Characterizing Genes Of *Candida albicans* Involved In Invasiveness, Mating And Genotoxic Stress Response

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Abstract

Characterizing Genes Of *Candida albicans* Involved In Invasiveness, Mating And Genotoxic Stress Response

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Concordia University, 2018

In the life cycle of the fungal pathogen *Candida albicans,* the formation of filamentous cells is a differentiation process that is critically involved in host tissue invasion and in adaptation to host cell and environmental stresses. Here we have used the GRACE™ library to identify genes controlling invasiveness and filamentation; conditional repression of the library revealed 69 mutants that triggered these processes. Intriguingly, the genes encoding the SUMO E3 ligase Mms21 and all other tested members of the sumoylation pathway were both non-essential and capable of triggering filamentation upon repression, suggesting an important role for sumoylation in controlling filamentation in *C. albicans*. Both Mms21 nulls (*mms21Δ/Δ*) and SP domain (SUMO E3 ligase domain) deleted mutants displayed filamentation and abnormal nuclear segregation. Transcriptional analysis of *mms21Δ/Δ* showed an increase in expression from 2 to 8 fold above WT for hyphal specific genes (HSGs) including *ECE1*, *PGA13*, *PGA26, HWP1, ALS1, ALS3, SOD4*, *SOD5, UME6 and HGC1*. The Mms21 deleted mutants were unable to recover from DNA damaging agents like methyl methane sulfonate (MMS), hydroxyurea, hydrogen peroxide and UV radiation, suggesting that the protein is important for genotoxic stress responses.

Mating in *C. albicans* requires switching from mating sterile white form to mating competent opaque form and this is only achieved in mating type-like (*MTL*) homozygous strain. In a heterozygous *MTL* (*MTLa/α*), protein heterodimer of a1-α2 will bind and repress Wor1, a master regulator of white-opaque switching. We have screened the GRACE library for genes that can be involved in *MTL a/α* mating and found 10 mating positive strains but half of them had become homozygous at *MTL*. Among the heterozygous mating positive mutants, Mms21 was identified which upon deletion in other strain background did mate with 3315*αα* testers. By performing phenotypic and genetic analysis, we have found that Mms21 deleted mutants, due to increased cellular and genotoxic stress, have increased homozygosis at *MTLaa* followed by increased white-opaque switching and subsequent mating. This analysis provides a direction of how natural Candida *MTL a/α* cells inside a stressful host environment can achieve mating.

We have performed a large scale GRACE library screen for MMS sensitivity and have identified 56 genes sensitive to MMS upon tetracycline shut off. About half of those sensitive mutants were involved in DNA replication, DNA damage and cytokinesis. Among the cytokinesis sensitive mutants was Hof1. Deletion of CaHof1 renders cells sensitive to MMS and generates genome instability that partially activates checkpoint kinase CaRad53. CaRad53 down-regulates CaHof1 in response to MMS. Genetic interaction analysis shows that CaHof1 is involved in the CaRad53 pathway and epistatic to nuclear excision repair pathway. Our data suggests a novel function of Hof1 in DNA damage response.

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List of Abbreviations

Small Ubiquitin like Modifier	SUMO
Siz/PIAS domain	SP
Structural Maintenance of Chromosome	SMC
Cyclic AMP/ Protein Kinase A	cAMP/PKA
5-fluoroorotic acid	5-FOA
Methyl Methane Sulfonate	MMS
Gene Replacement And Conditional Expression	GRACE
N-Acetylglucosamine	GlcNAc
Yeast Nitrogen Base	YNB
Loss of heterozygosity	LOH
Mitogen Activated Protein Kinase	MAPK
Mating Type-Like	MTL
Nourseothricin	NAT
Phosphate-Buffered Saline	PBS
Polymerase Chain Reaction	PCR
Room Temperature	RT
Tetracycline	TET
Hydroxy Urea	HU
Hydrogen Peroxide	H_2O_2
Ultra Violet Radiation	UV
Wild Type	WT
Yeast extract, Peptone, Dextrose	YPD
Differential Interference Contrast	DIC
4’,6-DiAmidino-2-PhenylIndole	DAPI
Carbon Dioxide	CO_2
Clustered Regularly Interspred Short Palindromic Repeats	CRISPR
CRISPR associated protein 9	Cas9

Chapter 1: Introduction

1.1 A Brief Introduction to *Candida albicans*

The fungal kingdom comprises over 100,000 species throughout the world. Up to 150 species have been documented to cause human infection (MARICHAL AND VANDEN BOSSCHE 1995). The severity of fungal diseases varies from superficial infections to life threatening systemic infections. Most of the infections are opportunistic because the infecting agents are commonly found as commensal organisms residing in the human host as part of their microbial flora. *Candida albicans,* which can cause serious infections in immunocompromised individuals (ODDS 1987), is a member of the normal microflora of the human oral, gastrointestinal and urinogenital tracts. Normally, Candida is present in the yeast form, but when it becomes pathogenic, it switches the phenotype to the hyphal form to invade the host cell epithelium. Interactions between C. *albicans* cells and mammalian host tissues are known to be highly complex. Candida infections arise as a sequence of time scaled steps-

- Adhesion to an epithelial surface is required to initiate the colonization of the actual surface.
- Penetration into the epithelial surfaces is the limit of the infectious process in most cases, leading to the establishment of a superficial candidiasis; normally the fungus is incapable of further invasion into the immunologically intact host.
- When the fungi reach to the blood stream, they must face the blood-borne cellular host defense system. In a candidemia, Candida cells must be able to penetrate the

endothelial surfaces and invade tissues to gain access to the blood stream and spread to other organs.

Fungal infections are treated with antifungal agents. An antifungal agent is a drug that selectively eliminates fungal pathogens from a host with minimal toxicity to the host (DIXON AND WALSH 1996). The prevalence of resistance to antifungal agents has significantly increased in the past few decades, and this resistance has important implications for mortality, morbidity and health care in the community. Until recently, fungi were not recognized as important pathogens because the annual death rate due to Candidiasis was steady and low between 1950 and 1970 (MISHRA *et al.* 2007). Since 1970 however, this rate increased significantly due to more widespread use of immunosuppressive therapies, indiscriminate use of broad-spectrum antifungal agents, the increase in immunosuppressive viral infections and the common use of indwelling intravenous devices (MISHRA *et al.* 2007). These developments and the associated increase in fungal infections necessitated the search for new, safer and more potent agents to combat serious fungal infections (MISHRA *et al.* 2007). For nearly 30 years, amphotericin B, which has side effects of nephrotoxicity, was the sole drug available to treat serious fungal infections. The imidazoles and the triazoles in the late 1980s and the early 1990s were major advances in the safe and effective treatment of local and systemic fungal infections. Triazoles, and in particular fluconazole, have high safety profiles, and this has led to their extensive use in both treatment and prophylaxis. Fluconazole has been used to treat more than 16 million patients, which includes 300,000 AIDS patients, in the United States alone since the launch of this drug but due

to selective pressure and widespread use of these few antifungal drugs, there have been increasing reports of antifungal resistance (REVIE *et al.* 2018). Among these cases, infections caused by the fungus *C. albicans* are the most common and some of the strains of *C. albicans* have developed multidrug resistance (PRASAD AND KAPOOR 2005).

A majority of the *C. albicans* genome is still not well characterized. According to the Candida Genome database, only about one quarter of *C. albicans* genes with *Saccharomyces cerevisiae* orthology have been characterized specifically in the pathogen. Therefore, a detailed characterization of the remaining genome is required for a better understanding about the biology of this organism.

1.2 *Candida albicans* and Morphogenesis

C. albicans has to adapt to different host niches that provide very different environments for growth, and the pathogen does so by using different signaling mechanisms and adaptations that allow it to survive and proliferate in these environments (HILLER *et al.* 2011). One of the most important adaptations is switching among a variety of cellular forms in response to host cell niches and different environmental stresses (STAIB *et al.* 2001; GASCH 2007). Different phenotypic forms of *C. albicans* include white, opaque (SLUTSKY *et al.* 1987), gray (TAO *et al.* 2014) GUT (PANDE *et al.* 2013) and a filamentous form. As well, yeast and filamentous forms can develop extracellular communities called biofilms under certain conditions (CHANDRA *et al.* 2001). The different morphogenetic forms play crucial roles in aspects of *C. albicans* biology including survival inside the host, reproduction, virulence and resistance to antifungal drugs.

1.2.1 Yeast to Filamentous Switching

C. albicans has the ability to switch from small round yeast cells to large filamentous hyphal and pseudohyphal cells. Pseudohyphae and hyphae are called the "filamentous" morphologies, because cells typically grow in a polarized manner, are elongated in form, and are attached to each other. Pseudohyphal cells are generally ellipsoidal and have constrictions at the septal junctions while hyphal cells generally have parallel sides, are uniform in width, and possess true septa lacking constrictions (SUDBERY *et al.* 2004). Yeast to hyphal switching is the most frequent morphological transition in *C. albicans* (BRAND 2012). Both yeast and filamentous forms are critical for *C. albicans* virulence (LO *et al.* 1997). The white yeast form is important for pathogen dissemination via the blood stream and therefore involved in Candida virulence (JACOBSEN *et al.* 2012). The filamentous form has the ability to invade host tissues *in vivo* or agar medium *in vitro*. The invasive filamentous form is induced in response to different environmental signals such as increased temperature, nutrient limitation or the presence of serum (BROWN AND GOW 1999). Upon proper signal induction, yeast cells can initiate the invasion of tissues and organs via formation of hyphae or pseudohyphae (CSANK *et al.* 1998; FELK *et al.* 2002). After host tissue invasion, the pathogen then enters the blood stream and spreads to other parts of the host thus resulting in a systemic infection (FELK *et al.* 2002).

Two key pathways play central roles in the regulation of filamentous growth: the cyclic-AMP/ protein kinase A (cAMP/PKA) pathway and a mitogen activated protein kinase (MAPK) pathway (SONNEBORN *et al.* 1999; CULLEN AND SPRAGUE 2012; SI *et al.* 2013).

The cAMP/PKA pathway acts as a major regulator of filamentation where Cyr1 is activated by external signals and the activated Cyr1 then converts ATP to cAMP (HOGAN AND SUNDSTROM 2009). cAMP acts as a secondary messenger by binding to Bcy1, the regulatory subunit of PKA, causing conformational changes in PKA thus releasing the two catalytic subunits Tpk1 and Tpk2 (Fig 1.1). These activated catalytic subunits then activate downstream filamentation regulators such as Efg1 via phosphorylation (STOLDT *et al.* 1997; BOCKMUHL AND ERNST 2001). The MAPK pathway, on the other hand, signals through a cascade of MAP kinases (Cst20, Hst7, Cek1) (Fig 1.1 yellow colored) and ends on in the activation of transcription factor Cph1 which then induces the filamentous response genes (LIU *et al.* 1994).

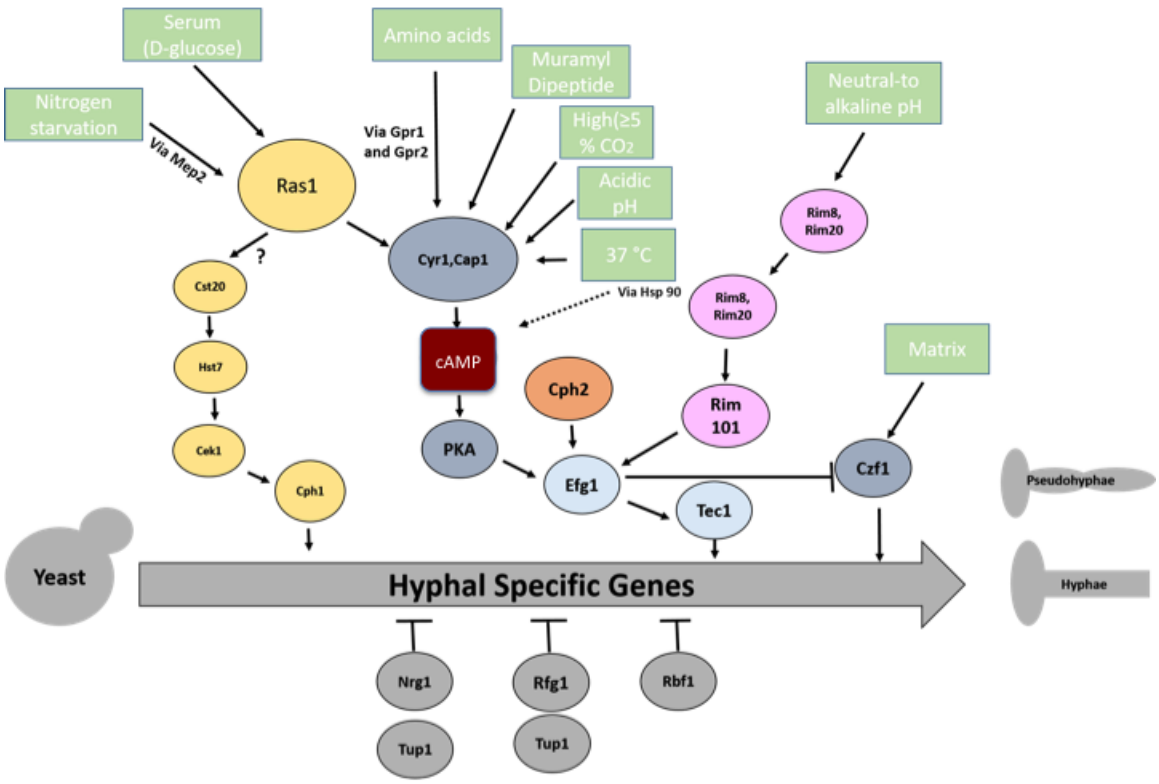


Figure 1.1 Schematic representation of cAMP/PKA (centre greyish scheme) and MAPK (left yellowish scheme) pathways in filamentation (BERMAN AND SUDBERY 2002). The arrows represent activation and the bars represent repression. External signals (green boxes) feed into Ras1 and Cyr1. Activated Cyr1 converts ATP to cAMP. The cAMP (red box) acts as a secondary messenger on PKA (Bcy1), changes its conformation to release catalytic subunits Tpk1 and Tpk1, which then activate downstream filamentation regulators. MAPK pathway signals activate Cph1, which then induces filamentation response genes. Efg1, Cph1, Nrg1, Tup1, Rfg1 and Rbf1 are main downstream transcription factors and they act as activators or repressors of hyphal specific genes.

1.2.2 White-opaque Switching and Mating

C. albicans has the ability to switch from the mating sterile white form to the mating competent opaque form. White cells are small and round in shape and form white dome-shaped colonies while opaque cells are slightly larger with an elongated shape, and their colonies give a greyish and flatter appearance on agar medium. As well, white and opaque forms have other distinguishable characteristics; opaque colonies are stained pink with phloxine B while white form colonies are not, the white form is better in systemic or cutaneous infections, while the opaque forms are good in adhesion to the skin, opaque cells are more resistant to different drugs, the two forms have different lipid contents and show significantly different gene expression profiles (ANDERSON AND SOLL 1987; KENNEDY *et al.* 1988; ANDERSON *et al.* 1990; GHANNOUM *et al.* 1990).

Wor1 is the key player in Candida white-opaque switching (HUANG *et al.* 2006). *C. albicans* is a diploid organism and most of the wild type isolates are heterozygous at the Mating Type-Like (*MTL*) locus designated as *MTL a/α*. However, in order to switch to opaque form the cells need to be homozygous at the *MTL* locus. The *MTL* locus is located on chromosome 5 and it consists of two heteroallelic loci, *MTLa* and *MTLα.* The

MTLa locus contains genes for two transcriptional regulators, *a1* and *a2* where the a2 protein is for activation of *MTLa* specific genes. The *MTLα* locus also contains two transcriptional regulator genes*, α1* and *α2* where α1 is involved in the activation of *MTLα* specific genes. The a1 and α2 proteins will form a heterodimer that can bind to the promoter region and repress expression of Wor1. Therefore, homozygosity at the *MTL* locus is required for Wor1 expression and white to opaque transition. Homozygosity at the *MTL* locus is achieved either through gene conversion or chromosome loss followed by duplication of the retained copy (SOLL 2014). Besides these regulatory genes, each of these loci contains non-sex genes (NSGs) designated as *PIK*, *OBP* and *PAP* that code for phosphatidylinositol kinase, an oxysterol binding protein-like protein and a poly(A) polymerase respectively (HULL AND JOHNSON 1999). The *OBP* locus has been implicated in *C. albicans* biofilm formation and in fluconazole resistance (SRIKANTHA *et al.* 2012).

The MAPK pathway is the main regulatory pathway of Candida mating (CHEN *et al.* 2002). The pathway signaling starts when pheromones bind to the transmembrane receptors; Ste2 (the 'α' pheromone receptor) and Ste3 (the 'a' pheromone receptor) (DOHLMAN *et al.* 1998). Upon binding of pheromone to the receptor, the signal is transduced to a heterotrimeric G protein which causes dissociation of Gα from Gβγ (KURJAN 1993; DIGNARD *et al.* 2008; LU *et al.* 2014). The Gβγ subunits transfer the signal through a cascade of MAP kinases, that include Cst20, Ste11, Hst7 and Cek1, to activate the transcription factor Cph1 via release of the inhibitory

activity of Dig1 (BARDWELL *et al.* 1998; REGAN *et al.* 2017). Finally, the activated Cph1 induce the pheromone and mating response genes (MONGE *et al.* 2006).

1.3 DNA Damage and Morphogenesis in *C. albicans*

DNA replication checkpoints had been implicated in the development of filamentation in the presence of DNA damaging agents (SHI *et al.* 2007). Cell division defects or damage to the DNA repair and stress response machinery has been implicated in increased polarized and hyphal growth (BACHEWICH *et al.* 2003; BACHEWICH *et al.* 2005; BACHEWICH AND WHITEWAY 2005). *C. albicans* also tends to switch more from white to opaque form with increases in genotoxic stress or defects in DNA repair system (ALBY AND BENNETT 2009). In *C. albicans*, deletion of the DNA repair protein Rad52p caused filamentation and increased expression of hyphal specific genes (HSGs) (ANDALUZ *et al.* 2006). Rad52 acts downstream of the Smc5/6 DNA damage repair complex and the Rad52 protein is regulated by Smc5/6 complex mediated sumoylation (TORRES-ROSELL *et al.* 2007). Moreover, deletion of Candida Smt3, an ortholog of the *S. cerevisiae* SUMO protein, resulted in cellular morphological defects (LEACH *et al.* 2011). Large-scale filamentation studies also revealed that filamentation occurred when genes involved in cell division, DNA repair and SUMO pathway were repressed (O'MEARA *et al.* 2015).

1.3.1 Sumoylation Pathway and Mms21

Sumoylation is the covalent attachment of SUMO (Small Ubiquitin-like Modifier) to a lysine (K) of a target proteins. Sumoylation is an important post-translational modification that is involved in transcription regulation, stress responses, cellular

differentiation and protein transport, unlike the related ubiquitination where the main purpose of the protein modification is to target the protein for degradation (SEELER AND DEJEAN 2003; WOHLSCHLEGEL *et al.* 2004; ZHOU *et al.* 2004). Binding of SUMO to a target protein is a reversible process and happens in several steps (Fig 1.2). Initially, SUMO specific proteases (SUP) cleave, via hydrolase activity, the C-terminus of a precursor SUMO (Smt3) protein exposing its terminal diglycine residues. The mature SUMO is then activated by the E1 enzyme heterodimer (Aos1-Uba2) in an ATP dependent two-step process by initial C-terminal adenylation and then by thioester bond formation between a glycine of SUMO and the catalytic cysteine of Uba2 (OLSEN *et al.* 2010). The activated SUMO is directly transferred to the E2 conjugating enzyme (Ubc9) by E1 thus forming a thioester bond between the SUMO C-terminus and the E2-catalytic cysteine. SUMO is then conjugated via E2 to the lysine in the consensus sequence ΨKXE (Ψ a hydrophobic amino acid, X any amino acid, K lysine, E glutamate) of target proteins resulting in SUMO-target isopeptide bond formation (GONG *et al.* 1997; JOHNSON AND BLOBEL 1997; JOHNSON 2004; DYE AND SCHULMAN 2007; FLOTHO AND MELCHIOR 2013). Conjugation of SUMO to the target proteins via E2 is facilitated by specific SUMO E3 ligases (MEULMEESTER *et al.* 2008). Finally SUMO can be cleaved from the target protein by SUMO specific isopeptidases such as Ulp1 in yeast (LI AND HOCHSTRASSER 1999).

Mms21 is an important E3 ligase that contains a C-terminal SP (Siz/PIAS) domain required for its ligase activity. In *C. albicans*, the *MMS21* gene is located on chromosome 3 and encodes a 272 amino acid protein. Besides being a member of the SUMO pathway with its importance in post-translational modifications, Mms21 has also been reported

to play major roles in DNA repair via homologous recombination (as a member of the Smc5/6 complex) and in the maintenance of chromosomal integrity and telomeric length (McDonald *et al.* 2003; Pebernard *et al.* 2004; Andrews *et al.* 2005; Potts and Yu 2005; Zhao and Blobel 2005; Potts and Yu 2007; Duan *et al.* 2009b).

