daniels et al. 2005) were selected as the reference set for assessing the pheromone response of various strains. RNA-Seq results were normalized against the WT CAI4 strain not treated with pheromone. Selected genes were defined and were counted as upregulated if they were located in the top 200 genes ranked by fold increase over control for the strain.

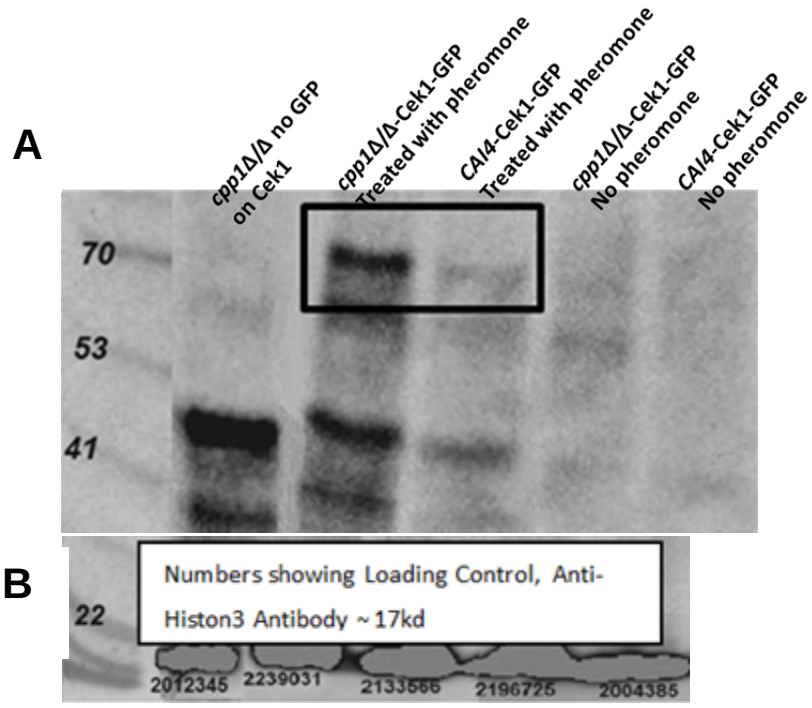


Figure 9. Western-blotting using Anti-GFP antibody. (A) This figure shows an accumulation of the phosphorylated form of *Cek1-GFP* MAPK in the *Cpp1*Δ/Δ strain compare to the wild-type *CAI4* strain. The mating pathway was activated in both strains by treating the cells with α-pheromone for 3 hours. The protein size of the *cek1* (~48kd) plus GFP (27kd) is 75kd in total. Other MAP kinases (*cek2*) protein sizes are ~48kd. (B) Anti-histon3 antibody was used as a loading control; its protein size is ~17kd. Proteins were quantified using Bradford method and equal amounts of proteins were loaded.