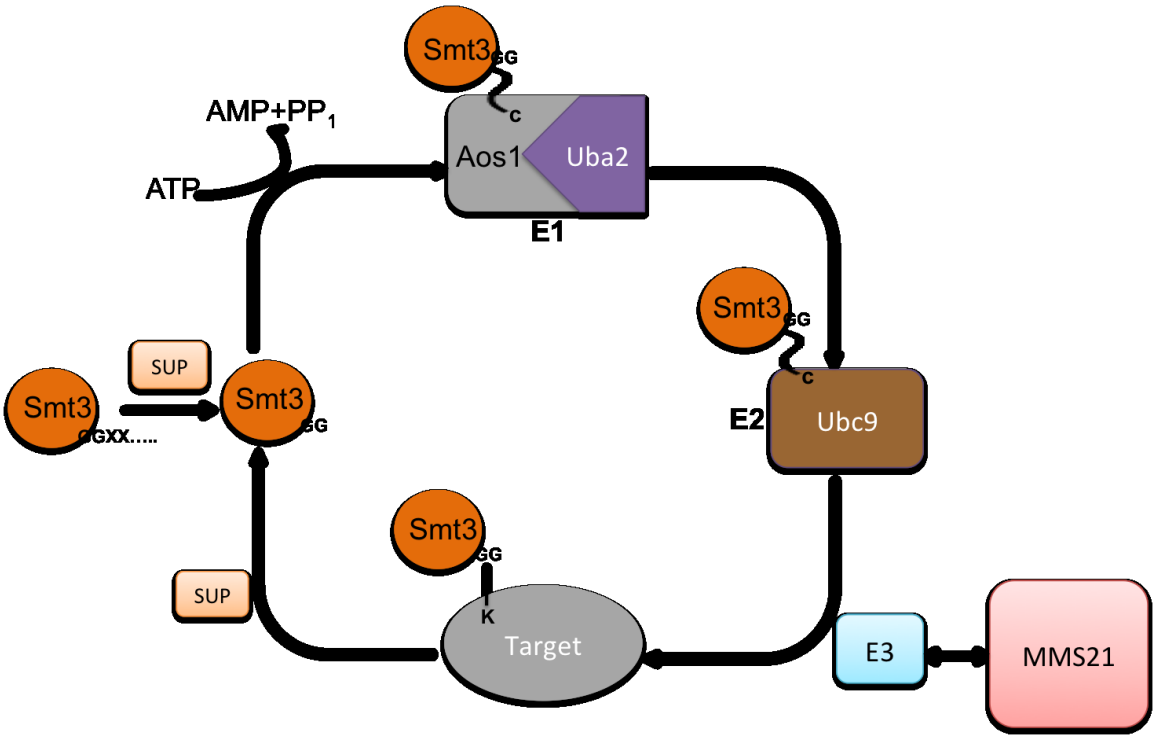


Figure 1.2 Schematic representation of the sumoylation pathway in *Saccharomyces cerevisiae* (Flotho and Melchior 2013). A nascent Smt3 (SUMO) is proteolytically cleaved by SUMO-proteases (SUP) to expose its C-terminal Glycine-Glycine (GG) motif. A mature Smt3 is then activated by the SUMO E1 heterodimer Aos1-Uba2 in an ATP consuming step forming a thioester bond between the GG motif of SUMO and the catalytic cysteine of Uba2. Smt3 is then transferred to the catalytic cysteine of a SUMO E2 enzyme Ubc9, again forming a thioester bond. Ubc9, usually with the help of specific E3 ligases (e.g. Mms21), catalyzes the formation of an isopeptide bond between the C-terminal glycine of SUMO and a lysine (K) residue of the target protein. This whole process of sumoylation is reversible where as SUMO can be cleaved off the target protein by SUMO specific isopeptidases.

1.3.2 Mms21 is Part of The DNA Repair Smc5/6 Complex

Mms21 in *S. cerevisiae* is part of the essential DNA repair structural maintenance of chromosome 5/6 (Smc5/6) complex (Fig 1.3). Mms21 interacts (via its N-terminus) with the Smc5 subunit of the complex where in *S. cerevisiae* it is involved in sumoylation of Smc5 while in *S. pombe* it is involved in sumoylation of Smc6, Nse3 and itself (autosumoylation) (FOUSTERI AND LEHMANN 2000; HO *et al.* 2001; MCDONALD *et al.* 2003; PEBERNARD *et al.* 2004; ANDREWS *et al.* 2005). In *S. cerevisiae,* mutations in the N-terminus of Mms21 result in failure of the protein to interact with Smc5 and cause several growth defects (DUAN *et al.* 2009a). It has been suggested that Mms21-mediated sumoylation of the Smc5/6 complex helps the complex to interact with DNA damage repair proteins like Rad52, Rad50 and Yku70/80. The Smc5/6 complex in yeast is required to regulate Rad52 function thus allowing nucleolar exclusion of Rad52 foci and preventing rDNA hyper-recombination (TORRES-ROSELL *et al.* 2007).

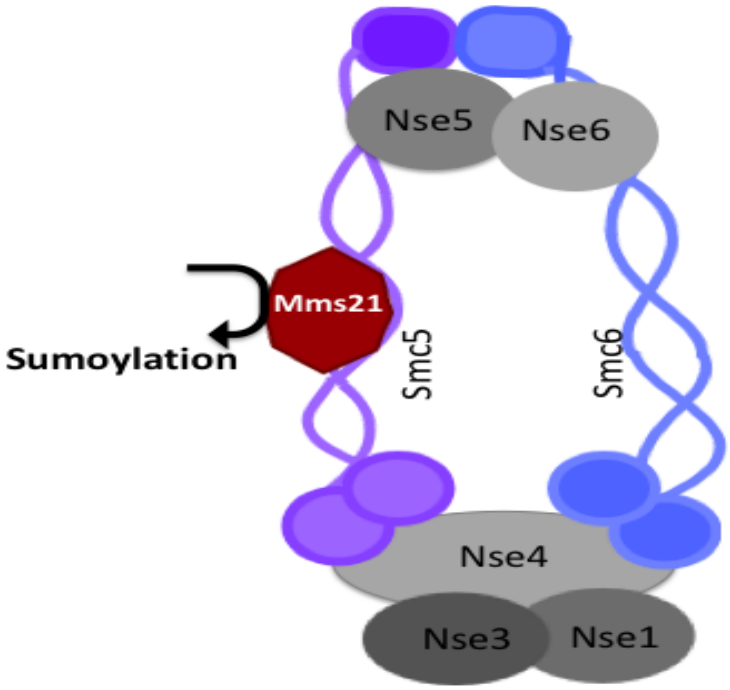


Figure 1.3 Schematic representation of the *S. cerevisiae* Smc5/6-Mms21 subunits complex. Mms21 N-terminus directly interacts with Smc5 providing structural stability to the complex while the C-terminus of Mms21 is involved in sumoylation via its SP domain.

1.4 Genotoxic Stress

Genotoxic (Genome-Toxic) stress compromises the genome integrity of an organism. There are several mutagens that can induce genotoxic stress: these include the alkylating agent Methyl Methane Sulfonate (MMS), the DNA replication stalling chemical hydroxyurea (HU), the oxidative stress agent hydrogen peroxide (H_2O_2) and thymine-dimer-causing ultra-violet (UV) radiation. MMS is a frequently used mutagen in molecular studies related to DNA repair and cancer. MMS causes methylation of DNA bases particularly guanine to 7-methylguanine and adenine to 3-methyladenine thus resulting in base mispairing and ultimately causing double strand breaks (DSBs) in DNA (LUNDIN *et al.* 2005; SHI *et al.* 2007). In response to DNA damaging agents, cells recognize the damage and use different checkpoint signals to stop the cell cycle and DNA replication and then repair the damage using different repair systems. (ZHOU AND ELLEDGE 2000; SIRBU AND CORTEZ 2013). Though MMS and other genotoxic agents have been studied extensively in *S. cerevisiae* much less is known in *C. albicans* about genotoxic stresses and the genes involved in response to these stress. Therefore, a detailed analysis of genotoxic stress response in *C. albicans* is required.

1.5 Hof1, Cytokinesis and Genotoxic Stress Response

Hof1 (Homolog Of cdc Fifteen) is a well-studied cytokinesis related protein in *S. cerevisiae* and *S. pombe*. In *C. albicans* the *HOF1* gene is located on chromosome 4 and

it codes for a 607 amino acid (aa) protein. The Candida Hof1 shares about 15 % similarity with ScHof1 and 26 % similarity with *S. pombe* Cdc15. CaHof1 contains 2 domains: an N-terminal F-Bar domain which contains a FCH domain (13-102 aa) and a C-terminal SH3 domain (539-605 aa). The *S. cerevisiae* Hof1 interacts, via its SH3 domain, with the FH1 domain of formin Bnr1 in the bud neck for actin cytoskeleton organization (KAMEI *et al.* 1998). This interaction of Hof1 with Bnr1 is required to inhibit actin elongation thus maintaining proper actin cable geometry at the bud neck (GRAZIANO *et al.* 2014). The N-terminus of ScHof1, on the other hand, is required for plasma membrane ingression and for actomyosin ring (AMR) contraction thus facilitating primary septum (PS) formation for a proper cytokinesis progression (MEITINGER *et al.* 2011; DEVREKANLI *et al.* 2012). In *C. albicans* deletion of Hof1 leads to severe cytokinesis defects with cells being unable to separate properly (LI *et al.* 2006). Large-scale gene deletion assays in yeast have found *hof1* mutants to be sensitive to MMS (CHANG *et al.* 2002; SVENSSON *et al.* 2011) but a proper reasoning for this sensitivity was not provided. Since Hof1 mainly functions in late cell division where it has reported roles in the G2/M phases of the cell cycle, studying Hof1 for MMS sensitivity and genotoxic stress response is very compelling and may lead to some interesting new findings about Hof1 roles in Candida damage response and cell cycle control.

1.6 GRACE™ Library

The GRACE™ (Gene Replacement And Conditional Expression) library is a collection of approximately 2500 conditional mutants created in the CaSS1 strain background with the purpose of finding essential genes and effective drug targets in *C.*

albicans (ROEMER *et al.* 2003). The parental CaSS1 strain and all the derivatives constitutively express a chimeric transactivation fusion protein consisting of the *E. coli tet*R binding domain and the *S. cerevisiae GAL*4 activation domain (Fig 1.4). Conditional repression of individual mutant strains can be achieved either by supplementing the medium with tetracycline or by growing them on 5 Fluororotic acid (5-FOA) containing medium. The GRACE library contains *URA3* linked to the tet transactivator, and in the presence of 5-FOA cells can avoid making toxic 5-fluorouracil if they lack the *URA3* gene. Thus 5-FOA treatment can select for cells that have lost the transactivator, and thus shut off tet promoter-activated genes in the treated strain (BOEKE *et al.* 1987; ROEMER *et al.* 2003).

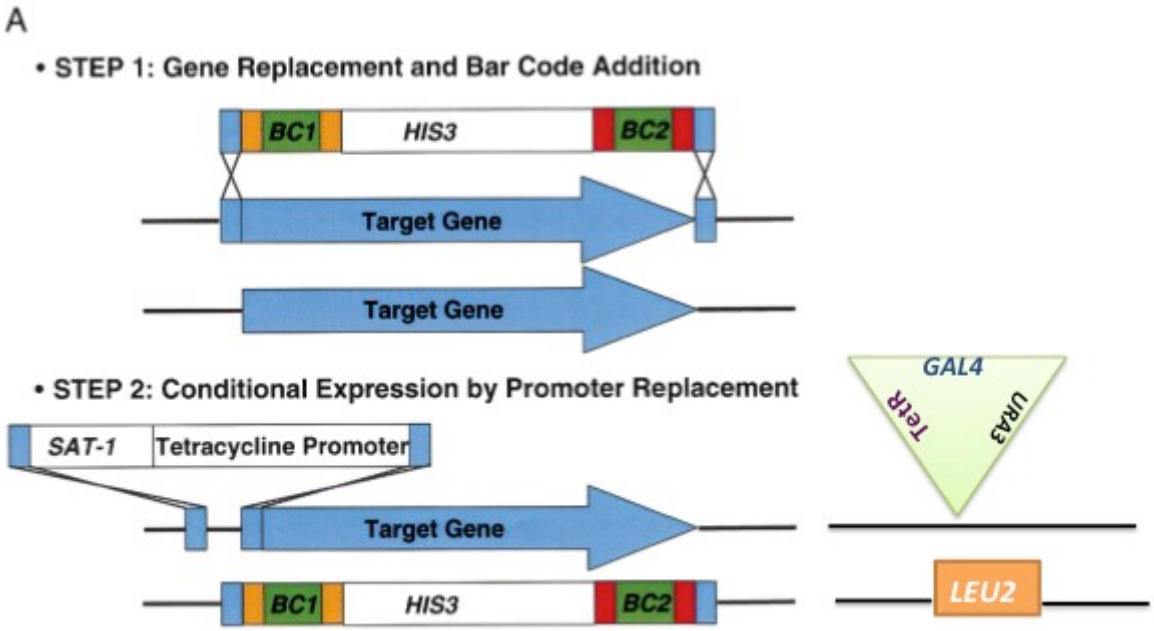


Figure 1.4 Schematic representation of GRACE Library construction by Roemer *et al.* (BOEKE *et al.* 1987; ROEMER *et al.* 2003).

In this study we have characterized Mms21 that is involved in *C. albicans* invasiveness, filamentation, biofilm formation and mating control. Deletion of the Mms21 protein or its SP domain caused filamentation and other cellular effects. The mutants were highly sensitive to different genotoxic agents as well as to different drugs used in treatments of Candida infections that damage either DNA, the cell wall or the cell membrane, indicating that Mms21 plays crucial roles in stress and drug resistance. Moreover, we have also found, via GRACE library screening, many genes that are involved in the *C. albicans* stress response to MMS. In particular, we have performed detailed phenotypic and genetic analysis of CaHof1 for its role in the genotoxic stress response.

Chapter 2: Mms21: A Putative SUMO E3 Ligase in *Candida albicans* that Negatively Regulates Invasiveness and Filamentation, and is Required for Genotoxic and Cellular Stress Response

2.1 Introduction

Candida albicans is one of the major causes of hospital-acquired infections in the USA, and the cost in treatment is nearly 2.6 billion USD annually (STEVENS *et al.* 2014). This opportunistic fungal pathogen has evolved to survive inside its human host, and switching among a variety of cellular forms appears important for allowing it to adapt to host cell responses and different environmental stresses (STAIB *et al.* 2001; GASCH 2007). These different phenotypic forms include white, opaque (SLUTSKY *et al.* 1987), GUT (PANDE *et al.* 2013) and gray (TAO *et al.* 2014) yeast forms, and a filamentous form that appears critical for the fungus to invade host tissues (CALDERONE AND FONZI 2001). The white yeast form, with small rounded budding yeast cells, is thought to be important for pathogen dissemination via the blood stream, and to thus have a role in virulence (JACOBSEN *et al.* 2012). The invasive or filamentous form is induced in response to environmental cues such as nutrient limitation or the presence of serum. When an appropriate signal is transduced, yeast cells can initiate invasion by forming either hyphae or pseudohyphae (CSANK *et al.* 1998; FELK *et al.* 2002). These filamentous cells can invade host tissues and ultimately gain entry into the blood stream to cause life threatening systemic infections (FELK *et al.* 2002). Thus, in addition to the yeast form, the filamentous form is also critical for *C. albicans* virulence (LO *et al.* 1997).

In order to combat this pathogen we need a better understanding of its biology including the regulation of yeast-filamentous switching. To date only about one quarter of the *C. albicans* genes with *Saccharomyces cerevisiae* orthology have been significantly characterized (http://www.candidagenome.org/), so the majority still need to be investigated in order to have a good understanding about the physiology of this organism, an understanding that can ultimately help find improved treatments for *C. albicans* infections.

Because invasiveness, the ability of *C. albicans* cells to invade host tissues *in vivo* or agar medium *in vitro*, is critical for fungal pathogenicity, we have explored, using the GRACE™ library (ROEMER *et al.* 2003), the *C. albicans* genome to identify genes controlling invasiveness and filamentation. Through tetracycline-induced gene repression of the GRACE library, we have identified 69 negative regulators of the invasiveness and filamentation. Intriguingly, the genes encoding the SUMO protein Smt3 and all other tested members of the sumoylation pathway (Uba2 and Aos1 (SUMO E1), Ubc9 (SUMO E2) and Mms21 (SUMO E3)) were both non-essential and capable of triggering filamentation upon repression. The SUMO protein Smt3 and the E1 enzyme Aos1 have been previously implicated as repressors of filament formation in *C. albicans* (O'MEARA *et al.* 2015).

Sumoylation, the covalent attachment of SUMO (Small Ubiquitin-like Modifier) to target proteins, is an important post-translational modification that plays key roles in processes as distinct as transcription regulation, stress response, cellular differentiation and protein transport (SEELER AND DEJEAN 2003; WOHLSCHLEGEL *et al.* 2004; ZHOU *et al.*

2004). The sumoylation-process steps are very similar to those of ubiquitination. Initially a precursor SUMO protein, for example Smt3 in *S. cerevisiae*, is cleaved at its C-terminus by specific SUMO-proteases to expose diglycine followed by SUMO activation by a SUMO E1 enzyme heterodimer (Aos1-Uba2) in an ATP dependent step resulting in thioester bond between Uba2 and SUMO. The E1 enzymes then transfer activated SUMO to the E2 conjugating enzyme (Ubc9) forming a thioester bond between E2 and the SUMO diglycine. The E2 can directly transfer activated SUMO to the lysine (K) of many target proteins recognizing the consensus sequence ΨKXE (Ψ a hydrophobic amino acid, X any amino acid) through the formation of an isopeptide bond (GONG *et al.* 1997; JOHNSON AND BLOBEL 1997; JOHNSON 2004). Sumoylation of a majority of proteins are carried out by specific SUMO E3 ligases (MEULMEESTER *et al.* 2008). Mms21 in *S. cerevisiae* and its ortholog Nse2 in both human and *Schizosaccharomyces pombe* is an E3 ligase containing the so-called SP-RING (Siz/PIAS RING) domain that is exclusively found in SUMO E3s. Mms21 has important roles in post-translational modification, homologous recombination, DNA repair, and maintenance of chromosomal integrity and telomeric length (MCDONALD *et al.* 2003; PEBERNARD *et al.* 2004; ANDREWS *et al.* 2005; POTTS AND YU 2005; ZHAO AND BLOBEL 2005; POTTS AND YU 2007; DUAN *et al.* 2009b).

Mms21 is also a member of the DNA repair Smc5/6 complex and our GRACE screening also revealed Smc6 and Qri2 of this complex to be non-essential filamentation repressors. In *S. cerevisiae*, the Smc5/6 complex is required for nucleolar exclusion of Rad52 foci to prevent rDNA hyper-recombination (TORRES-ROSELL *et al.* 2007). In *C. albicans,* deletion of Rad52 caused filamentation and cell cycle related defects with

increased hyphal-specific gene (HSGs) expression that was independent of Efg1 (ANDALUZ *et al.* 2006). The SUMO protein Smt3 deletion also resulted in similar cell cycle and growth defects (LEACH *et al.* 2011). In *C. albicans,* DNA replication checkpoints are critical for filament formation in the presence of DNA damaging agents (SHI *et al.* 2007). Defects in the cell division, DNA damage and stress response machinery have been implicated in increased polarized growth and white-opaque switching (BACHEWICH *et al.* 2005; BACHEWICH AND WHITEWAY 2005; ALBY AND BENNETT 2009). Wos1, a newly characterized SUMO E3 ligase in *C. albicans*, affects white-opaque switching through sumoylation of Wor1 (YAN *et al.* 2015b). As Mms21 is an essential part of the Smc5/6 complex in *S. cerevisiae* where this complex is critical for DNA replication checkpoints and damage repair, we explored the roles of Mms21 in *C. albicans* physiology to shed some light into the yeast-filamentous switching control, and to investigate the importance of Mms21 in response to different genotoxic and cellular stresses.

2.2 Materials and Methods

2.2.1 Strains and Culture Conditions

Strains used in this study are described in Appendix 1. For general growth and maintenance, strains were cultured in fresh yeast-peptone-dextrose YPD medium (1 % w/v yeast extract, 2 % w/v Bacto peptone, 2 % w/v dextrose, 80 mg/L uridine with the addition of 2 % w/v agar for solid medium) at 30°C. Cell growth, transformation and

DNA preparation were carried out using standard yeast procedures. Different types of phenotypic analyses were carried out in yeast growth conditions. The conditions used for microscopy and stress susceptibility are described below.

2.2.2 GRACE Library and Invasive Assay

The GRACE™ (Gene Replacement And Conditional Expression) library is a collection of approximately 2500 conditional mutants of created in the CaSS1 strain background with a purpose of finding essential genes and effective drug targets in *C. albicans* (ROEMER *et al.* 2003). A parental CaSS1 strain was engineered in the *C. albicans* CAI4 strain background by deleting and replacing one allele of each individual gene with a *HIS3* gene flanked by two gene-specific bar codes up-stream and down-stream of the *HIS3* gene. The parental CaSS1 strain and all the derivatives constitutively express a chimeric transactivation fusion protein consisting of the *E. coli tet*R binding domain and the *S. cerevisiae GAL*4 activation domain. Conditional repression of individual mutant strains can be achieved by supplementing the medium with tetracycline.

The GRACE library was transferred from frozen glycerol stocks into 96 well plates containing liquid YPD with 1 mg/ml nourseothricin using sterile 96 pin replicators and incubated overnight at 30°C. For the invasive assay, overnight grown copies of the library were inoculated onto YPD agar with added tetracycline at a concentration of 100 µg/ml and incubated at 30°C for 4 days followed by a 10 second wash of each colony with a stream of water. The colonies that stayed on the plates after washing were scored invasive and those washed away were considered non-invasive. Images of plates

and colonies were scanned at 600 dots per inch (dpi) using an Epson Perfection v500 photo scanner.

2.2.3 Deletion of *MMS21* and Mms21 SP Domain

Oligonucleotides and plasmids used in this study are shown in Appendix 2. The two alleles of the *MMS21* were deleted in a SN148 strain (wild type) background by standard gene deletion method using a two-step PCR disruption (DIGNARD *et al.* 2007). The first allele was replaced with *HIS1* and the second allele with *ARG4* from pFA-HIS1 and pFA-ARG4 plasmids respectively (GOLA *et al.* 2003). Since both plasmids have a common DNA backbone, oligonucleotides Amj37Fw and Amj38Rv (appendix 2) were used to prepare the cassettes for the deletion of the *MMS21* gene. The correct insertion of *HIS1* was confirmed using genomic DNA of positive colonies by PCR analysis and the proper strain was named *MMS21/mms21Δ*. The second allele was deleted with *ARG4* in strain *MMS21/mms21Δ* and was confirmed by PCR to generate *mms21Δ/Δ* or null *mms21* mutant.

Deletion of the Mms21 SP domain in strain *MMS21/mms21Δ* was performed using plasmid pFa-TAP-Arg4 (LAVOIE *et al.* 2008) using a similar strategy. The SP domain was deleted from strain *MMS21/mms21Δ* with a PCR generated cassette, using oligonucleotides Amj73Fw and Amj72Rv and subsequently confirmed by PCR. A Western analysis of the mutant protein using anti-TAP antibodies was carried out to confirm the accurate sizes.

2.2.4 Reintegration of *MMS21* into The Null *mms21* Mutant

The null *mms21* mutant was complimented at the RP10 locus with a copy of *MMS21* from a wild type strain using the CIp10 plasmid (MURAD *et al.* 2000). For reintegration, a 1,465-bp *MMS21* fragment from genomic DNA was amplified by PCR using oligonucleotides AmjHindIIIFw and AmjXhoIRv and Expand high-fidelity polymerase (Roche). The amplified PCR fragment was digested with restriction enzymes *Hin*dIII and *Xho*I and cloned into plasmid CIp10 in *Escherichia coli* strain DH5α. A correct integration of *MMS21* into the plasmid was confirmed by sequencing; this construct was designated CIp10-*MMS21*. CIp10-*MMS21* was then linearized by digestion with *Stu*I prior to transforming the null *mms21* mutant. The *URA* positive colonies were analyzed by PCR and a successful *MMS21* integration into the null *mms21* mutant was confirmed.

2.2.5 Microscopic Analysis

For cell morphology, overnight cultures grown in YPD at 30°C were subjected to phase differential interference contrast (DIC) microscopy. For nuclear segregation analysis, overnight cultures were resuspended at a starting optical density of 600 nm (OD_{600}) = 0.1 in synthetic complete medium (0.67 % w/v yeast nitrogen base with ammonium sulfate, 2 % w/v dextrose, 1.59 % w/v complete amino acids mixture, pH 7.0) and incubated for 3 1/2 to 6 hours (until successful completion of first cellular division of both wild type and mutant strains) followed by the addition of 2 µg/ml DAPI (Sigma-Aldrich) into each tube. Cells were examined by DIC at 63X and fluorescent microscopy at 100X magnification using a Leica DM 6000 microscope mounted with a Hamamatsu Orca ER CCD camera.

2.2.6 Stress Phenotypes

To test sensitivities of strains to different agents and to temperature, overnight cultures grown in YPD at 30°C were serially diluted in 10 fold stages at room temperature to a dilution concentration of 10^6 and 10^3 cells per ml and 5 µl of each dilution was spotted on YPD agar containing one of the following; methyl methane sulfonate (MMS, 0.01 % v/v), hydroxyurea (HU, 10 mM), hydrogen peroxide (H_2O_2, 5 mM), Congo red (150 µg/ml), amphotericin B (1 µg/ml), clotrimazole (0.5 µg/ml), fluconazole (10 µg/ml), posacanozole (10 µg/ml), NaCl (1 M) and 90 seconds UV light exposure. Plates were then incubated at 30°C for 4 days except for caspofungin (0.75 µg/ml) containing plates that were incubated for 7 days at 30°C. Heat sensitivity of strains was tested on YPD agar at 37°C and 42°C for a period of 4 days.

2.2.7 Micorarray Profiling

The *mms21Δ/Δ* and SN148 cultures were grown in YPD overnight at 30°C, diluted to OD_{600} of 0.1 in YPD at 30°C, grown to an OD_{600} of 0.8 to 1.2, then harvested and stored at -80°C until RNA extraction. Total RNA isolation and cDNA preparation were performed as previously described (TEBBJI *et al.* 2014). *Candida albicans* whole genome arrays were obtained from the NRC-BRI facility and were hybridized in a hybridization chamber at 42°C for overnight incubation. Microarray slides were scanned using a GenePix4000B microarray scanner, and the images were analyzed in GenePix Pro 7; the output data were processed with Microsoft Excel 2013 and MultiExperiment Viewer (MeV). Transcription profiling data are available at GEO with the accession number: GSE116544.

2.2.8 RNA-seq

The *mms21Δ/Δ* and SN148 cultures were grown in YPD overnight at 30°C, diluted to OD_{600} of 0.1 in YPD at 30°C and then grown to an OD_{600} of 0.8 to 1.2 on a 220-rpm shaker. Total RNA was extracted using the Qiagen RNeasy minikit protocol, and RNA quality and quantity were determined using an Agilent bioanalyzer. Paired-end sequencing (150bp) of extracted RNA samples was carried out at the Quebec Genome Innovation Center located at McGill University using an Illumina miSEQ sequencing platform. Raw reads were pre-processed with the sequence-grooming tool cutadapt version 0.4.1 (MARTIN 2011) with the following quality trimming and filtering parameters (`--phred33 --length 36 -q 5 --stringency 1 -e 0.1`). Each set of paired-end reads was mapped against the *C. albicans* SC5314 haplotype A, version A22 downloaded from the Candida Genome Database (CGD) (http://www.candidagenome.org/) using HISAT2 version 2.0.4. SAMtools was then used to sort and convert SAM files. The read alignments and *C. albicans* SC5314 genome annotation were provided as input into StringTie v1.3.3 (PERTEA *et al.* 2015), which returned gene abundances for each sample. Raw and processed data have been deposited in NCBI's Gene Expression Omnibus (EDGAR *et al.* 2002). RNA-seq data are accessible through GEO Series accession number: GSE121210.

2.3 Results

2.3.1 Large-scale Invasive Growth Screening in *Candida albicans*

To determine a subset of genes that play roles in *C. albicans* invasive growth, the GRACE strains (ROEMER *et al.* 2003) were conditionally repressed on tetracycline-containing (100 μg ml^{-1}) YPD agar at 30°C for 96 h followed by a 10 second wash of each

colony with a stream of water. We identified those strains that were not washed off the plates and displayed wrinkled colonies during growth under the repressing conditions as potentially containing repressed genes that were involved in the regulation of invasive growth. We identified 69 strains from the 2,357 single mutants of GRACE collection that were invading the agar medium during tetracycline repression, suggesting those genes code for functions that inherently repress invasiveness and filamentation. The genes observed included several transcription regulators and overall were implicated in a variety of cellular processes including cell division, chromosome maintenance, DNA repair, transport, ubiquitination and sumoylation (Fig 2.1).

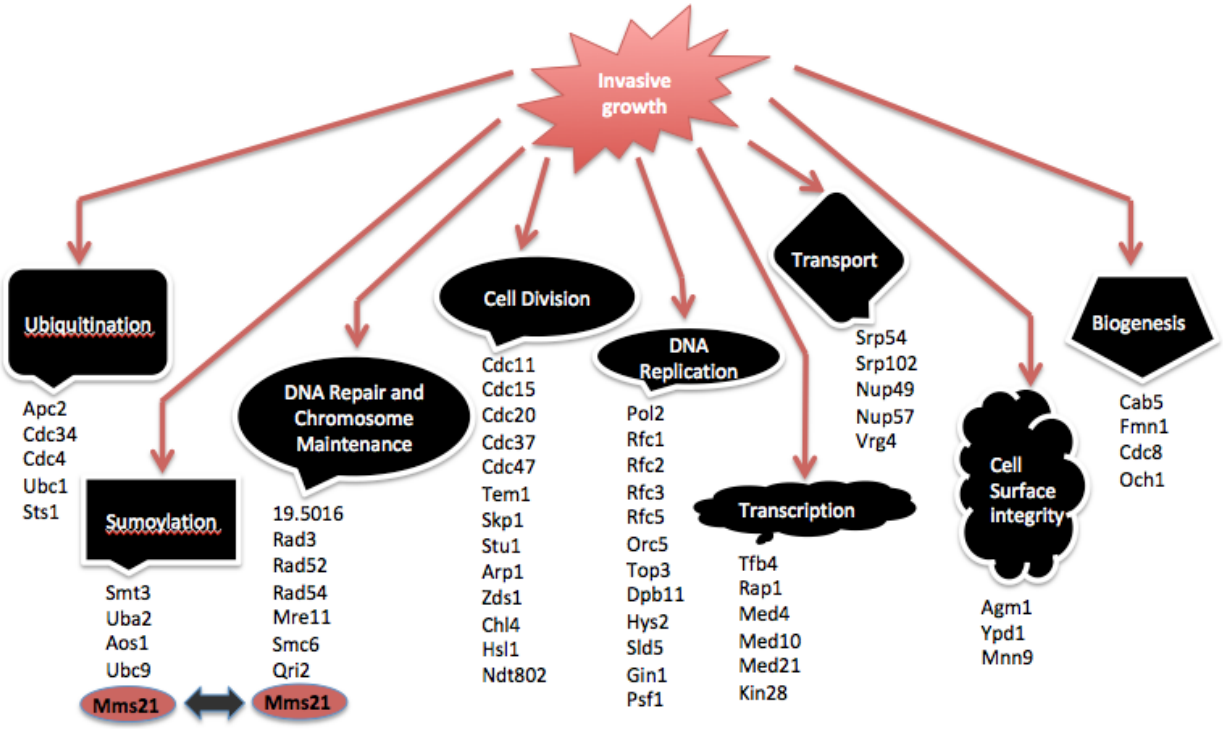


Figure 2.1 Invasive mutants collection from GRACE screening. A total of 69 mutants were scored invasive from the GRACE library screening of which 60 mutants are shown here with their respective cellular processes.

These data emphasized the inherent complexity involved in controlling invasiveness and filamentation in *C. albicans*. Many of the negatively acting components were also involved in the control of cell cycle and division, highlighting a correlation between normal cell division and filamentation. Some of the invasive growth repressors, specifically those involved in cell division, chromosome maintenance and DNA repair, have been previously implicated in negative control of filamentation (O'MEARA *et al.* 2015). We also found a considerable number of genes that have not been previously implicated in control of filamentation that include transcription regulators, genes involved in cellular transport and biogenesis, and some members of the ubiquitination and sumoylation pathways.

2.3.2 Members of The Sumoylation Pathway Negatively Regulate Filamentation

Sumoylation is involved in the processing of many proteins including elements involved in cellular differentiation (ZHOU *et al.* 2004). We examined all members of the SUMO regulatory circuit in the GRACE collection for invasive growth and filament formation following their repression with tetracycline. All tetracycline-repressed strains containing members of the SUMO pathway were viable, and all showed invasiveness and filamentation, forming highly invasive wrinkled colonies on agar medium (Fig 2.2A). The cellular morphology of all the repressed mutants was highly filamentous, with cells displaying both hyphal and pseudohyphal phenotypes while the parental strain cells remained in the yeast form (Fig 2.2B). The SUMO protein Smt3 and the E1 enzyme Aos1 have been previously implicated as repressors of filament formation (O'MEARA *et al.*

2015) while Uba2 (a second E1), Ubc9 (an E2) and Mms21 (an E3) had not been previously reported; this suggests that the SUMO pathway as a whole has a critical role in the regulation of filamentous growth. Since we also found Smc6 and Qri2 of the Smc5/6 complex to be filamentation and invasiveness repressors, and Mms21 is also part of this complex, we investigated Mms21 as a key player of filamentation control.

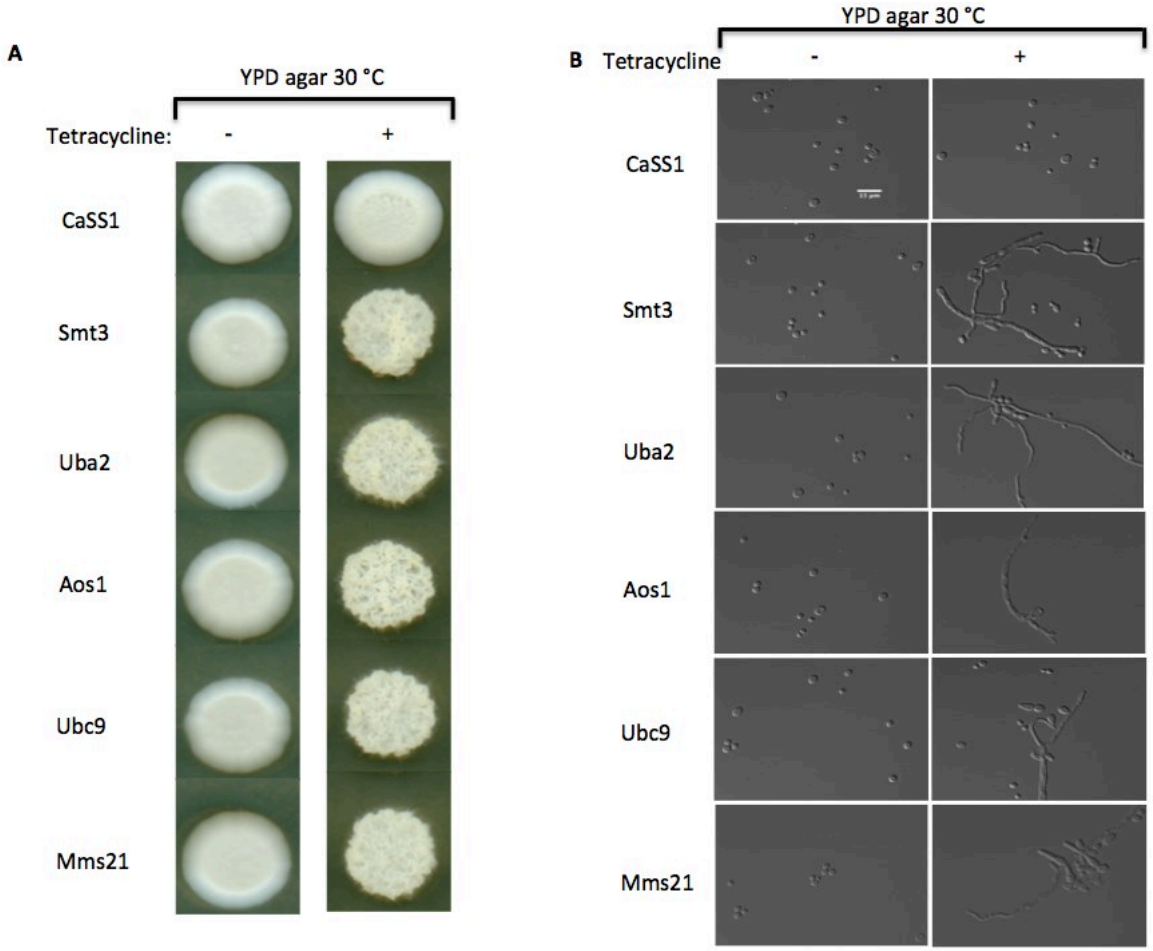


Figure 2.2 Members of the sumo pathway negatively regulate filamentation**.** (A) Putative members of the sumoylation pathway in *C. albicans* were grown at 30°C on YPD agar with and with out 100 µg/ml Tetracycline for conditional repression. Each mutant repression caused fuzzy colonies except for the wild type CaSS1. (B) Representative DIC images of repressed and non repressed SUMO mutants at 63 X magnifications using LEICA DM 6000 microscope, mutants cells displayed filamentous (hyphal, pseudohyphal)

morphology while the wild type CaSS1 showed normal yeast morphology. Scale bar is set at 15 µm.

2.3.3 Mms21 Contains a Conserved SUMO SP Domain

The *C. albicans MMS21/C3_06200C_A* gene resides on chromosome 3 (http://www.candidagenome.org/), and encodes a 272 amino acid protein that is 32% identical to its *S. cerevisiae* ortholog, 39% identical to *S. pombe* Nse2 and 32% identical to human Nse2. Mms21 contains an SP (Siz/PIAS) RING domain that is exclusively found in SUMO E3s and is involved in the final conjugation step of SUMO to the lysine of the substrate protein. The SP domain of *C. albicans* Mms21 is predicted to start at S^{192} and run until L^{239} and is highly conserved among different organisms (Fig 2.3). This domain contains the motif CXH/CX_4C that is required for zinc ion (Zn^{2+}) binding to create protein stability, and is important for E3 ligase function (YUNUS AND LIMA 2009).

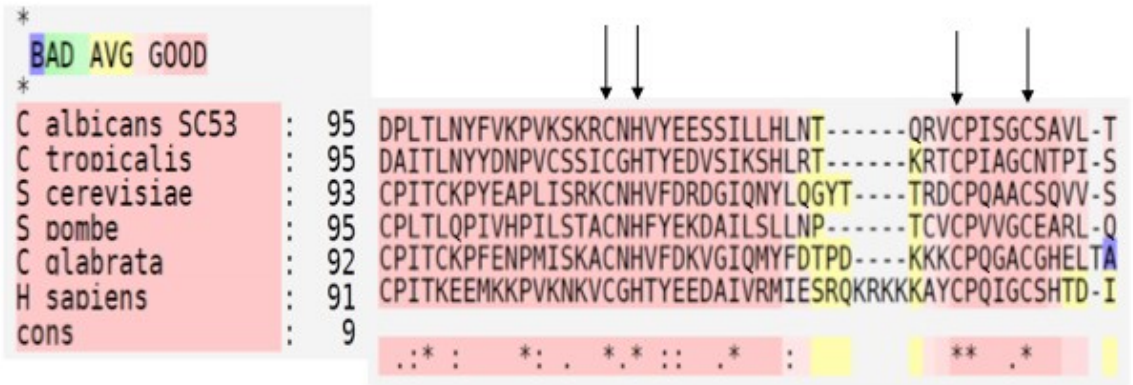


Figure 2.3 T-COFFEE structural protein alignment (Expresso) of the Siz-PIAS (SP) domain among different organisms. The arrows indicate the residues of CXH/CX_4C required for Zn^{2+} binding. Red regions represent conserved residues while the * asterisk is used to label residues that are identical in the consensus sequence. Color scheme of BAD AVG

GOOD represents the level of consistency between the final alignment and the library used by T-Coffee. A score of 9 (dark red regions) represents highly consistent alignment.

2.3.4 Mms21 Deleted Cells are Viable and Highly Invasive

Mms21 is an essential SUMO E3 ligase in both *S. cerevisiae* (ZHAO AND BLOBEL 2005) and *S. pombe* (MCDONALD *et al.* 2003). Mms21 is also part of the chromosome maintenance and the DNA repair Smc5/6 complex; some members of this complex (O'MEARA *et al.* 2015) have been previously implicated in controlling filamentous growth in *C. albicans*. We have confirmed from screening of the GRACE library that inactivation of Mms21 de-repressed invasiveness, suggesting Mms21 may be a link between invasive growth and important cellular processes like chromosome maintenance, DNA repair and sumoylation.

To confirm our finding of Mms21 as a non-essential protein in *C. albicans* that is required to repress invasiveness and filamentation, we generated a complete knock out of both the alleles in the strain SN148 using the standard two-step deletion approach. One copy of the gene was replaced with the selection marker *HIS1* or *URA3* while the other was replaced by *ARG4*. The complete removal of both functional alleles of *MMS21* and their replacement with the selection markers was confirmed by PCR. These *mms21 Δ/Δ* (null) mutants, both with and without *URA3*, grew slowly in liquid YPD medium and formed small but highly invasive wrinkled colonies on YPD agar under standard yeast growing conditions (Fig 2.4A). A functional copy of *MMS21* from the wild type SN148 strain was reinserted into the RP10 locus of the *mms21 Δ/Δ* mutants; this recovered the normal smooth yeast phenotype from the mutant-specific wrinkly invasive phenotype.

Thus Mms21 is a non-essential protein in *C. albicans,* but a key player in morphogenesis control.

2.3.5 Mms21 SP Domain Deleted Mutants are Also Invasive

Screening of the GRACE library suggested an important role for sumoylation in repressing invasiveness and filamentation. To test whether specific E3 ligase catalytic activity is involved in this control, we replaced the 92 amino acids of the C-terminal SP domain of the functional *MMS21* allele in a deletion heterozygote strain with a C-terminal TAP and an *ARG4* selectable marker. The *mms21Δ/SPΔ* mutant was confirmed by both PCR and western blot analysis. These *mms21Δ/SPΔ* mutants also formed highly invasive and slow growing small wrinkled colonies on YPD agar plates under yeast growth conditions (Fig 2.4A), suggesting that Mms21-mediated sumoylation plays a major role in *C. albicans* invasive growth control.

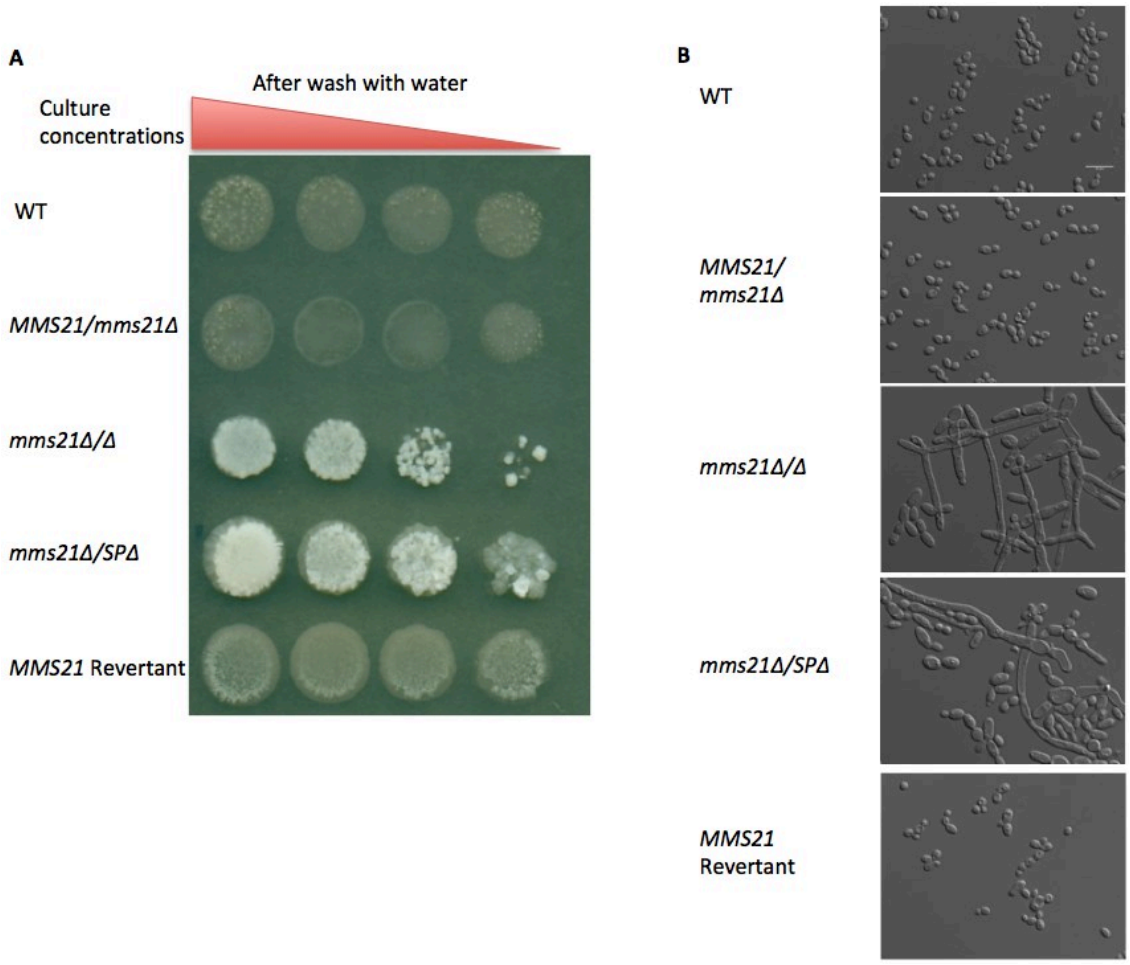


Figure 2.4 Colony and cell morphologies of *MMS21* mutants. (**A**) 1:10 serial dilution of overnight culture of mutants were spotted on to YPD agar and then incubated at 30°C for 4 days. Individual colonies were washed with a stream of water for 10 seconds; *mms21Δ/Δ* and Mms21 SP deleted mutants were found invasive. (**B**) DIC images of mutants grown under yeast conditions at 63X magnification with a scale bar of 15 µm. The *mms21Δ/Δ* and *mms21Δ/SPΔ* mutants displayed large filamentous cells.