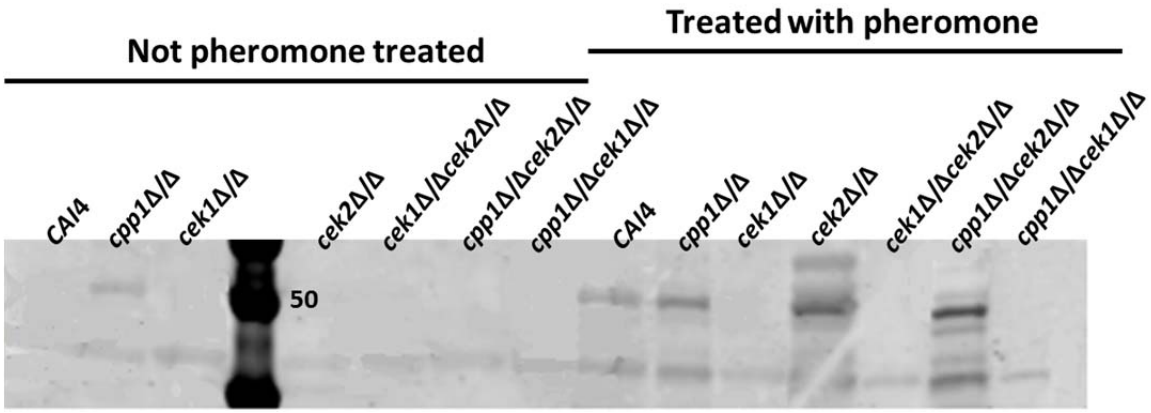


Figure 10. Western-blotting using F223-5H1-1 antibody against phosphorylated MAPKs. The mating pathway was activated by treating the cells with α-factor for 3 hours. The protein size of the *cek1* and *cek1* is ~48kd. We used the antibody against the phosphorylated form of the MAPKs (Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody). The phosphor-MAPK band disappears in the absence of Cek1 MAPK which indicates no phospho- Cek2 MAPK. There is an increased in accumulation of phosphorylated Cek1 MAPK in *cpp1*Δ/Δ, *cek2*Δ/Δ, and *cpp1*Δ/Δ *cek2*Δ/Δ. (The accumulation of phosphorylated Cek1 increases from *cpp1*Δ/Δ through *cek2*Δ/Δ to its maximum in *cpp1*Δ/Δ *cek2*Δ/Δ). Proteins were quantified using Bradford method and equal amounts of proteins were loaded.

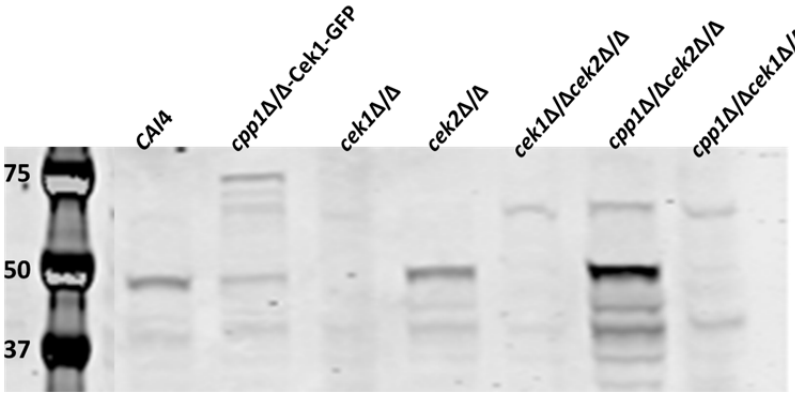


Figure 11. Western-blotting. The mating pathway was activated by treating the cells with α-factor for 6 hours. The protein size of the *cek1* and *cek1* is ~48kd. We used the antibody against the phosphorylated form of the MAPKs (F223-5H1-1 antibody). The phosphor-MAPK band disappears in the absence of Cek1 MAPK in *cek2*Δ/Δ strain, but there is a faint band in *cpp1*Δ/Δ (Cek1-GFP) and *cpp1*Δ/Δ *cek1*Δ/Δ which needs further investigation. There is an increased in accumulation of phosphorylated Cek1 MAPK in *cpp1*Δ/Δ, *cek2*Δ/Δ, and *cpp1*Δ/Δ *cek2*Δ/Δ. (The accumulation of phosphorylated Cek1 increases from *cpp1*Δ/Δ through *cek2*Δ/Δ to its maximum in *cpp1*Δ/Δ *cek2*Δ/Δ). Proteins were quantified using Bradford method and equal amounts of proteins were loaded.