2.3.6 *mms21Δ/Δ* and *mms21Δ/SPΔ* Cells Displayed Yeast to Filamentous Phenotypes, Increased Cell Size and Cell Cycle-related Defects

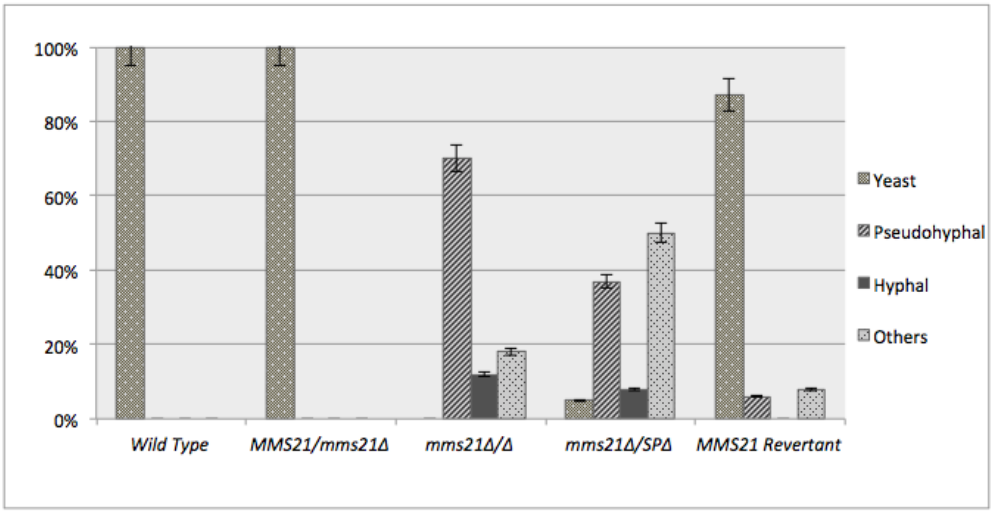
A structurally and enzymatically functional Mms21 is required for normal growth. Mms21 deletion in *S. cerevisiae*, *S. pombe* and humans is lethal while mutations

in its C-terminal SP domain caused severe growth defects (ANDREWS *et al.* 2005; ZHAO AND BLOBEL 2005; HOCH *et al.* 2008). Moreover, mutations in the N-terminal region of the *S. cerevisiae* Mms21 affected its interaction with Smc5 (Smc5/6 complex) and also caused growth defects (DUAN *et al.* 2009a). We have found that the *mms21Δ/Δ* and *mms21Δ/SPΔ* cells displayed abnormal phenotypes at 30°C when cultured in liquid YPD. Individual mutant cells were morphologically aberrant ranging from enlarged and elongated yeast-like cells to pseudohyphal or hyphal cells (Fig 2.4B). There were 12 % hyphal, 70 % pseudohyphal and 18 % abnormal (large irregular yeast-like) phenotypic variants in the *mms21Δ/Δ* mutants with none of the cells displaying true normal yeast morphology despite growing at 30°C (Fig 2.5A). The *mms21Δ/SPΔ* mutants, on the other hand, were 8 % hyphal, 37 % pseudohyphal, 50 % abnormal and 5 % yeast type. In the WT background 100% of the cells exhibited the normal yeast form. In both the complete deletion and the SP deleted strains, the mutant cells grew as clusters of attached cells that were difficult to dissociate. We measured and compared (see materials and methods) the cell size of the mutants in log phase yeast growth conditions. The yeast-form-like *mms21Δ/Δ* cells ranged from 15.9-63.6 μm^2 in size while *mms21Δ/SPΔ* yeast-like cells were in the range of 13.6 μm^2 to 62 μm^2 (Fig 2.5B). The size of these mutants was nearly twice the size of the wild type and the *mms21* revertant strain cells, which ranged from 7.9 μm^2 to 32.8 μm^2. Thus either Mms21 deletion or its SP domain removal has a significant impact on the cellular morphology of *C. albicans.*

We measured the cell density of cultures of these mutants after a 24 hour incubation period by counting the number of cells using a haemocytometer to avoid

issues caused by cell filamentation during spectrophotometric growth measurements. We used a starting cell count of 1×10^7 cells/ml. The *mms21Δ/SPΔ* mutants reached 25×10^7 cells/ml while the *mms21Δ/Δ* mutants acquired cellular densities of only 20×10^7 cells/ml after 24 hours incubation at 30°C in liquid YPD. The wild type and the revertant strains reached much higher cell densities near or above 80×10^7 cells/ml (Fig 2.5C). Furthermore, measuring cell densities using a spectrophotometer (OD 600 nm) revealed that the WT strain reached an OD of 1 from 0.1 within 6 hours of incubation under yeast growth conditions while *mms21Δ/Δ* mutants took around 14 hours to acquire an OD of 1. These data highlight the impact of a structural and enzymatically functional Mms21 on the growth of *C. albicans*.

A



B

Strains	Area (μm²)	
	Smallest cell	Largest cell
WT	8.3	29.6
MMS21/ mms21Δ	7.9	27.8
mms21Δ/Δ	15.9	63.6
mms21Δ/SPΔ	13.6	62
MMS21 revertant	9.4	32.8

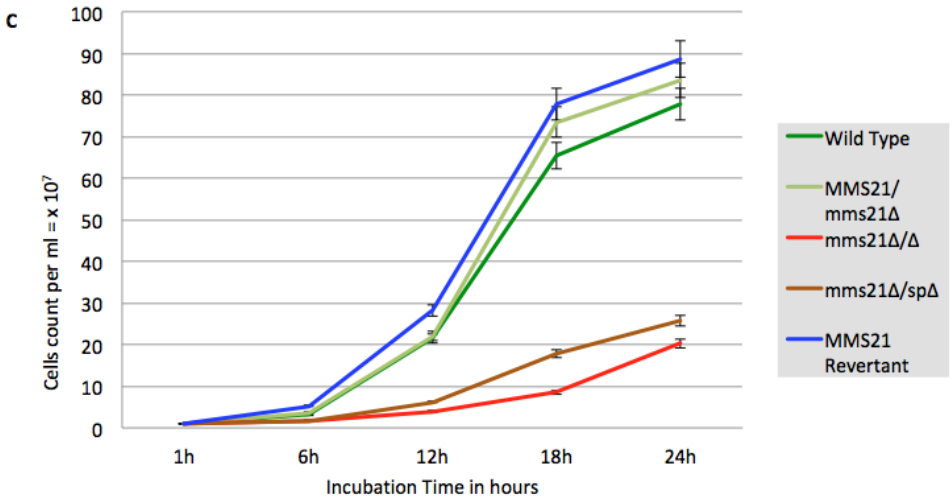


Figure 2.5 Cell division and growth defects in Mms21 mutants (**A**) A clustered column graph showing percentage of different cell morphologies displayed by mutants. The *mms21Δ/Δ* and *mms21Δ/SPΔ* mutants have increased filamentous phenotypes. (**B**) A table of the cell size area of the log phase growing strains measured in DAPI channel using ImageJ Fiji 1.0. (**C**) A graph showing cell counts of mutants and wild type cultures in YPD at 30°C for 24 h. The initial cell count was started at 1×10^7 cells per ml. Both *mms21* mutants doubled their first cell population at the 6 hour mark while the revertant and the wild type doubled every 2 hours. Error bars are based on the standard deviation from two biological replicates of each data point reading taken every 6 h.

In *S. cerevisiae*, the SUMO E3 ligase activity encoded by *MMS21* is essential for nuclear integrity and proper organization of telomeres (ZHAO AND BLOBEL 2005). Deletion of the *C. albicans* SUMO protein Smt3 resulted in significant nuclear segregation and chitin distribution defects (LEACH *et al.* 2011). To examine the effects of the Mms21 null and its SP domain deletion on nuclear segregation, cells (*n* = 200 for each) of the mutants and wild type strains were stained with DAPI and observed under the microscope. Interestingly, a high proportion of cells of both null and SP deleted mutants showed variable nuclei (Fig 2.6A). In case of the wild type strain, 92 % (*n* = 62) of large budded cells have 2 nuclei, one in each of bud and mother cell while 91 % (*n* = 62) of

small budded cells have nuclei at the junction of mother and bud cell separation. For the heterozygote strain with one functional *MMS21* allele similar numbers were observed, i.e. 94 % (*n* = 68) for large budded cells and 91 % (*n* = 66) for small budded cells, respectively. Both of these strains showed a normal pattern of yeast cell morphogenesis where large budded cells with 2 nuclei were in late M while small budded cells were in the late G2 phase of the cell cycle (BERMAN 2006). Interestingly, both null and SP deleted mutants showed variable defects in nuclei segregation with large budded cells containing only one nucleus either in the mother or in the bud cell, or containing non-budded cells having two nuclei prior to bud formation. Unlike the wild type cells with more prominent circular nuclei, a large number of mutant cells have irregular shaped nuclei (Fig 2.6A). These abnormal nuclear variations were 68 % (*n* = 136) in null and 47 % (*n* = 96) in SP deleted mutant cells respectively, indicating cells might be in late S/G2 arrest or have a defective M cycle due to a failure in the DNA repair machinery thus causing abnormal nuclear content and the respective increase in cell size.

Septal ring formation is central to faithful nuclear segregation between mother and daughter cells (BERMAN 2006). We tested all of our strains for chitin composition of the cell wall and septa using calcofluor staining. All of the wild type cells (n = 214) showed a normal chitin distribution in their cell walls and at septal junctions. The null and SP deleted mutant cells displayed uneven chitin distribution, while the septum separating a bud and a mother cell were not as prominent and distinct as in wild type cells (Fig 2.6B). The null mutants had 46 % (n = 201) while SP deleted cells had 40 % (n = 194) of cells showing chitin and septal abnormalities suggesting that septa and chitin

deposition were delayed in the mutants rather than occurring at the start of nuclear and cellular division. These results are consistent with previous findings that the Mms21 protein is required for proper nuclear and cell segregation in budding yeast. All these defects in cellular morphology, (slow growth, enlarged cell size, filamentation and abnormal nuclear segregation), were eliminated by complementing the null mutant with a functional copy of *MMS21*, showing Mms21 is required for normal cellular physiology in *C. albicans*.

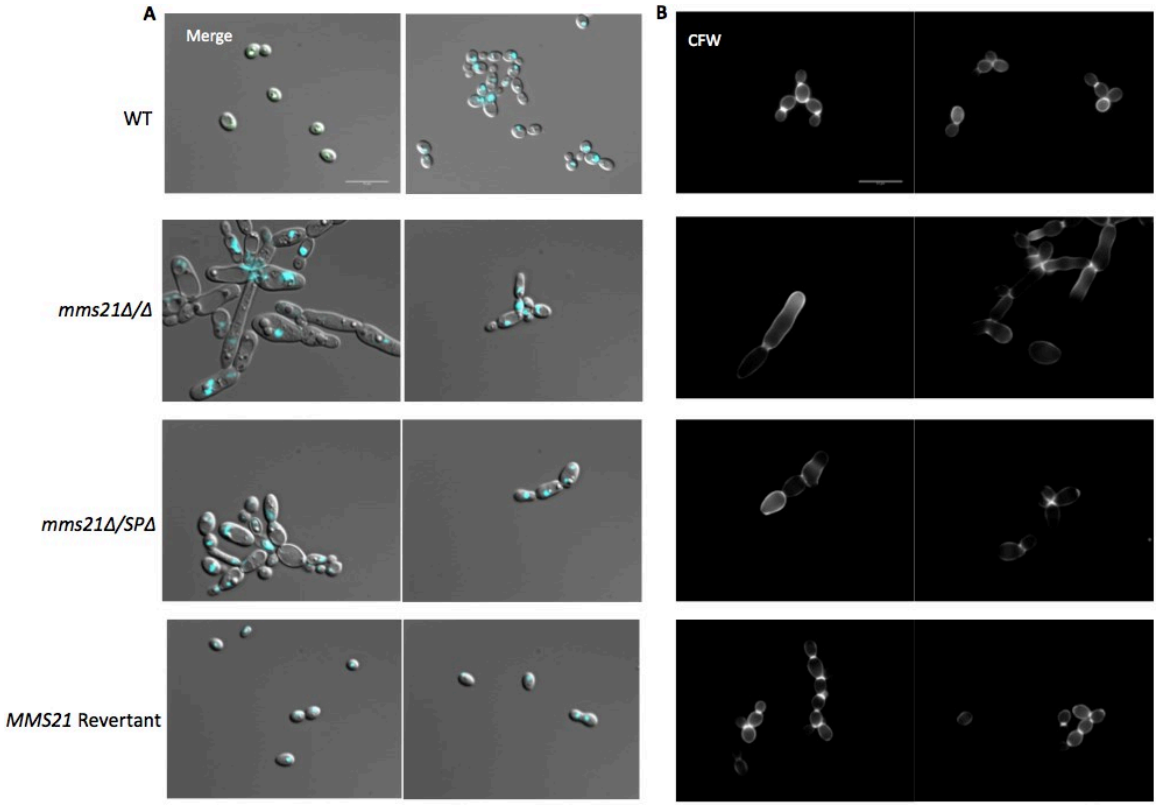


Figure 2.6 Nuclear segregation and cytokinesis defects in Mms21 mutants (**A**) Wild type and strains with one wild type allele of *MMS21* showed normal nuclear segregation. The *mms21Δ/Δ* and *mms21Δ/SPΔ* mutants displayed considerable variation in the number of nuclei present in the individual cells. (**B**) Calcoflour stained cells where wild type cells displayed even chitin distribution and prominent septa while both *mms21* mutants have abnormal chitin composition and inconspicuous septa.

2.3.7 Mms21 is Required for Genotoxic and Cellular Stress Response

MMS21 was first identified as a gene in *S. cerevisiae* whose inactivation conferred sensitivity to methyl methane sulfonate (MMS), X-rays and ultraviolet radiation (PRAKASH AND PRAKASH 1977). In yeast and other organisms Mms21 has been found to be part of the Smc5/6 DNA repair complex where it is required for sumoylating other complex members (such as Smc5) as well as for providing structural stability to the complex (MCDONALD *et al.* 2003; ANDREWS *et al.* 2005; POTTS AND YU 2005; ZHAO AND BLOBEL 2005). Genotoxic stress compromises the genome integrity of an organism and in *C. albicans* defects in response to such stresses have been implicated in increased polarized growth and white to opaque switching (BACHEWICH *et al.* 2005; SHI *et al.* 2007; ALBY AND BENNETT 2009). In *C. albicans* several proteins have been reported to be sumoylated during hydrogen-peroxide-induced oxidative stress (LEACH *et al.* 2011). As our phenotypic screening revealed that there is an increase in filamentation in Mms21 deleted mutants we assessed whether these mutants were defective in response to different types of stresses.

We assayed the response of *mms21Δ/Δ* and *mms21Δ/SPΔ* to genotoxic stress causing agents including the alkylating agent MMS, the DNA replication stalling chemical hydroxyurea, the oxidative stress agent H_2O_2 and thymine-dimer-causing UV radiation (Fig 2.7A and D). Both *mms21* null and SP deleted mutants were highly sensitive to MMS showing that the loss of the Mms21 protein or its putative SUMO SP domain function blocks cells from recovering from MMS induced cellular stress. The null mutant, but not the SP deleted mutant, never recovered from HU, UV and H_2O_2 induced stresses,

highlighting the importance of Mms21 for a potential structural role in maintaining the Smc5/6 complex intact and fully functional for its subsequent DNA damage response. The reinsertion of the *MMS21* gene into the null mutant resulted in the wild type response to these stresses.

Global sumoylation analysis has revealed several sumoylated proteins including various heat shock proteins in both *S. cerevisiae* and *C. albicans* (ZHOU *et al.* 2004; LEACH *et al.* 2011). The null mutants and cells lacking the SP domain necessary for E3 activity were unable to grow at 42°C, consistent with Smt3 being implicated in sumoylating heat shock proteins in *C. albicans*. As well as not growing at 42°C, cells lacking both alleles of Mms21 showed extremely poor growth at 37°C (Fig 2.7B). The fact that *mms21Δ/MMS21* and the Mms21 revertant grew normally at 37°C implies that at least one fully functional allele of the Mms21 gene may be required for *C. albicans* thrive under normal human body temperature.

We also surveyed the effects of different cellular stress causing agents in the Mms21 defective mutants. The cell wall is the first line of defense for most of the unicellular organisms including bacteria and fungi. The cell wall of *C. albicans* contains 10% chitin that is important for the rigidity of the cell to the external harsh factors (RUIZ-HERRERA *et al.* 2006). Congo red and the antifungal drug caspofungin cause cell wall stress by targeting glucan and chitin synthase (RONCERO AND DURAN 1985; GHANNOUM AND RICE 1999). The *mms21* null mutant was highly sensitive to both Congo red and caspofungin (Fig 2.7B). Mutants were also tested against cell-membrane-stress-causing ergosterol inhibitors (GHANNOUM AND RICE 1999). The null and SP deleted mutants were

highly sensitive to amphotericin B as they were unable to recover from drug treatment. Ergosterol biosynthesis inhibitors including fluconazole, clotrimazole and posaconazole were highly effective against the null mutant, while intriguingly the SP deleted mutant showed an essentially normal response (Fig 2.7C). As well, the null mutant also grew slowly under salt stress (Fig 2.7D). Altogether, these results show that Mms21 plays important roles in *C. albicans* physiology by helping the pathogen cope with genotoxic and cellular stresses.

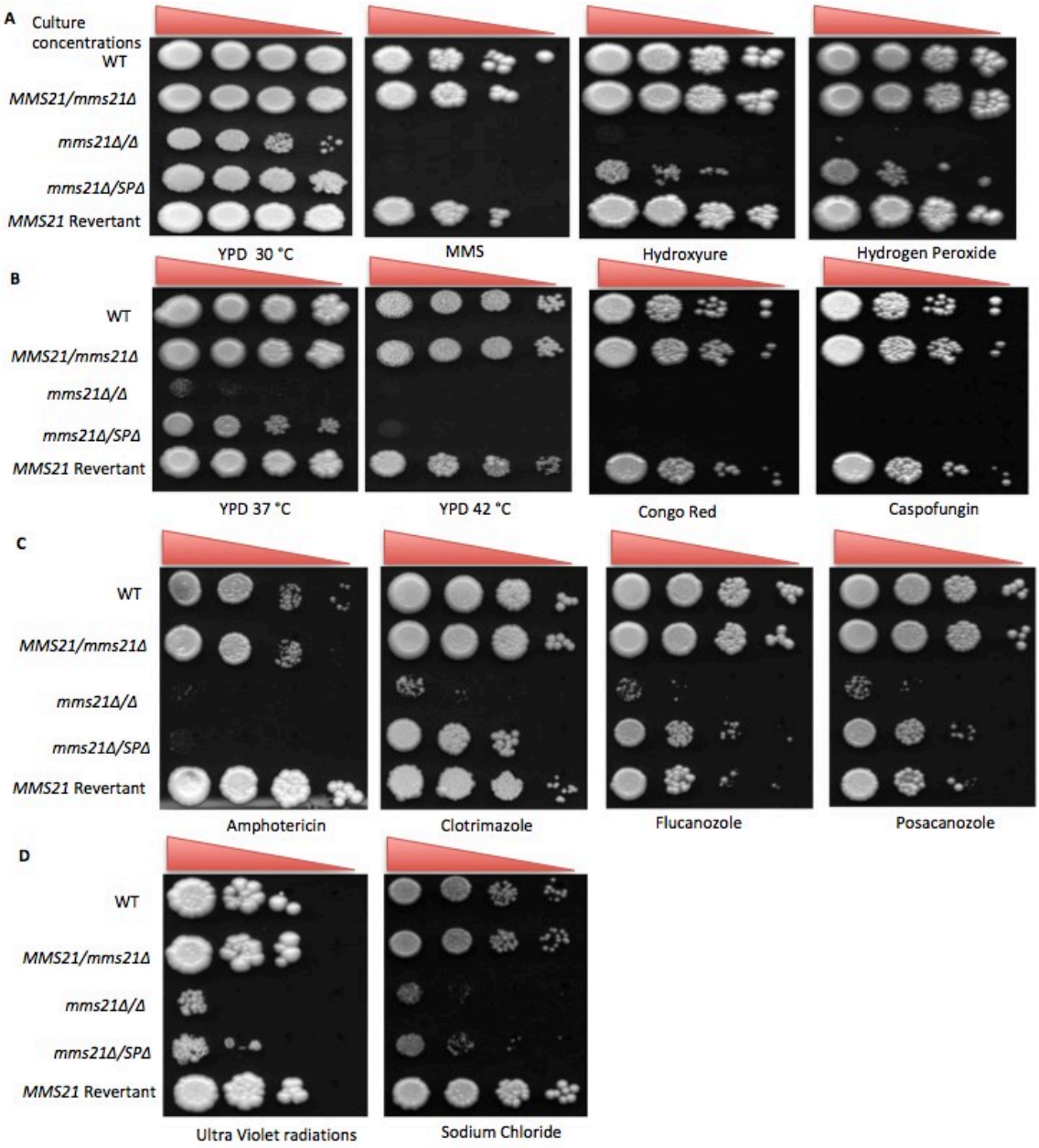


Figure 2.7 Genotoxic and cellular stress assay. A 1:10 serial dilution of Overnight cultures grown in yeast growth conditions were spotted (highest cell concentrations being 1×10^6 cells per ml while lowest being 1×10^3 cells per ml) onto YPD agar plates containing different chemicals and were incubated at 30°C for 4 days except for caspofungin (7 days); (**A**) strains were subjected to genotoxic stress of Methyl Methane sulfonate (0.01% v/v), Hydroxy Urea (10mM) and Hydrogen peroxide (5mM), (**B**) strains response to heat (37°C and 42°C) and cell wall stress caused by congo red (150 µg/ml) and caspofungin (0.75 µg/ml), (**C**) to determine resistance to different cell membrane damaging drugs, amphotericin B (1 µg/ml); clotrimazole (0.5 µg/ml), fluconazole (10

µg/ml), posacanozole (10 µg/ml), and (**D**) sensitivity to UV radiation and NaCl (1 M). Experiment was repeated 3 times for each sample for consistent results.

2.3.8 Expression Profiling of *mms21Δ/Δ*

Since the null *mms21* mutant has a significant affect on the physiology of *C. albicans* through an increase in invasiveness and filament formation, and displays slower growth rates, aberrant nuclear segregation, and weak stress responses, we assayed the overall differences in gene expression between the *mms21Δ/Δ* mutant and the wild type under yeast growth conditions using custom microarrays. Using a statistical-significance analysis with a *P* value of less than 0.05 and a cutoff of 2 fold, we have identified 373 genes whose levels of expression were significantly affected in *MMS21* inactivation; 151 genes were up and 222 were down regulated. The majority of up-regulated genes were involved in ribosome and ribonucleoprotein biogenesis, in RNA processing, and in response to reactive oxygen species, while many were associated with nucleic acid and heterocycle metabolic processes. Around 15 % of the up genes were associated with *C. albicans* filamentation and virulence. Among the down-regulated genes were genes involved in oligo-peptide transport, hexose transport, stress response, nitrogen utilization, oxidation-reduction processes and fatty acid metabolism. An increase in the expression of ribosomal protein genes and a decrease in the expression of oligo-peptide and hexose transport genes have been implicated in *C. albicans* infection of epithelial tissues (SPIERING *et al.* 2010).

There are a number of significant genes that are up and down regulated during *C. albicans* yeast-hyphal transition (NANTEL *et al.* 2002; KADOSH AND JOHNSON 2005), and

when the cells are grown under hypoxia (STICHTERNOTH *et al.* 2011). We have found many genes that increase or decrease in their expression correlated with the yeast-hyphal transition and virulence (Table 2.1). Important genes in this list include *PGA13*, *PGA26*, *PGA31*, *ALS1*, *ALS3*, *ECE1*, *HWP1*, *SOD4* and *SOD5*. The genes for two important GPI (Glycosyl Phosphotidyl Inositol) mannoproteins that constitute 30-40 % of the cell wall by weight (Pga13 and Pga26), were highly up-regulated in our mutant (5.8 and 3.9 fold respectively); these are key players in *C. albicans* morphogenesis and virulence (LAFORET *et al.* 2011; GELIS *et al.* 2012). Similarly, there is upregulation of genes for Hwp1 (hyphal wall protein 1), Als1 (agglutinin like sequence 1) and Ece1 (extent of cell elongation1), all proteins highly important for fungal adherence to the host cells, development of hyphae, and virulence (STAAB *et al.* 1999; TSUCHIMORI *et al.* 2000; SHEPPARD *et al.* 2004; MOYES *et al.* 2016). The expression of the *SOD5* (superoxide dismutase 5) gene was 2.3 fold up-regulated in the *mms21* null and this gene is also up-regulated during hyphal development (MARTCHENKO *et al.* 2004). This increase in expression of *SOD5* could be related to the adaptation to the reactive oxygen species in the absence of Mms21.

Table 2.1 A list of genes that are highly significant for hyphal development and virulence and their fold increase and decrease in expression in *mms21 null* against WT using microarray and RNA-seq analysis.

Genes (Increase expression in yeast-hyphal switch)	Fold Increase (Microarray) 8 5 3 2.5 2	Fold Increase (RNA Seq.)	Genes (Decrease expression in yeast-hyphal switch)	Fold Decrease (Microarray) -2 -2.5 -3 -5 -8	Fold Decrease (RNA Seq.)
PGA13	5.7	3.7	*FGR41 (PGA35)*	-2.0	-2.9
PGA26	3.9	2.2	*RIB3*	-2.1	-1.7
SOD4 (PGA2)	3.6	4.8	*RHD3 (PGA29)*	-2.2	-1.0
ALS1	3.6	2.0	*HMO1*	-2.4	-2.1
RBT1 (ECE99)	3.0	2.5	*MAF1*	-2.4	-1.0
RFX2	2.9	2.4	*STF2*	-2.8	-2.5
CSH1	2.8	1.5	*SOD6 (PGA9)*	-4.3	-3.9
ALS3	2.7	2.1	*IFE2 (BDH2)*	-4.6	-2.8
PGA31	2.7	1.7	*UCF1*	-8.9	-1.8
IHD1 (PGA36)	2.5	1.1			
SOD5 (PGA3)	2.3	2.7			
ECE1	2.1	8.7			
HWP1	2.1	4.5			
UME6	1.7	8.5			
HGC1	1.5	4.8			

We followed up on our microarray profiling by performing whole genome transcriptome sequencing (RNA-seq) analysis of *mms21Δ/Δ* and WT under yeast growth conditions. By using a P value of less than 0.05 and a fold cut off 2.0 we found 188 up and 97 down expressed genes. Analysis using the Candida Genome Database Gene Ontology Slim Mapper revealed that among upregulated genes, 21.3 % represent for RNA metabolic process and 16 % represent genes involved in ribosome biogenesis.

Among the downregulated genes 17.5 % were related to carbohydrate metabolic processes, 16.5 % to transport and 14 % to stress response. Our RNA-seq data, for the aforementioned HSGs expressions, were consistent with our microarray data (Table 2.1). Furthermore, the RNA-seq analysis revealed Ume6, a true hyphae transcription factor (ZEIDLER *et al.* 2009), to be more than 8 fold, and Hgc1, a hyphal specific cyclin (ZHENG *et al.* 2004), to be 4.8 fold up respectively.

Transcription profiling also revealed several genes of the stress response machinery were down-regulated including *DAP1*, *RAD2*, *RAD4*, *BUB3*, and *HNT2* that support our findings of low genotoxic and cellular stress response in null *mms21* mutants. Overall, our transcription data suggest that in the absence Mms21 there is a definite change in the expression levels of several important genes that contribute to *C. albicans* morphogenesis and virulence.

2.3.9 Filamentation in *mms21Δ/Δ* is Independent of Efg1

Efg1 is required for hyphal protein expression under hyphal inducing conditions (SOHN *et al.* 2003). Intriguingly, our microarray data show no change in the level of expression of important filamentation transcription factor Efg1 in the null *mms21* mutant. Efg1 lies upstream of Hwp1 and its deletion eliminates the yeast-hyphal transition (SHARKEY *et al.* 1999). To test whether filamentation shown by the *mms21* null mutant is independent of Efg1, we deleted *MMS21* in the *efg1Δ/Δ* strain as previously described (REGAN *et al.* 2017). As shown in Figure 2.8, the *efg1Δ/Δ* mutant cells were locked in yeast form but deleting both alleles of *MMS21* (*efg1Δ/Δ mms21Δ/Δ*) resulted

in filament formation like the *mms21* null. Therefore, filamentation caused by the absence of Mms21 is independent of the pathway controlled by Efg1.

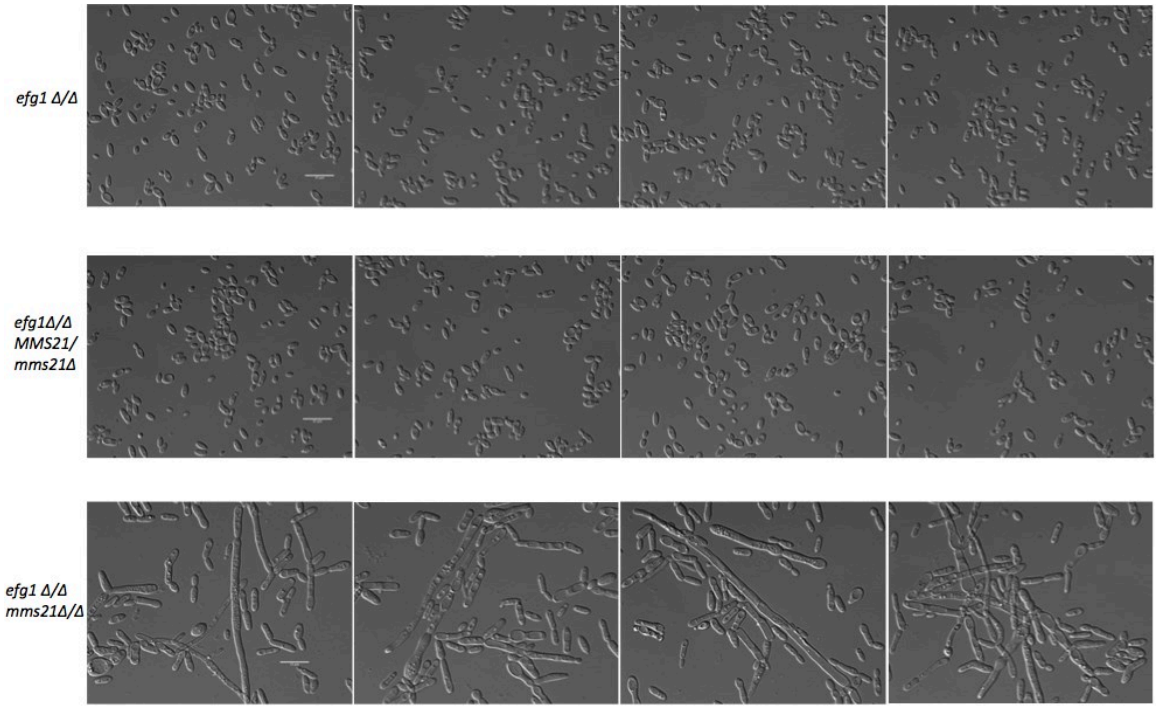


Figure 2.8 Representative DIC images of *efg1 Δ/Δ, efg1 Δ/Δ MMS21/mms21Δ* and *efg1 Δ/Δ mms21Δ/Δ* mutants at 63 X magnifications using LEICA DM 6000 microscope. *efg1 Δ/Δ* mutants were locked in yeast form but deleting both alleles of *MMS21* in *efg1 Δ/Δ* mutants relieved cells from yeast form resulting in filamentation. Scale bar is set at 15 µm.

2.4 Discussion

We have characterized, as a component of invasiveness, the *C. albicans* ortholog of Mms21, initially identified as part of the essential DNA damage repair SMC5/6 complex of yeast and also as an E3 ligase of the sumoylation pathway in humans, *S. cerevisiae* and *S. pombe* (McDonald *et al.* 2003; Andrews *et al.* 2005; Potts and Yu 2005; Zhao and Blobel 2005). We found *MMS21* to be non-essential in *C. albicans,* which is consistent with the findings that the unique SUMO protein-encoding locus *SMT3* is non-

essential as well (LEACH *et al.* 2011). Although the SUMO pathway is dispensable in *C. albicans,* the loss does generate growth related defects. Either deleting *MMS21* or removing its active site domain directs *C. albicans* to form invasive filaments under normal yeast growing conditions. Intriguingly, microarray analysis of the *mms21Δ/Δ* mutant compared with wild type cells under yeast growth conditions revealed a 2 to 6 fold increase in the expression of several genes involved in filamentation and virulence. Previously, large-scale homozygous mutants study in *C. albicans* has found Mms21 deleted mutants showed normal competitive fitness in mouse (NOBLE *et al.* 2010) though they could not find any change in cell morphology upon deletion.

The yeast-hyphal (and hyphal-yeast) transition in *C. albicans* can be triggered by both environmental conditions and internal stimuli. There are a number of filamentation transcription factors and repressors important for this transition. The hyphal G cyclin 1 (Hgc1) is a key protein involved in hyphal induction (ZHENG *et al.* 2004); this protein is regulated by the cyclic AMP (cAMP) pathway that is one of the key pathways controlling hyphal morphogenesis (HARCUS *et al.* 2004; LU *et al.* 2013). This pathway also involves transcription factor Efg1 and is repressed by Nrg1-Tup1 (KADOSH AND JOHNSON 2005). Ume6 is another transcription factor required for true hyphal elongation as it acts downstream of Efg1 and other activators like Czf1 and Cph1 while it is also repressed by Nrg1-Tup1 (ZEIDLER *et al.* 2009). We found that the null *mms21* mutant formed filaments in the absence of Efg1, and our microarray and RNA-seq data also showed no significant changes in the expression of Efg1, Czf1, Cph1 and repressors Nrg1-Tup1 in the null mutant. However, Hgc1 and Ume6 were both upregulated

indicating these two proteins and the HSGs were transcriptionally induced independent of Efg1. The activated Ume6, in particular, may have derepressed the HSGs thereby affecting the cell wall architecture to form hyphae as previously proposed (ZEIDLER *et al.* 2009).

In *S. cerevisiae*, Mms21 is part of the DNA repair Smc5/6 complex and mutations in this complex result in G2/M phase cell arrest (MENOLFI *et al.* 2015). Moreover, the Smc5/6 complex interacts and sumoylates the DNA damage repair Rad52 protein for recombination repair at rDNA (TORRES-ROSELL *et al.* 2007). In *C. albicans*, Rad52 deletion resulted in filamentation with cells arrest in the G2/M phase (ANDALUZ *et al.* 2006). Since an unperturbed Smc5/6 complex is required to regulate the Rad52 protein and subsequent recombination, it is possible that Mms21 deletion is effecting this regulation thus resulting in G2/M cycle arrest. In *S. cerevisiae*, it has been proposed that extending the G2 phase or delaying the formation of active cyclin dependent kinase complex would result in pseudohyphae formation (LEW AND REED 1995). Therefore, this increased filamentation in Mms21 absence appears to be a result of defects in cell division and DNA damage repair machinery as seen for other genes (BACHEWICH *et al.* 2003; BACHEWICH AND WHITEWAY 2005). In fact, often the input signals that causes filamentation impose some sort of cell stress whether these signals are changes in temperature or pH or use of N-acetylglucosamine (GlcNAc) or serum (BROWN AND GOW 1999). Mms21 deleted cells are also under stress thus inducing filamentation response genes and eventual filamentation.

Besides the hyphal and pseudohyphal morphology displayed by the mutant strains, many of the cells were enlarged, elongated and irregular shaped and growing at a slow rate when compared to the wild type. We also observed many of these mutant cells were multi-nucleated, suggesting a defect in proper nuclear segregation. Similar defects had been previously attributed to an improper SUMO ligase activity of Mms21 in other organisms (ANDREWS *et al.* 2005; SERGEANT *et al.* 2005; ZHAO AND BLOBEL 2005; DUAN *et al.* 2009a). Moreover, there were abnormal chitin content and inconspicuous septa in these mutant cells. These defects in our mutants likely arise from the inability of these cells to carry out proper sumoylation of the septin system as in *S. cerevisiae* (JOHNSON AND BLOBEL 1999); specific septin related proteins, although not the septins themselves, have been reported to interact with the SUMO Smt3 in *C. albicans* (MARTIN AND KONOPKA 2004; LEACH *et al.* 2011). Mms21 is also part of the DNA repair Smc5/6 complex which is required for the timely removal of DNA-mediated sister chromatid linkages in *S. cerevisiae* (BERMUDEZ-LOPEZ *et al.* 2010). All these abnormal cellular morphologies could have arisen due to mutant cell problems in late G2 or M phases of the cell cycle because mutations in Mms21 caused defects in the Smc5/6 complex and in the sumoylation pathway.

We have observed that both *mms21Δ/Δ* and *mms21Δ/SPΔ* mutants showed impaired response to different genotoxic stress causing agents. The Smc5/6 complex in humans repairs DNA double strand breaks by homologous recombination by recruiting the Smc1/3 cohesin complex to the sites of double strand breaks (POTTS *et al.* 2006). Both *mms21Δ/Δ* and *mms21Δ/SPΔ* mutants were highly sensitive to the DNA alkylating

MMS indicating that not only a whole functional Mms21 but also its active C-terminal SP domain is required to overcome the DNA damage caused by MMS. These findings were consistent with previous reports that the SUMO ligase function of Mms21 is required for DNA damage repair in *S. cerevisiae* (ZHAO AND BLOBEL 2005; RAI *et al.* 2011). In case of other genotoxic stresses, the null *mms21* mutant cells were more sensitive to the genotoxic stress than the mutation specifically defective in the sumoylation active site. This is possibly because the N-terminus of Mms21 is interacting with Smc5 in the Smc5/6 complex and this interaction (to provide structural stability to the complex) is required for the normal DNA repair function of Smc5/6 complex. Compromise of this interaction has been found lethal in *S. cerevisiae,* while altering the Mms21 C-terminus containing the E3 ligase domain has less severe consequences (DUAN *et al.* 2009a). Based on our results and previous findings that the Smc5/6 complex is required to cope with genotoxic stresses we suggest that the structural stability role of Mms21 is more important than its E3 ligase activity in cellular recovery from different types of genotoxic stresses.

The *mms21Δ/Δ* and *mms21Δ/SPΔ* mutants were found to be highly sensitive to a temperature of 42°C. Leach *et al.* showed several heat shock proteins to be sumoylation targets in *C. albicans* including Hsp60, Hsp104, Ssc1. Ssb1 and Sse1. We detected no temperature sensitivity when we deleted Siz1, a second putative SUMO E3 ligase (unpublished work). There is thus a possibility that these proteins are modified via Mms21 mediated sumoylation to help *C. albicans* cope with increasing temperatures. Moreover, Mms21 was also found to be important for the tolerance of *C. albicans* to

amphotericin B and ergosterol biosynthesis inhibitors thus maintaining the cell membrane permeability and pathogen resistance to these drugs.

Overall, from our GRACE library screening we have found Mms21 to be non-essential and a repressor of invasiveness. We have further investigated the roles of Mms21 in *C. albicans* by deleting both the entire gene as well its C-terminal SUMO SP domain. By creating *mms21* null (*mms21 Δ/Δ*) and Mms21 SP domain deleted (*mms21Δ/SPΔ*) mutants, we have found out that the full Mms21 protein as well its C-terminal SUMO activity act as repressors of invasiveness and filamentation. Both these mutants have also shown cell cycle related defects with increases in division time and in defective nuclear segregation. Furthermore, we have shown that Mms21 is critical for *C. albicans* response to genotoxic stresses, and thus plays a vital role in preserving genome integrity. As well, Mms21 is also important for the normal response of cells to the cell wall, cell membrane and thermal stresses. Our data show that Mms21 plays important roles in cellular differentiation, genotoxic and cellular stress responses thus contributing to the pathogenicity and survival of this opportunistic fungal pathogen. It will be exciting to identify the molecular targets of Mms21-mediated sumoylation and establish how they impact on these various cellular processes.

Chapter 3: Mms21 Deletion in *Candida albicans* Triggers Increased Homozygosis at the Mating Type-Like Locus, Mating and Biofilm Formation.

3.1 Introduction

White to opaque switching is a prerequisite for *C. albicans* mating (SOLL 2014). This switching is under epigenetic control and it requires cells to be homozygous at the mating type like (*MTL*) locus. Mating in *S. cerevisiae* is relatively simple where a diploid *MAT a/α* cells can undergo meiosis to *a* or *α* and then mate (HABER 1998). In contrast, the white to opaque switching requirement in *C. albicans* makes its mating more complex than that of its relative. The *C. albicans MTL* locus resides at chromosome 5 and is comprised of two loci designated as *MTLa* and *MTLα* (HULL AND JOHNSON 1999). The *MTLa* locus contains transcription regulator genes *a1* and *a2* while *MTLα* contains *α1* and *α2.* The a2 protein activates a-specific genes while α1 activates α-specific genes (TSONG *et al.* 2003). The a1 and α2 proteins form a heterodimer that acts as a repressor of a variety of genes including Wor1.

In *C. albicans* Wor1 has been identified as a master regulator of white to opaque switching (HUANG *et al.* 2006). *MTL* homozygous strains were not able to switch to opaque upon Wor1 deletion whereas ectopic expression of Wor1 allowed *MTL* heterozygotes to switch to the opaque form (HUANG *et al.* 2006; ZORDAN *et al.* 2006). Homozygosity at the *MTL* locus is achieved either through gene conversion or chromosome loss followed by duplication of the retained copy (SOLL 2014). After homozygosis the cell has to switch to the opaque form to mate (PORMAN *et al.* 2011).

The opaque state is promoted by some environmental factors such as room temperature (25°C), the presence of N-acetylglucosamine (GlcNAc), 5 % carbon dioxide (CO_2), low pH and low oxidative stress (HUANG 2012). Finally, mating between two opaque opposite *MTL* strains is achieved through activation of the mitogen-activated protein kinase (MAPK) signaling pathway (CHEN *et al.* 2002).

Opaque cells, in addition to being mating competent, can also signal white cells to form biofilms (DANIELS *et al.* 2006). Biofilms are a surface associated matrix of cells that involve both yeast and filamentous cells associating into stable, complex structures formed on biotic and abiotic surfaces (NOBILE AND MITCHELL 2006; FINKEL AND MITCHELL 2011). Biofilms provide a controlled environment for fungal cells to thrive while avoiding host immune responses (XIE *et al.* 2012). Filamentous growth is directly associated with Candida biofilm formation. Moreover, *C. albicans* biofilms can also help the pathogen in either causing serious blood stream infections or by providing resistance to antifungal drugs (LIU *et al.* 1994; NOBILE AND MITCHELL 2006; FINKEL AND MITCHELL 2011).

A fundamental question that remains is whether *C. albicans MTL a/α* wild isolates mate in their natural host environment. Recently Ofr1 (opaque formation repressor 1) has been implicated in *C. albicans MTL a/α* white to opaque switching and mating (SUN *et al.* 2016) while deletion of CaDig1, a repressor of the MAPK pathway, resulted in both homothallic (same sex) mating and biofilm formation (REGAN *et al.* 2017). Furthermore, increased genotoxic stress or deletion of DNA repair genes caused an increase white-opaque switching in *MTL* homozygotes (ALBY AND BENNETT 2009). We have recently identified Mms21 to be involved in Candida filamentation and genotoxic

stress response. Here we report low-frequency mating of *C. albicans MTL* a/α strains upon Mms21 deletion. The mating resulted from an increase in white-opaque switching after increased homozygosis at *MTLa*. Moreover, Mms21 mutants also triggered robust biofilm formation indicating a link between *C. albicans* filamentation and biofilm formation upon Mms21 deletion.

3.2 Materials and Methods

3.2.1 Strains and Culture Conditions

Strains used in this study are described in Appendix 1. For general growth and maintenance, strains were cultured in fresh yeast-peptone-dextrose YPD medium (1 % w/v yeast extract, 2 % w/v Bacto peptone, 2 % w/v dextrose, 80 mg/L uridine with the addition of 2 % w/v agar for solid medium) at 30°C. Cell growth, transformation and DNA preparation were carried out using standard yeast procedures. For mating and phenotypic switching screening, YNB (Yeast Nitrogen Base) medium with dextrose (2 % w/v) or GlcNAc (1.25 % w/v) was used. Other carbon sources, including galactose, fructose or mannitol (each 2 % w/v) were used. For opaque colony identification, Phloxine B (5 µg per ml) was added to the agar medium.

3.2.2 GRACE Library Shut off Assay

The 69 invasive GRACE library (ROEMER *et al.* 2003) strains were transferred from frozen glycerol stocks into 96 well plates containing liquid YPD and incubated overnight at 30°C. Next day cultures were serially diluted in 10 fold stages at room temperature to a dilution concentration of 10^3 cells per ml and then 100 µl were spread onto YNB (plus

uridine) dextrose agar containing 1 % (w/v) 5-fluroorotic acid (5-FOA). Plates were incubated at 30°C for 4-7 days and then each colony was picked and subsequently streaked onto YPD and YNB without uracil to confirm ura auxotrophic GRACE switch off. Positive Ura auxotrophs from YPD were picked, grown overnight at 30°C in liquid YPD and then stored as 22-25% (final concentration) of glycerol stocks in −80°C freezer.