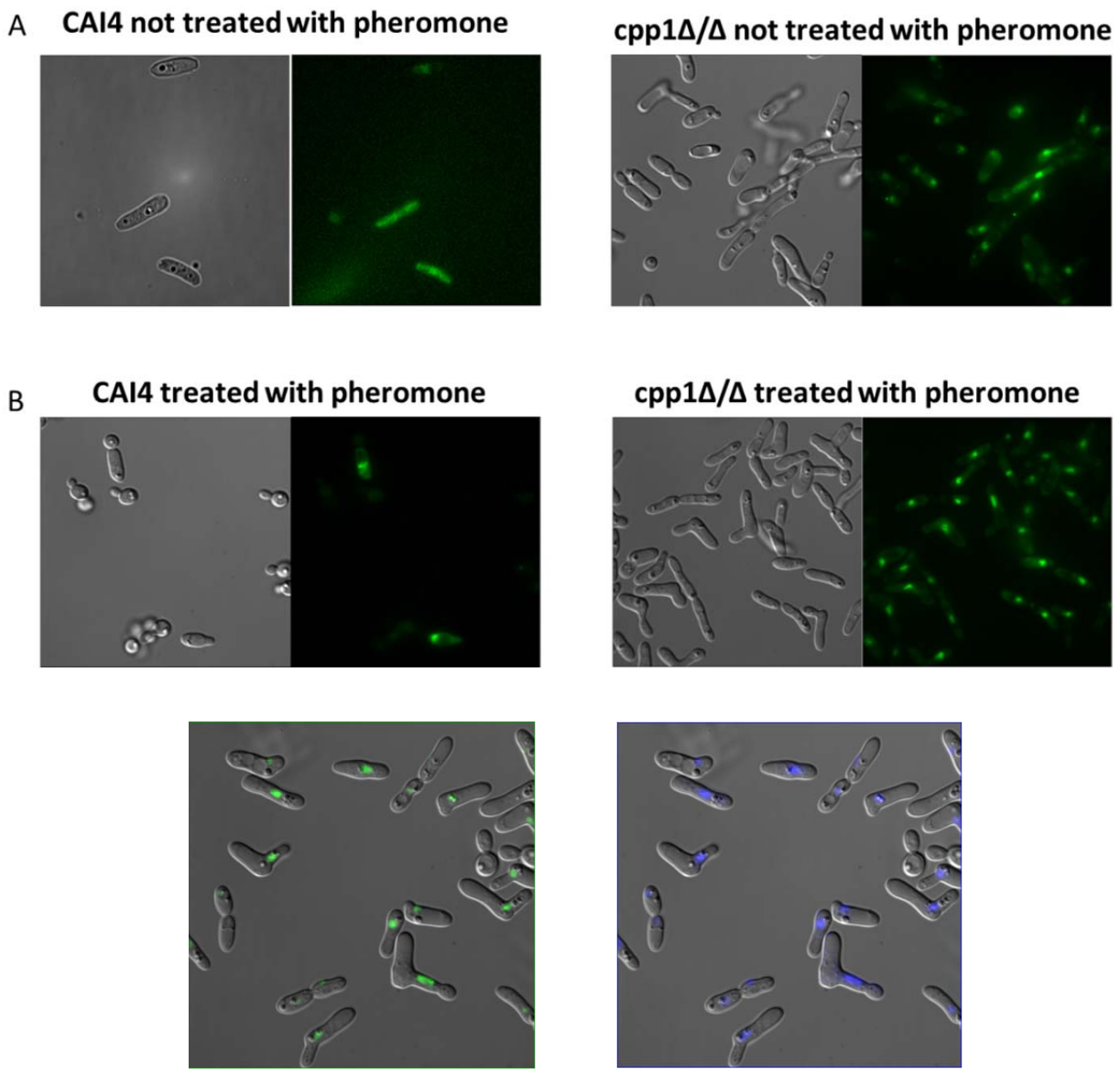


Figure 12: **Fluorescent Microscopy**. (A) Opaque cells of the wild-type CAI4 (Cek1-GFP) and *cpp1* Δ/Δ (Cek1-GFP) strains were observed under the Tirf microscope before treating them with pheromone. GFP-Cek1 fluorescence is detected all over the cell for CAI4 (Cek1-GFP) and both in nucleus and cytoplasm in *cpp1* Δ/Δ strain before pheromone treatment. (B) Cells are treated with pheromone for 3 hrs. GFP signal concentrated in one point which then by DAPI staining (C) we confirmed that it corresponds to the nucleus. Therefore, there is co-localization of Cek1-GFP and the Nucleus.

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