3.2.3 Mating Assays

Cells were streaked on YNB-GlcNAc agar medium containing phloxine B for 5-7 days at RT to select opaque colonies. Opaque cells of strains 3315*α* and 3745**a** were used as the tester strains for mating. Colonies of the GRACE switch off strains, grown on YNB GlcNAc at 25°C, were patched as squares on YNB GlcNAc agar as experimental strains. For *mms21Δ/Δ* mutant mating, *mms21* null and negative (WT a/α) and positive (WT aa) controls were streaked as horizontal straight lines on separate YNB GlcNAc agar plates. For the GRACE library mating assay, opaque tester strains were grown in liquid YNB dextrose overnight at RT and then spread onto YNB agar plates. For *mms21Δ/Δ* mating, opaque cells of tester strains were streaked as vertical straight lines on YNB GlcNAc plates. The two sets of tester and experimental streaks were replica plated onto the same YNB GlcNAc agar plates after 48 hours of incubation at RT. After 48 hours incubation on YNB GlcNAc plates at RT, cells were replica plated onto different YNB dextrose selection media (-ura, -leu, -his, -trp, -lys). All plates were incubated at 30°C for 3 days before scanning and restreaking on the selection medium for further confirmation of stable prototrophic colonies (DIGNARD *et al.* 2008).

Quantitative mating assays were done in liquid YNB GlcNAc medium. Opaque cells of testers and experimental strains were grown in liquid YNB GlcNAc at RT with shaking at 220 rpm for 24 hours. Cells were counted with a haemocytometer at 100x magnification and then testers and experimental strains were mixed in 5 ml fresh YNB GlcNAc liquid medium in 50 ml Falcon tubes at a final concentration of each strain of 1x 10^7 cells per ml. Cells were incubated at RT at 220 rpm shaking for 48 hours before plating onto YNB Glucose medium for prototrophic selection; selection plates were incubated at 30°C for 3 days before counting the colonies (Lu *et al.* 2014).

3.2.5 Phenotype Switching

White and opaque cells were all selected from single colonies on YNB-GlcNAc medium after five days at room temperature. Cells then were suspended in water, the cell concentration was adjusted, and the suspensions plated on agar medium containing 5 μg/ml phloxine B and different carbon sources. Plates were incubated at room temperature. Data were collected and plates were scanned on the 7^{th} day and the frequency of sectored colonies calculated by standard statistical methods.

3.2.6 Microscopic Analysis

For white and opaque cell morphologies, overnight cultures grown in YCB-GlcNAc or other carbon source medium at RT were subjected to phase differential interference contrast (DIC) microscopy. Cells were examined by DIC at 63X and fluorescent microscopy at 100X magnification using a Leica DM 6000 microscope mounted with a TIR camera.

3.2.7 Immunofluorescence Assay

Single colonies of experimental strains growing for 5 d at RT on YCB-GlcNAc plates were inoculated into fresh YNB dextrose liquid medium and grown overnight at RT. After cultures reached saturation at 5×10^6 cells per ml, cultures were centrifuged and each pellet was washed 3 times with 0.01 M PBS. Washing was followed by cells blocking with 1 ml blocking buffer (2 % BSA, 1 % goat serum, 0.01 M PBS) for 30 mins before incubation with 100 µl of opaque-specific primary antibodies for another hour at RT. Cells were then washed 3 times with PBS containing 0.05 % Tween 20. Washed cells were incubated with 100 µl of the secondary antibody - Texas red conjugated goat anti-mouse antibody (1/100 dilution in blocking buffer) for 1 hour in the dark at RT. After incubation, cells were washed 3 times with PBS containing 0.05 % Tween 20 followed by once with PBS only, five mins each time. Cells were finally suspended in 50 µl PBS and 5 µl was applied to each slide then covered and sealed with a cover slip. Slides were placed in a microscope slide box for protection from light before observation at red spectrum under the Leica DM6000 fluorescence microscope (Texas red excitation 596 nm and emission 620 nm).

3.2.8 *URA3* Loss Assay

To confirm genomic instability the following assay was designed. Homozygous mutant strain (*mms21Δ::URA3/mms21Δ::ARG4*) and heterozygous WT (*MMS21/mms21Δ::URA3*), created as discussed in Chapter 2.2.3, were grown overnight in liquid YPD at 30°C. The next day cells were counted using a haemocytometer at 100X magnification and 1×10^6 cells per ml for each strains were taken and 10-fold serial

dilutions were performed. Diluted and undiluted cultures were then washed 3 times with sterile distilled water to remove any YPD residue and then 100 µl of 1×10^6 cells per ml were spread onto YNB (plus uridine) dextrose agar containing 1 % (w/v) 5-FOA while 1×10^3 cells per ml of each culture were plated onto YPD plates for viable cell counts. Cultures were grown on 5-FOA plates for 4-7 days at 30°C and then ura auxotrophs were counted and statistical analysis was performed.

3.2.9 Loss of Heterozygosity (LOH) at *MTL* Locus

Strains were grown overnight at 30°C in YPD and then 20 µl of each culture was added into 1 ml of sterile distilled water followed by centrifugation. Pellets were washed 3 times with 1 ml of sterile distilled water while centrifugation was performed in between each wash. Finally, 100 µl of washed cultures were spread onto YNB sorbose (2 % w/v) and incubated at 30°C for 4-7 days. After incubation, small colonies were selected and patched onto YPD agar, incubated for a few days at 30°C followed by PCR confirmation of the *MTL* genotype using *MTLa* and *MTLα* specific primers (Appendix 2).

3.2.10 Biofilm Assay

Biofilm assays were performed as previously described (DANIELS *et al.* 2006). The experiment was run in triplicate for each strain. Single colonies of the experimental strains were inoculated into 5-10 mL of YNB dextrose and grown overnight at RT. After 24 hours, the absorbance of each culture was measured at OD600nm and then final volume of 2 ml of each overnight culture in YNB at OD 2.0 was added into a 12-well polystyrene plate. Plates were swirled to distribute cells evenly and then incubated statically for 48 hours at RT. The YNB medium was then decanted and the remaining

cells were washed three times in 700 µL of 0.01 M PBS. Samples were allowed to air-dry for 45 minutes before staining with 385 µL of 0.4 % w/v aqueous crystal violet for a further 45 minutes. Wells were washed 4 times with 1 mL of sterile distilled water and then plates were scanned at 300pi by an Epson Perfection v500 photo scanner. Each culture was then de-stained by addition of 700 µL of 95 % v/v ethanol. After 45 minutes at room temperature, 200 µl of each destaining solution was transferred to a 96 well plates and then 10 fold dilutions were performed in 95 % ethanol (final volume of 200 µl) and the absorbance at OD_{595} was measured and averaged.

3.3 Results

3.3.1 GRACE Library Mating

The GRACE™ strains are prototrophs and in order to make them auxotrophic to allow further genetic manipulation, the 69 invasive strains were put on 5-FOA containing solid medium. *C. albicans* with *URA3* accumulates toxic fluorouracil in the presence of 5-FOA, so for cells to survive in the presence of 5-FOA they have to get rid of *URA3* (BOEKE et al. 1987). Through inter or intra-chromosomal recombination, the GRACE strains will typically also lose the tet transactivator of the regulated promoter along with the *URA3*. Therefore, to identify loss of the *URA3* marker as well the transactivator, 47 strains with uracil auxotrophy were recovered with all showing the invasive phenotype. Cross-patch mating experiments with testers 3315*αα* and 3745*αα* was performed. The mating assay confirmed 10 of these strains were mating competent with the testers when the regulated gene was inactivated even though these strains still

should have the non-mating a/α genotype. Mating was scored as good, decent or weak. Only 4 strains exhibited good mating efficiency while the remaining 6 were decent or weak maters. However, growth on 5-FOA medium can result in random mutations and aneuploidy so we tested these strains for loss of heterozygosity (LOH) at the *MTL* locus prior to mating using PCR amplification with *MTLa* and *MTLα* specific primers. Half of these strains had become homozygous for the mating type locus, while the *MMS21*, *CDC20*, *CDC47, KIN28* and *GIN1* strains were still *MTLa/α* (Appendix 3). GRACE *MMS21* did mate weakly (Figure 3.1) but growth on 5-FOA did not cause either LOH at the *MTL* locus or loss of other markers (Appendix 3) in the strain suggesting mating was due to the gene shut off itself.

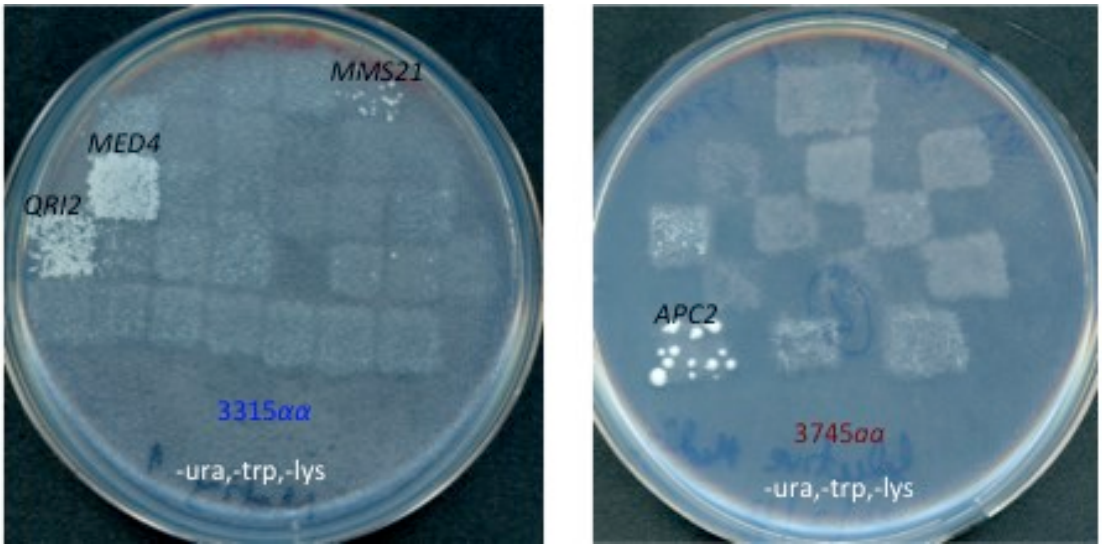


Figure 3.1 Mating ability of GRACE strains**.** We recovered 47 GRACE –Ura strains and patched them at RT on YNB GlcNAc medium as experimental strains. Strains 3745**a** and 3315α, in the opaque state, were used as mating type testers. They are Trp and Lys auxotrophs. These testers were spread on GlcNAc medium and grown at RT for two days and then replica plated to the same medium. After 2 days of growing strains were again replica plated on to the selection medium YNB dextrose (-ura, -trp, -lys) and grown at 30 ^{o}C for 3 days to detect auxotrophic mating products.

3.3.2 Mms21 Deletion Allows *MTLa/α* Cells to Mate

We first checked the mating ability of both *mms21Δ/Δ MTL a/α* and *mms21Δ/SPΔ MTLa/α* growing on YNB GlcNAc with 3315*αα* and 3745*aa* testers. We observed some phototrophic mating progenies of *mms21Δ/Δ* and the 3315*αα* tester growing on different YNB dextrose selection media (-ura, -trp, -lys, and/or –his, -trp, -lys) as shown (Figure 3.2 A.B). In addition strain *mms21Δ/SPΔ* was unable to mate with 3315*αα*, and both *mms21Δ/Δ* and *mms21Δ/SPΔ* were unable to mate with the 3745*aa* tester. We next investigated whether deletion of Mms21 caused increased mating with 3315αα by performing quantitative mating assays (data not shown). We found no increase in the mating frequency in the *mms21Δ/Δ* strain suggesting this mating is a rare event limited to Mms21 deletion strains.

3.3.4 *mms21Δ/Δ* Mating is Carbon Source Dependent

White to opaque switching in *C. albicans* occurs under the influence of some environmental factors such as the use of GlcNAc as carbon source, low pH and 5 % CO_2 (HUANG 2012). Since we found that the *mms21Δ/Δ a/α* strain growing at RT on GlcNAc was able to mate with 3315*αα*, we asked whether this mating is only happening on GlcNAc medium that favors white-opaque switching, or if it also happens on normal YNB dextrose. We tested the mating ability of *mms21Δ/Δ aa* (*MTL* homozygous) strains grown at RT on YNB GlcNAc and YNB dextrose with 3315*αα.* As shown in Figure 3.2 C, only GlcNAc growing mutant strains were able to mate, indicating opaque switching and mating happens in Mms21 deleted cells under normal opaque-favorable conditions.

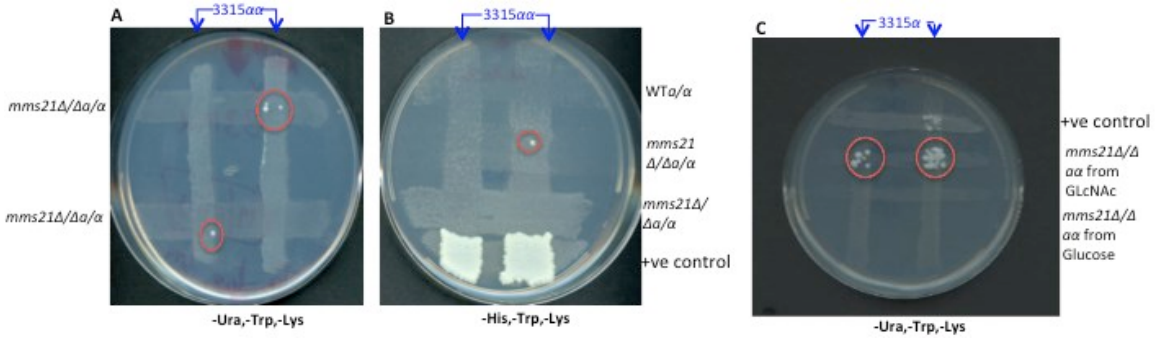


Figure 3.2 Mating of *mms21Δ/Δ a/α and mms21Δ/Δ aa* with 3315*αα* testers**.** Experimental and testers strains were grown for 5 days at RT on YNB GlcNAc then streaked in straight lines on YNB GlcNAc. After 2 days of growth both experimental and tester strains were replica plated on to YNB dextrose plates and allowed to grow further for 2 days at RT. Then plates were replicated on selection medium (YNB dextrose). (**A**) 4 colonies of mating progeny of *mms21Δ/Δ a/α* (*HIS1::ARG4*) appeared on YNB dextrose selective medium after 3 days at RT (**B**) A colony of mating progeny (encircled red) of *mms21Δ/Δ a/α* (*URA3::ARG4*) with tester strain appeared (**C**) Mating ability of *mms21Δ/Δ aa* grown on YNB GlcNAc and dextrose. Only the GlcNAc grown mutant strain was able to mate with the 3315αα tester (encircled red).

3.3.4 *mms21Δ/Δ* Cells have an Increase in White to Opaque Switching

An increase in genotoxic stress or deletion of DNA repair genes caused an increase in white-opaque switching on GlcNAc (ALBY AND BENNETT 2009). Since Mms21 is required for the genotoxic stress response in *C. albicans* and because Mms21 depleted cells could mate on GlcNAc, we asked whether *mms21Δ/Δ* has increased white-opaque switching. Identification of true opaque colonies in the Mms21 deletion strains was complicated by the fact that *mms21Δ/Δ* colonies are readily stained with phloxine B under all culture conditions (Fig 3.3 A). Phloxine B is typically used for opaque colony identification since it can penetrate opaque colony cell wall giving the opaque colony its characteristic pink color (ANDERSON AND SOLL 1987; KENNEDY *et al.* 1988; ANDERSON *et al.* 1990), but cannot be used to detect opaque *mms21* strains due to their inherent

staining. Moreover, opaque cells are large oval shaped single cells rather than small round true yeast/white cells but under a microscope most of *mms21Δ/Δ* cells are much more irregular and filamentous shaped making it hard to confirm true opaque cells (Fig 3.3 B).

We found two types of *mms21Δ/Δ* colonies on GlcNAc plates at RT; one flatter, spread out and fuzzy, and the other also fuzzy, but less spread out. We performed immunofluorescence microscopy of both types of colonies using opaque cell specific antibody blotting. We found that the flatter spread out colony cells showed more opaque signal than the other type (3.3 C). Thus we considered only the former colonies as true opaque and performed statistical white-opaque switching analysis against WT on GlcNAc at RT (Fig 3.3 D). We found a 5 % increase in white to opaque switching upon Mms21 deletion than the WT confirming that *C. albicans* tend to switch more due to defects in DNA repair machinery.

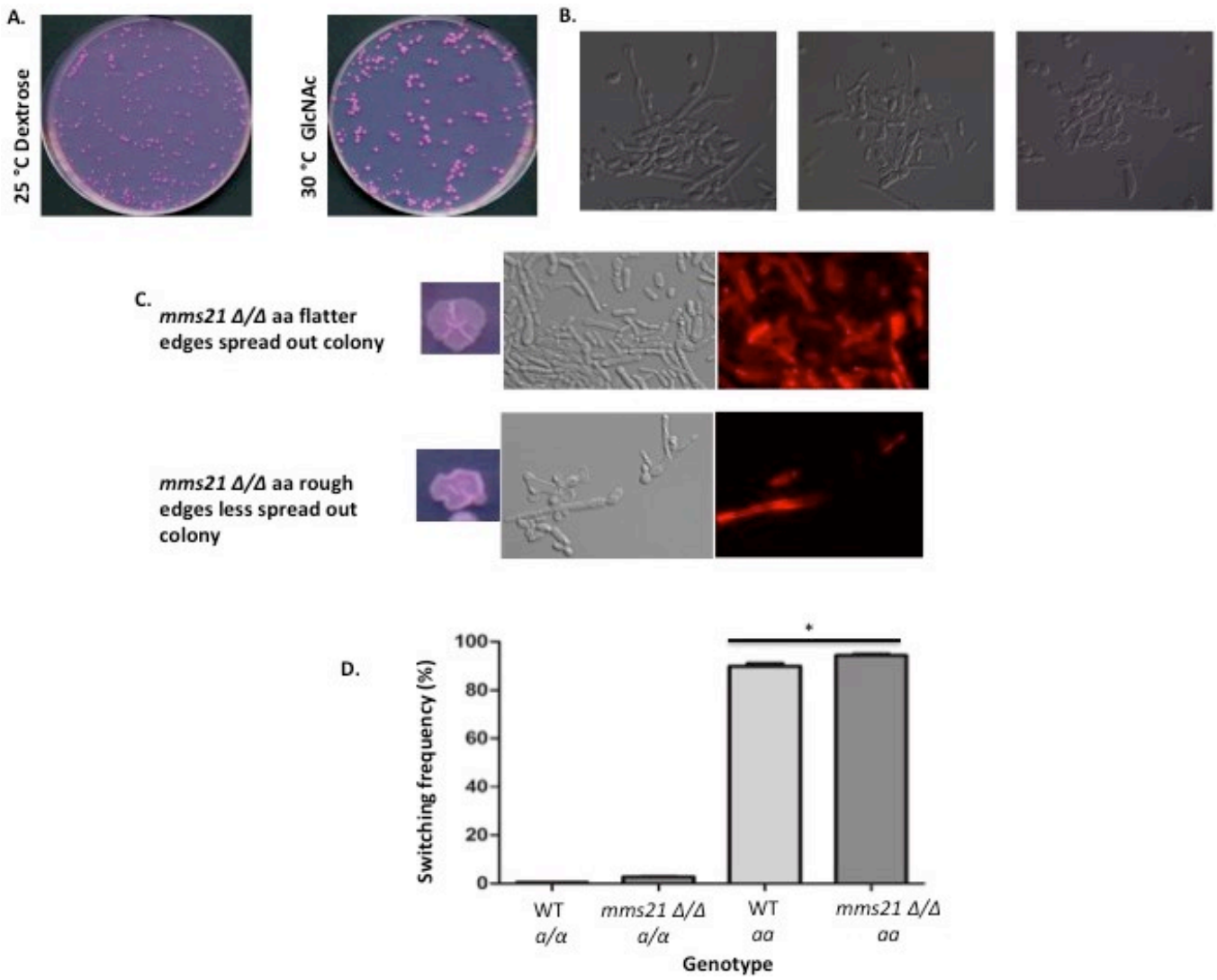


Figure 3.3 White-opaque switching frequency in *mms21Δ/Δ* mutants (**A**) *mms21Δ/Δ* mutant colonies can easily get stained with phloxine B using any carbon source at both RT and 30 °C making it difficult to find true opaque colonies (**B**) *mms21Δ/Δ* cells are filamentous and have large irregular shapes (**C**) mutants growing on GlcNAc phloxine B at RT form 2 types of colonies. Flatter spread out colonies showed more opaque signal under immunofluorescence microscopic analysis (**D**) mutant displayed an increase in white opaque switching, N=1000, *= $p < .05$, Student's t-test.

3.3.5 Mms21 Deleted Cells are Genomically Unstable

There is a possibility of increased recombination in *mms21Δ/Δ* mutants as they are defective in DNA repair and thus may be enhanced in homozygosis at the *MTL* locus.

We checked for the loss of *URA3* at the *MMS21* gene locus in both *mms21Δ/Δ* and WT strains in the presence of 5-FOA (BOEKE *et al.* 1987). We observed a 6-fold increase in *URA3* loss in Mms21 deficient mutants when compared with the WT (Table 3.1). This suggests that *mms21Δ/Δ* strains are genomically unstable and are prone to more recombination than the WT. A generally increased recombination frequency would be predicted to increase homozygosis at *MTL* locus and enhance subsequent mating.

Table 3.1 Frequency of loss of *URA3* in *mms21Δ/Δ* and WT when cells were grown on YNB 5-FOA medium for 5-7 days at 30°C.

Strain	**Medium**	**Culture concentration and dilution**	**Volume plated (ml)**	**Colony count (cfu)**	**Viable cells (cfu/ml)**	**Frequency of *URA3* loss**
WT	YPD	10^{-3} dilution	0.1	201	2×10^{6}	5×10^{-5}
	5-FOA	1×10^{6} cells/ml	0.1	100		
mms21Δ/Δ	YPD	10^{-3} dilution	0.1	128	1.2×10^{6}	3.1×10^{-4}
	5-FOA	1×10^{6} cells/ml	0.1	402		

3.3.6 Mms21 Deletion Resulted in Increased Homozygosis at *MTL*a

Mms21 deleted *MTL a/α* cells were able to mate with 3315*αα* testers and not with 3745*aa* so there may be an increased homozygosis to *MTLaa* causing *mms21Δ/Δ* cells to mate with the the alpha tester. To test this we used a sorbose growth LOH assay. When provided with L-sorbose as the only carbon source, to convert L-sorbose to D-sorbitol for effective utilization, *C. albicans* requires overexpression of the *SOU1* gene

and this is achieved by monosomy of chromosome 5 through random loss of a copy of chromosome 5 (RUSTCHENKO *et al.* 1994; JANBON *et al.* 1998; WANG *et al.* 2004; GREENBERG *et al.* 2005). It had been suggested that negative regulators of Sou1 reside at chromosome 5. Since the *MTL* locus also resides at chromosome 5, growth on L-sorbose will randomly select for loss of either *MTLa* or *MTLα*. We allowed mutants and WT strains to grow on YNB sorbose for 5-7 days and then colonies were picked and subjected to PCR analysis. We found that LOH did not increase in the mutants (Table 3.2) but Mms21 deleted colonies did show, of total homozygosis, 90 % homozygosis at *MTLa* and only 10 % homozygosis at *MTLα*, while the wild type colonies were 53 % *MTLa* and 47 % *MTLα* respectively. This confirms our observations of *mms21Δ/Δ a/α* mating with 3315*αα* is due to increased homozygosis at *MTLa*.

Table 3.2 Rate of loss of heterozygosity and increase homozygosis at *MTLa* in Mms21 deleted mutants.

Strain	**% Loss of *MTLα***	**% Loss of *MTLa***	**% Homozygosis at MTL*a***
WT *a/α*	9	8	53
mms21Δ/Δ a/α	14.5	1.5	90
mms21Δ/SPΔ a/α	8	6	57

3.3.6 *mms21Δ/Δ* and *mms21Δ/SPΔ* Mutants Showed Increase Biofilm Formation

Biofilms are surface associations of yeast and hyphal cells arranged into stable complex structures on biotic and abiotic surfaces (NOBILE AND MITCHELL 2006; FINKEL AND

MITCHELL 2011). The *mms21Δ/Δ* and *mms21Δ/SPΔ* cells tend to flocculate and stick to the walls of glass and propylene containers during routine culture growth. Moreover, microarray and RNA-seq analysis of *mms21Δ/Δ* as compared to WT revealed increased expression of *ALS1* and *ALS3* (Islam *et al.* 2018 accepted article Genetics). The *ALS1* and *ALS3* genes are highly expressed during biofilm formation (SHEPPARD *et al.* 2004). Therefore, we tested both types of mutants for biofilm formation. Strains were statically grown in 12 well plates in YNB dextrose at RT for 48 hours without addition of pheromone (Figure 3.4). We have found that both Mms21 mutants formed significantly more robust biofilms than the WT suggesting a role of Mms21 in modulation of Candida biofilm formation.

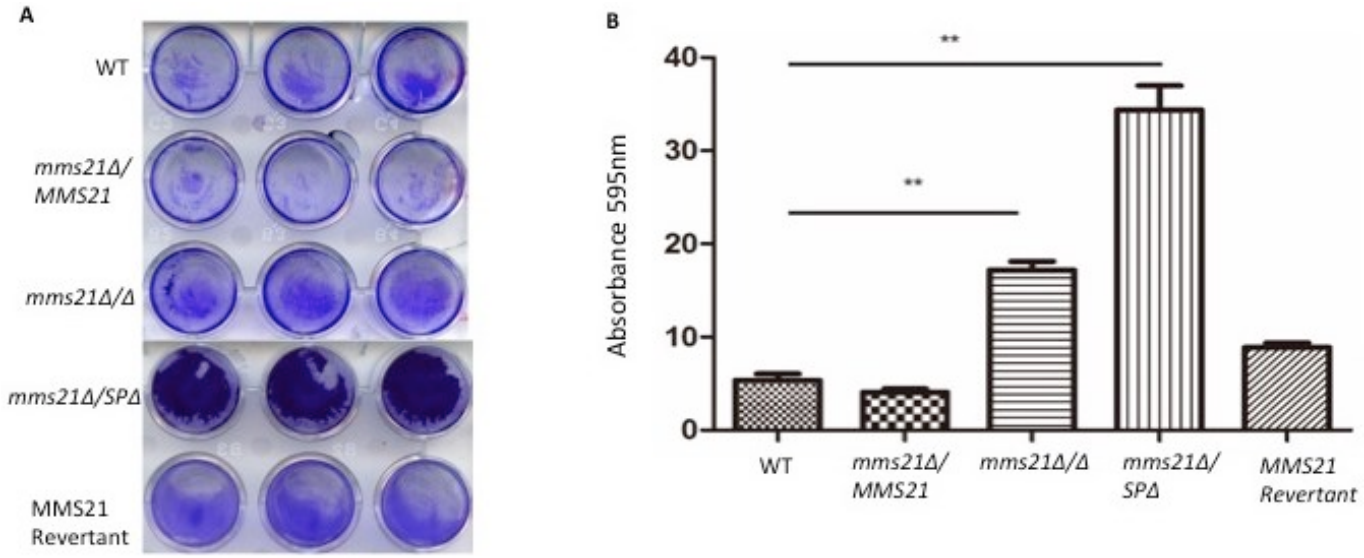


Figure 3.4 Increase in biofilm formation in Mms21 mutants. (**A**) Plate scan image of biofilm formation. Cells were grown in YNB dextrose for 48 hours at room temperature without shaking. Cells from strains were washed with PBS and stained with crystal violet. Plates were scanned after removing staining solution and washing individual plate wells 4 times with sterile distilled water (**B**) Quantification of biofilm formation in de-staining solution. error bars = standard deviation. **= p <.001, Student's t-test.

3.4 Discussion

Through screening of GRACE library and Mms21 deletion strain construction we have found that *C. albicans* can mate as *MTLa/α* but this mating still requires environmental and genomic induction. The Mms21 deletion strain showed an increase in white-opaque switching. Our finding was consistent with previous reports showing that *MTL* homozygous strains, defective in the DNA repair machinery had increased white to opaque induction (ALBY AND BENNETT 2009), although their findings did not provide a detailed analysis of how WT *MTL* heterozygous strains switch to opaque and subsequently mate. Here we suggest that upon increased genotoxic stress or due to a defective repair system, as in the case of *mms21* deleted mutants, Candida will lose heterozygosity at the *MTL* locus and then switch to opaque under favorable conditions. Our reasoning was supported by increases in *URA3* loss (Table 3.1) and an increase in homozygosis at the *MTLa* locus (Table 3.2).

The potential for aneuploidy may arise from defects in the DNA repair machinery. Deletion of the Candida DNA repair protein Rad53 resulted in an increase in chromosome loss and subsequent polarized growth (LOLL-KRIPPLEBER *et al.* 2014). Since Mms21 is one of the key players in DNA damage repair in other organisms, we tested for chromosome loss in *mms21Δ/Δ* mutants using a multiplex PCR assay that allows, eight bands of distinct sizes from the left and right arms of each of Candida eight chromosomes (LENARDON AND NANTEL 2012). The double deleted mutants showed normal-sized bands for each of the right and left arms of all eight chromosomes indicating that all 8 chromosomes are intact in null *mms21* mutants (data not shown). However there

were considerable variations in the reproducibility of this assay using different null mutants. In fact, there was a frequent occurrence of aneuploidy during *C. albicans* mutant strain production (ARBOUR *et al.* 2009).

Previously, we reported that Mms21 negatively regulates invasiveness and filamentation in *C. albicans* (Islam *et al.* 2018 accepted article Genetics). We, therefore, reasoned that Mms21 may also negatively regulate biofilm formation since filamentation is directly associated with Candida biofilms. We observed that both types of Mms21 mutants (*mms21Δ/Δ* and *mms21Δ/SPΔ*) formed increased biofilms. Intriguingly, the SUMO E3 SP domain deleted mutants generated more extensive biofilms than the null mutant. This is partly due to the fact that the *mms21* null grows more slowly than the SP deleted mutant as each cell division occurs after 6 hours incubation (Islam *et al.* 2018) so that the null mutant did not reach the level of growth saturation at the same point as did the SP mutants. Another possible reason is that Mms21-mediated sumoylation may target repression of genes required for biofilm formation. The transcription profiling of the null did reveal increased expression of *ALS1* and *ALS3*, two highly important biofilms formation genes (SHEPPARD *et al.* 2004; SILVERMAN *et al.* 2010), highlighting transcriptional induction of biofilm related genes upon Mms21 deletion.

Though the *mms21* null mutant displayed weak mating, we saw no changes in the expression profile related to mating or pheromone response genes (Islam *et al.* 2018). This could be due to conditions in which the profiling was performed where the cells were cultured under yeast inducing conditions rather than at room temperature.

There was clearly no up-regulation for *WOR1* whose expression is required for opaque cell formation and subsequent mating (MORSCHHAUSER 2010). We propose that mating in *MTL* heterozygotes happens due to increased homozygosis followed by white-opaque switching but this *MTL* homozygosis did not happen at a sub colonial level because the parental *mms21* null colonies that did mate were still *MTL* heterozygotes. Therefore, we suggest this homozygosis at the *MTL* locus happens at the cellular level due to stress and that those individual *MTL* homozygous cells will then grow into colonies. This claim is further supported by our findings that only few of mating progenies of *mms21* null with 3315*αα* testers appeared on selective media plates (Fig 3.2).

Since a majority of wild type Candida isolates are heterozygous at *MTL*, our findings suggest how these heterozygous strain may achieve mating. Increased genotoxic stress or DNA damage will induce WT strains to become homozygous at *MTL* and mating competent. In fact, the internal human host conditions do impose stress on Candida whether its 37°C temperature, low pH or serum (BROWN AND GOW 1999) and the presence of GlcNAc and higher CO_2 levels, especially in the gut, may help *C. albicans* to homozygose at the *MTL* locus leading to switching and mating.

Chapter 4: Methyl Methane Sulfonate Sensitivity in *Candida albicans* and The Role of CaHof1 in Genotoxic Stress Response.

4.1 Introduction

Cells must maintain their genomic integrity to function properly and propagate, but external factors like radiation and DNA damaging chemicals, and internal factors like replication errors and the production of reactive oxygen species, may cause DNA damage that can compromise this integrity (BERNSTEIN *et al.* 2013). The generation of DNA damage can disrupt normal cell function by generating chromosomal abnormalities, which in humans can lead to events such as cell death by necrosis or apoptosis, or to cancer (HOEIJMAKERS 2009). To defend against such events, cells have developed a set of molecular mechanisms to recognize and repair damaged DNA, thus ensuring the accurate transfer of genetic information from generation to generation (ZHOU AND ELLEDGE 2000; WOOD *et al.* 2001).

Because of the genetic and molecular tools available for such studies, the response to DNA damage has been well characterized in the ascomycete yeast *Saccharomyces cerevisiae* (SCHWARTZ *et al.* 2002; BOITEUX AND JINKS-ROBERTSON 2013). In general, different types of DNA damage have been found to induce distinct cellular responses. Methyl methane sulfonate (MMS) is a common DNA methylating agent that modifies both guanine (to 7-methylguanine), and adenine (to 3-methlyladenine), to cause base mispairing and ultimately double strand breaks (DSBs) in the DNA (LUNDIN *et al.* 2005; SHI *et al.* 2007). In the case of DNA damage, including MMS-induced base

alkylations, the cell must recognize the damaged DNA, pause the cell cycle and DNA replication, activate the proper repair machinery and finally restart a normal cell cycle after completion of the repair (SIRBU AND CORTEZ 2013).

The pausing of cellular processes to allow time for the repair of DNA damage results from the activation of signaling pathways regulating processes termed checkpoints (ZHOU AND ELLEDGE 2000). The activation pathway of the checkpoint kinase Rad53 has been well studied (PELLICIOLI AND FOIANI 2005; SWEENEY *et al.* 2005; BRANZEI AND FOIANI 2006; CONDE *et al.* 2010; CHEN *et al.* 2015). First, sensors such as Tel1 and Mec1 receive the signal from damaged DNA (SANCHEZ *et al.* 1996; BARONI *et al.* 2004) and, with the help of adaptor proteins Mrc1 or Rad9 (MURAKAMI-SEKIMATA *et al.* 2010; BERENS AND TOCZYSKI 2012; OHOUO *et al.* 2013; BACAL *et al.* 2018), phosphorylate Rad53. The phosphorylated Rad53 then further auto-phosphorylates and becomes the active form of the kinase (HEIDEKER *et al.* 2007). This activated Rad53 interrupts the cell cycle and regulates the downstream target proteins required to repair the damage. Deletion of checkpoint kinases like Rad53, Rad9 and Mrc1 generates strong sensitivity to DNA damaging reagents like MMS (WEINERT *et al.* 1994; HANWAY *et al.* 2002; KITANOVIC AND WOLFL 2006).

After checkpoint activation, distinct pathways are involved in repairing the DNA damage. The base excision repair (BER) pathway, the Rad52 related recombination epistasis group (KWON AND SUNG 2017), and the Rad6 epistasis group have been shown to take part in the response to MMS-induced DNA damage (SOMASAGARA *et al.* 2017). BER is mainly used to repair damage that creates minor disturbances in the DNA helix

(KROKAN AND BJORAS 2013). The BER pathway is initiated by a DNA glycosylase that recognizes and removes the damaged or abnormal base, leaving an abasic site that is further processed by either short-patch BER (for replacement of 1 nucleotide) or long-patch BER (for replacement of 2-13 nucleotides). The two processes largely use different proteins to complete the repair (MEMISOGLU AND SAMSON 2000). Short patch repair happens in both proliferating and non proliferating cells while long patch repair mainly happens in proliferating cells using replication proteins (KROKAN AND BJORAS 2013). The *RAD6* group represents a post-replication repair pathway, and includes genes encoding specialized translesion synthesis (TLS) polymerases, able to replicate through DNA damage (LAWRENCE 1994). As well, deletion of some components of other repair pathways like the Rad14-related nucleotide excision repair (NER) process (PRAKASH AND PRAKASH 2000), creates cells that are also sensitive to MMS. So overall the cellular response to MMS-induced alkylating events involves a number of repair proteins to recognize and correct the damage. After the DNA damage is repaired, the checkpoint kinase signal needs to be deactivated to ensure that the cell can restart a normal cell cycle. Protein phosphatases like Pph3 and Ptc2/Ptc3 (LEROY *et al.* 2003; O'NEILL *et al.* 2007; KIM *et al.* 2011), whose deletions also generate sensitivity to MMS, appear essential for the recovery from or adaption to MMS-induced DNA damage.

Candida albicans is one of the most common fungal pathogens in humans, and exists in three major morphological forms: yeast, pseudohypha and hypha (WHITEWAY AND BACHEWICH 2007). DNA damage in *C. albicans* cells causes genome instability and abnormal growth. *C. albicans* yeast cells treated with either the DNA-replication

inhibitor hydroxyurea (HU) or the DNA methylation agent MMS exhibited activation of the checkpoint kinase Rad53 accompanied with significant filamentous growth (SHI *et al.* 2007). Similarly, deletion of *RAD52* causes a strong sensitivity to MMS and also activates filamentous growth (ANDALUZ *et al.* 2006), and blocking the deactivation of CaRad53 by deletion of *CaPPH3* promotes filamentous growth and causes increased virulence (SUN *et al.* 2011; FENG *et al.* 2013; FENG *et al.* 2017). Other research also suggests that DNA repair-related proteins are involved in the regulation of morphogenesis and virulence (YAN *et al.* 2015a; YAO *et al.* 2016). Compared to the research in other organisms, the DNA damage response pathways have not been well studied in *C. albicans*.

To enhance our understanding of MMS-induced damage repair in *C. albicans*, we used the GRACE collection (ROEMER *et al.* 2003). We identified 56 conditional mutation strains that were sensitive to MMS. Among those potential DNA damage related genes we noticed that mutation of the *HOF1* gene, which was previously characterized to play a critical function in cytokinesis, led to sensitivity to MMS. To better understand the potential function of Hof1 in the DNA damage pathway, we used genetic approaches to characterize the function of Hof1. These studies suggest that Hof1 plays a role in the Rad53-mediated checkpoint.

4.2 Materials and Methods

4.2.1 Strains, Media and Reagents

C. albicans strains were grown in YPD medium with 50 mg uridine per liter as described (MACPHERSON *et al.* 2005). Strains and primers used in this study are listed in

Appendices 4 and 5 respectively. Methyl methane sulfonate (MMS) was purchased from Sigma (USA). Hydroxyurea (HU) and other chemicals were purchased from Bioshop (Canada). The Yeast Nitrogen Base (YNB) medium was supplemented with appropriate nutrients for plasmids selection and maintenance (Jiang et al., 2004). Solid medium contained 2 % agar.

4.2.2 GRACE Library MMS Sensitivity Assay

The GRACE library was transferred from frozen glycerol stocks into 96 well plates containing liquid YPD with 1 mg/ml nourseothricin using sterile 96 pin replicators and incubated overnight at 30°C. Overnight grown copies of the library were inoculated, using 96 pin replicator, onto YPD agar, YPD agar tetracycline (100 µg/ml) and YPD agar tetracycline with added MMS (0.01 % v/v). Plates were incubated at 30°C for 3 days and strains that were growing well on tetracycline but not on tetracycline+MMS plates were scored as sensitive. The sensitivity of those strains was further confirmed by making 10-fold dilutions of overnight cultures in sterile distilled water, and then plating 5 µl of each dilution on tetracycline and tetracycline+MMS plates. Images of plates and colonies were scanned at 300 dots per inch (dpi) using an Epson Perfection v500 photo scanner.

4.2.3 Gene Deletion, Rescue and Epitope Tagging of Proteins

To construct a *HOF1* deletion strain in *C. albicans*, we used a CRISPR/Cas9 system (Vyas *et al.* 2015). The sgRNA of *HOF1* was formed by annealing primers HOF1-sg-F and HOF1-sg-R, and cloning the resulting ds fragment into the *BsmB*I site of pV1093 to

generate plasmid pV1093-Hof1-sgRNA. A repair DNA fragment, containing the *HIS1* marker, was amplified with primers HOF1-Re-F and HOF1-Re-R using pFA-HIS1 as a template. The pV1093-Hof1-sgRNA was then linearized with *Sac*I and *Kpn*I and transformed into *C. albicans* strain SN148 together with the *HOF1* repair DNA. Transformants were selected on YNB -his plus 200 mg/mL ClonNAT. The knockout strains were confirmed by PCR with primers HOF1-Te-F and HOF1-Te-R. To rescue the phenotype of *HOF1* deletion strain, a 3031-bp DNA fragment containing the full-length *HOF1* gene was amplified with primers HOF1-Te-F and HOF1-Te-R and cloned into the *Kpn*I site of plasmid CIP10 (MURAD *et al.* 2000), generating pCIP10-HOF1. Then, pCIP10-HOF1 was linearized by *Stu*I and integrated into the RPS10 locus of the genome. The integration was confirmed using primers URA-TF and RPS-F.

Similarly, a transient CRISPR/Cas9 system (MIN *et al.* 2016) was used to knock out *MMS22*, *RAD18, RAD14, RAD4, RAD23, MMS21* and *UBC9* in SN148 or the *HOF1* deletion strain. Generally, a Cas9 gene was amplified with common primers P7 and P8. The sgRNA was amplified by annealing PCR with primers P5 and P6, using the products of two separate PCR reactions containing the sgRNA sequence, and repair DNA was amplified from pFA-ARG4. Finally, the Cas9, sgRNA and repair DNA products were transformed into either the wild type or the Hof1 deletion strain and selected on SD-Arg plates. The correct knockout strains were confirmed by PCR.

To construct the *PPH3 HOF1* double deletion strain, a *PPH3* deletion strain (FENG *et al.* 2013) was used as a background strain and similar CRISPR/Cas9 system for *HOF1*

was used. Similarly, the *MMS21* deletion strain constructed in Chapter 2 was used as a background strain to construct the *MMS21 HOF1* double deletion strain.

To test the expression of the Hof1 gene, a myc tag was amplified from the pFA-myc-URA plasmid and integrated at the 5' end of the *HOF1* gene using the CRISPR/Cas9 system, generating a homozygous *HOF1*-myc strain. Similarly, an HA tag was integrated into the 5' end of the *RAD53* gene using the CRISPR/Cas9 system. The integration was confirmed by both PCR and by Western blotting.

4.2.4 Protein Extraction, Western Blot and Immuno-Precipitation (IP)

Exponentially growing cells were harvested by centrifugation and ~100 mg of cells were suspended in 200 µL of ice-cold RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, EDTA-free protease inhibitor mix (Roche), and phosphatase inhibitor cocktail (Roche)) (GAO *et al.* 2014). After adding an equal volume of acid-washed glass beads, cells were broken by 5 rounds of 60 s of beating, with 5 min of cooling on ice between rounds, using a FastPrep-24 Disruptor. The supernatant was collected after centrifugation of the cell lysate at 12000 rpm for 10 min at 4°C. Protein concentrations were determined using a BCA protein assay (FENG *et al.* 2013). Western blot analysis was carried out as described (FENG *et al.* 2017).

For the IP assays, 10 µL anti-myc beads (QED Bioscience Inc) were washed twice with RIPA buffer and incubated with 5 mg total protein extract in 1 mL extraction buffer at 4°C overnight. Next morning, beads were harvested and washed three times with 1

mL RIPA buffer. Proteins were subjected to Western blot analysis as above, using rabbit monoclonal anti-myc antibody (QED Bioscience Inc) to detect CaHof1-myc.

To detect CaRad53-HA, a rabbit monoclonal anti-HA antibody (QED Bioscience Inc) and the goat anti-rabbit IgG secondary antibody were purchased from ODSEY, and processed as described above for the anti-myc antibody.

4.2.5 Genome Stability Assay

To assess genome stability (KUMARAN *et al.* 2013), the CIP10 plasmid (MURAD *et al.* 2000) containing a *URA* marker was linearized by *Stu*I and integrated into the RPS10 locus of both wild type and *CaHOF1* deletion strains, generating heterozygous *URA3*$^{+}$ strains. The strains were streaked in duplicates on YNB dextrose -ura and inoculated in YPD overnight at 30°C. Next day, cells were harvested and washed twice with distilled water before 10-fold dilutions were made for spotting. For each strain, 100 µl of 10^{-1} diluted cells were spotted on YNB dextrose 5-FOA plates while 20 µl of 10^{-4} diluted cells were spotted on YNB dextrose -ura plates to check the cell numbers. All the strains were grown as duplicates in three independent samples. Finally, the average numbers of cells on YNB dextrose 5-FOA plates and YNB dextrose -ura plates were calculated.

4.3 Results

Large-scale Identification of MMS Sensitive Strains in *C. albicans*

To identify MMS sensitive *C. albicans* strains, the GRACE strains (ROEMER *et al.* 2003) were conditionally repressed on tetracycline-containing (100 µg/ml) YPD agar with added MMS (0.01 % v/v) and grown at 30°C for 72 h. We identified 56 strains from the 2,357 single mutants of the GRACE collection that were sensitive to MMS upon repression (Fig 4.1). Approximately half of those sensitive mutants identified were parts of the cell division machinery, with roles in DNA replication, DNA damage repair and cytokinesis. Genes involved in transcription and RNA processing accounted for a further one third of the sensitive strains while the remaining genes were involved in translation, protein processing, cellular transport and biosynthetic pathways.

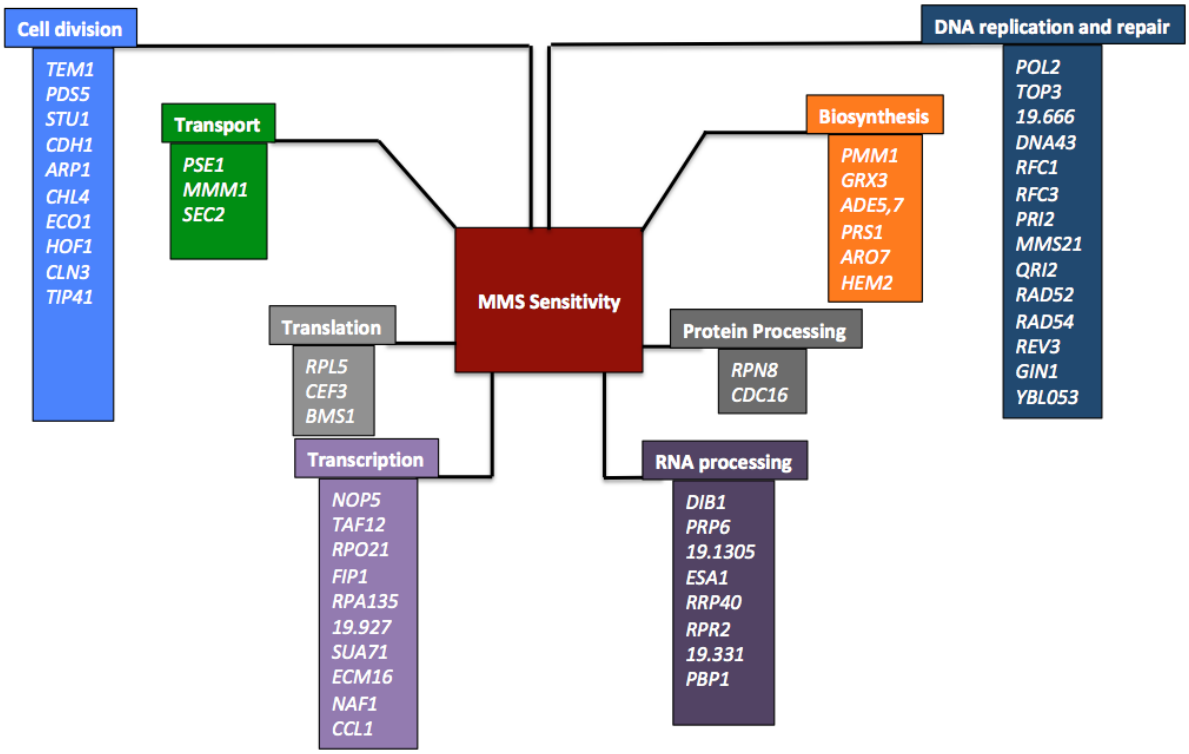


Figure 4.1 MMS sensitive mutants from GRACE screening. A total of 56 mutants were scored as sensitive to MMS from the GRACE library under tetracycline shut off. All 56 mutants with their respective predicted cellular processes are shown.

4.3.2 Deletion of Hof1 Renders Cells Sensitive to Genotoxic Stress

The GRACE library screening showed that conditional inactivation of Hof1 renders cells sensitive to MMS. In *S. cerevisiae,* ScHof1 is a well-studied protein implicated in regulating actin cytoskeleton organization, cytokinesis, actin cable organization, and secretory vesicle trafficking (VALLEN *et al.* 2000; BLONDEL *et al.* 2005; MEITINGER *et al.* 2011; WANG *et al.* 2018). In *C. albicans*, previous research has shown a function for CaHof1 in cytokinesis (LI *et al.* 2006). Since Hof1 is predicted to be a cytokinesis related protein, the MMS sensitivity of the *HOF1* deletion was somewhat unexpected. To investigate the DNA-damage related function of Hof1 in *C. albicans*, we deleted the *HOF1* gene in the SN148 background using the CRISPR/Cas9 system.

As expected, deletion of *HOF1* influenced cell morphology. As shown in Figure 4.2 A, most of the *HOF1* deletion cells showed an elongated shape and were connected together. We counted the number of cell chains with three or more cells. For *HOF1* deletion cells, over 80 % of the cells existed in cell chains, while less than 5 % of the cells existed in chains in the wild type strain (Fig 4.2 B). On solid medium, the *HOF1* deletion strain colony showed irregular surfaces and edges. The OD_{600} of wild type and *HOF1* deletion strains were tested at different time points to check the growth rate (Fig 4.2 C). We found that deletion of *HOF1* caused a somewhat slower growth rate as compared to that of wild type cells.

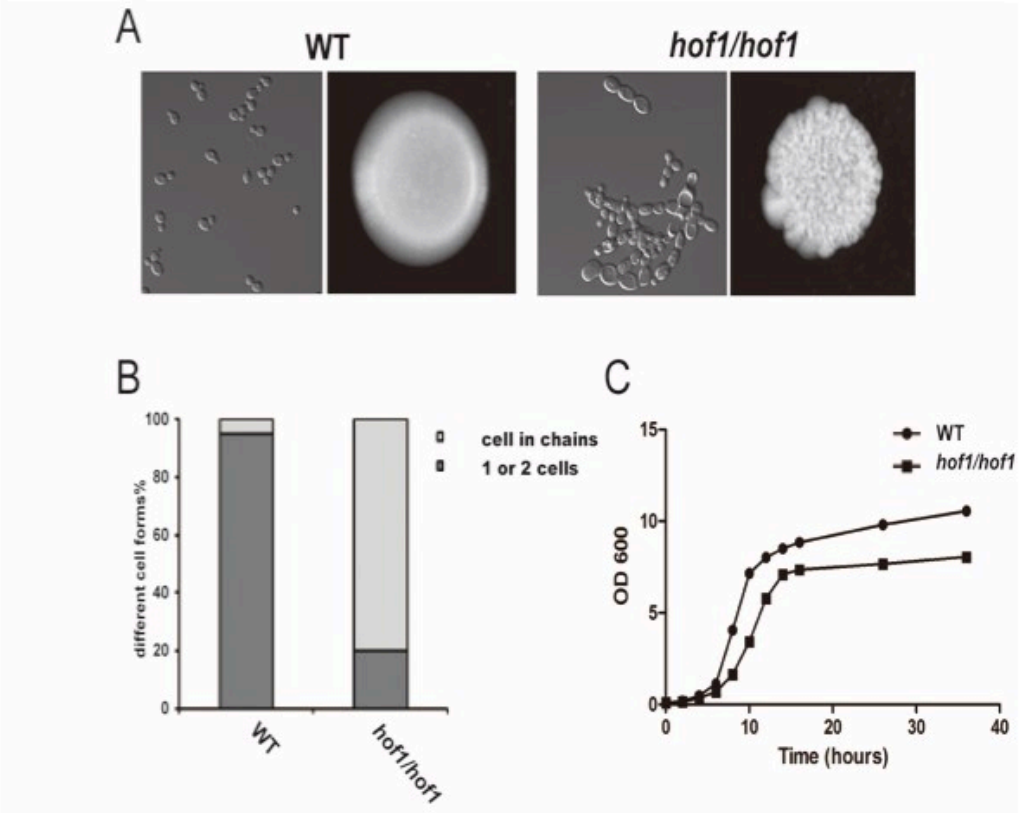


Figure 4.2 Deletion of *HOF1* is linked to a defect in cell division. (**A**) Both the wild type (SN148) and the *HOF1* deletion strain were grown in liquid YPD or on Solid YPD plates. Cell morphologies (DIC images) are shown. (**B**) Percentage of cells in chains was determined by dividing the sum of cells in chains by the total number of cells. (**C**) Growth curves of the wild type and the *HOF1* deletion strains were made by testing the OD_{600} at the indicated time points with appropriate dilutions.

Consistent with the results from the GRACE library screening, deletion of *HOF1* rendered *C. albicans* cells sensitive to MMS, and introduction of *HOF1* back into the homozygous mutant rescued the MMS sensitive phenotype (Fig 4.3 A). Further, we found that deletion of *HOF1* also resulted in sensitivity to both the DNA replication stress reagent HU, and to UV treatment. These results imply a potentially general function of Hof1 in the response to genotoxic stress.

To confirm that the function of Hof1 is related to a DNA damage pathway, we designed a heterozygous *URA* strain to spot on 5-FOA plates to assess genome stability. The result showed that the wild type strain has a low frequency of loss of the *URA* marker (0.37×10^{-4}), while the *CaHOF1* deletion strain has a considerably higher frequency of loss (6.67×10^{-4}), which is nearly 20 times that of the wild type strain (Fig 4.3 B). This result suggests that Hof1 has some role in maintaining genome stability.

In *S. cerevisiae,* mutation of Mms21 resulted in enhanced genome instability correlated with activation of the checkpoint kinase Rad53. Here, we checked the phosphorylation of *C. albicans* Rad53 in a *HOF1* deletion strain using a *MMS21* deletion strain as a control. Consistent with previous results, deletion of *MMS21* significantly increased phosphorylated and potentially activated Rad53, while deletion of *HOF1* also partly enhanced Rad53 phosphorylation as band shift (smear) in mutants was observed when compared to WT (Fig 4.3 C, similar results were also found in Figure 4.5 A). These data suggest that deletion of Hof1 enhances both genome instability and activation of the repair checkpoint kinase Rad53.

We further checked for a potential role of CaHof1 in response to other cellular stresses. We found deletion of CaHof1 rendered cells sensitive to 2.5 mM H_2O_2, 15 ng/ml Rapamycin, 4 μg/mL Fluconazole and 200 mg/mL Congo Red (Fig 4.3 D). These results show that CaHof1 may be involved in response to a variety of stresses.

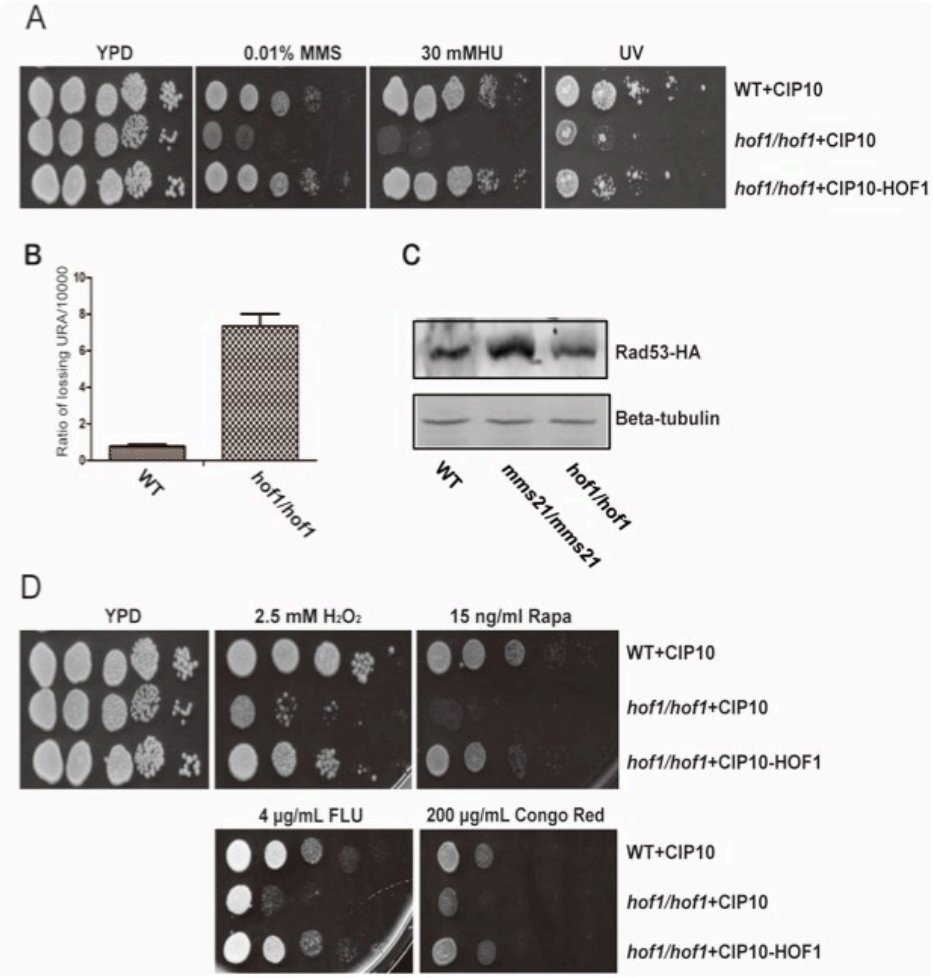


Figure 4.3 Deletion of *HOF1* causes sensitivity to genotoxic stress and increases genome instabilty. (**A**) Phenotype assay of the *HOF1* deletion strain to genotoxic stress. The wild-type (WT) , *HOF1* deletion strain (*hof1/hof1*), and recovery strain (*hof1/hof1: HOF1*) were grown overnight in YPD medium at 30°C, serially diluted and spotted onto YPD plate or YPD plates containing indicated reagents. Plates were incubated at 30 °C for 2–3 days. (**B**) The efficiency of losing the heterozygous *URA* marker was assessed by comparing the colonies on YNB 5-FOA and normal YPD plates. (**C**) The phosphorylation level of Rad53 was checked in the wild type (WT), *HOF1* deletion and *MMS21* deletion strains by Western blot analysis with anti-HA antibody as before. Beta tubulin was used as a loading control. (**D**) Phenotype assay of the *HOF1* deletion strain to H_2O_2, rapamycin, fluconazole and Congo red. The wild-type (WT), *HOF1* deletion strain (*hof1/hof1*), and recovery strain (*hof1/hof1: HOF1*) were grown overnight in YPD medium at 30°C, serially diluted and spotted onto YPD plate or YPD plates containing indicated reagents. Plates were incubated at 30°C for 2–3 days.

4.3.3 Domain Function Analysis of Hof1

In *S. cerevisiae*, the N terminus of Hof1 is implicated in the control of cell size and levels of actin cables, while the C terminus controls actin cable organization apparently via direct regulation of the formin Bnr1 (KAMEI *et al.* 1998; GRAZIANO *et al.* 2014). According to sequence alignment analysis, the amino acid (aa) sequence of Hof1 shows only 15 % identity with ScHof1. However, the proteins have a similar domain structure (Fig 4.4 A): an N terminal F-BAR domain (11-266 aa), containing a FCH domain (13-102 aa), and a C terminal SH3 domain (539-605 aa). In order to investigate the potential functions of Hof1 in the DNA damage response, we deleted these domains separately and checked the cell morphology and sensitivity to genotoxic stress.

As shown in Figure 4.4 B, cells containing a deletion of the SH3 domain showed nearly the same morphology as the WT strain, while cells with a deletion of the F-BAR domain exhibited numerous cell chains, and showed an overall similar cell morphology to that of the *HOF1* deletion cells. As well (Fig 4.4 C), we found that the F-BAR domain deletion, but not the SH3 deletion, rendered cells sensitive to both MMS and HU, suggesting a critical role for the F-BAR domain in both response to DNA damage and in the control of cell morphology.

We checked the expression of Hof1 and the Hof1 partial deletion versions through Western blot (Fig 4.4 D). Deletion of the F-BAR domain strongly reduced the level of the Hof1 protein in the cell. As well, the level of Hof1 decreased significantly in cells treated with MMS, and the level was reduced further with increasing

concentrations of MMS (data not shown). The CaHOF1 F-BAR domain deletion (Hof1ΔF-BAR) strain (Fig 4.4 D), also showed a further reduction in Hof1 levels when treated with MMS. Therefore, the F-BAR deletion may cause a significant perturbation in Hof1 function. Since the Hof1 level decreased in cells treated with MMS, we also checked the Hof1 expression in response to other stresses. We found that Hof1 protein level was down regulated in response to HU, H_2O_2, rapamycin and NaCl, while a significant up regulation of Hof1 was found in response to Congo red. (Fig 4.4 E).

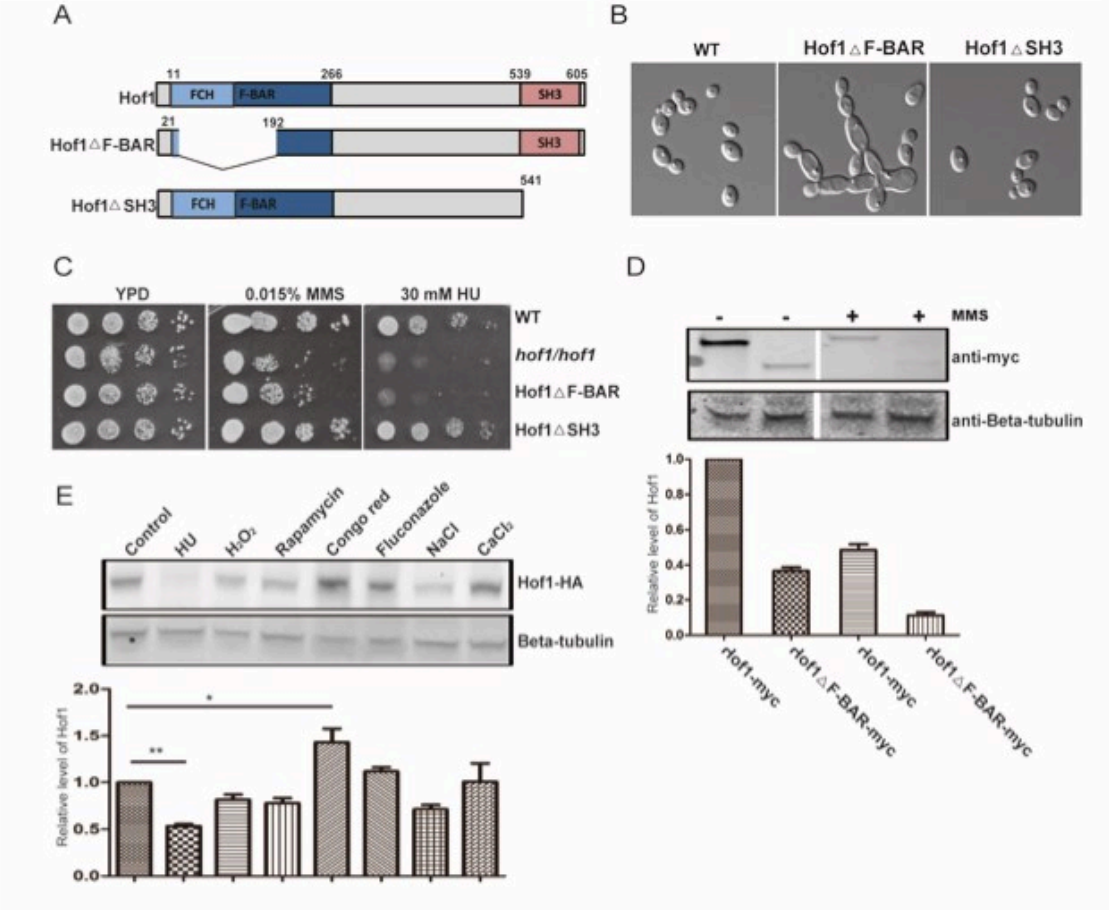


Figure 4.4 Function analysis of the F-BAR domain and the SH3 domain of CaHof1. (**A**) Illustration of Hof1 showing the F-BAR domain and the SH3 domain. (**B**) Cell morphologies of the Hof1 partially deleted strain. The wild type strain (WT), the *HOF1* F-BAR domain deletion (Hof1ΔF-BAR) strain as well as the SH3 domain deletion (Hof1ΔSH3) strain were grown in liquid YPD. Cell morphologies (DIC images) were

shown. (**C**) Genotoxic sensitivity assay of the *HOF1* partially deleted strain. The wild-type (WT) , the *HOF1* deletion strain, the Hof1ΔF-BAR as well as Hof1ΔSH3 were grown overnight in YPD medium at 30°C, serially diluted and spotted onto plates containg YPD or YPD plus the indicated reagents. Plates were incubated at 30°C for 2-3 days. (**D**) The expression of Hof1-myc and Hof1ΔF-BAR-myc were tested by Western blot. Cells were grown in YPD to log phase, treated with 0.02 % MMS for 90 mins, and then harvested for protein extraction, using untreated cells as control. The myc tagged proteins were pulled down by myc-beads and used for Western blotting. An anti-myc antibody was used for checking the expression of Hof1. Beta tubulin was used as a loading control. The expression was normalized on the bottom panel. (**E**) The expression of Hof1-HA was tested by Western blotting. Cells were grown in YPD to log phase, treated with 40 mM HU, 2.5 mM H_2O_2, 10nM rapamycin, 100 μg/mL Congo red, 5 μg/mL fluconzole, 1.5 M NaCl or 200 mM $CaCl_2$ for 90 mins, and then harvested for protein extraction, using untreated cells as control. An anti-HA antibody was used for checking the expression of Hof1. Beta tubulin was used as a loading control. The expression was normalized in the bottom panel.

4.3.4 Genetic Interaction Analysis of *HOF1* with DNA Damage Related Genes

In *S. cerevisiae*, Chang *et al.* (CHANG *et al.* 2002) and Svensson *et al.* (SVENSSON *et al.* 2011) have reported MMS sensitivity of a *HOF1* deletion strain based on high throughput screening results, but their analyses did not assign any specific function in the DNA damage pathway to Hof1. In order to study the function of Hof1 in response to DNA damage in *C. albicans*, we initially performed a genetic interaction analysis.

Rad53, a main checkpoint kinase in *C. albicans* (SHI et al. 2007), was chosen to address a potential role of Hof1 in DNA damage signal transduction. Rad18, an ortholog of ScRad18 involved in post replication repair (BAILLY *et al.* 1997), was chosen as a representative of the Rad6 epistasis group. Mms22, a putative adaptor subunit of an E3 ubiquitin ligase complex, was chosen to represent the replication repair pathway (YAN *et al.* 2015a). Pph3 is a catalytic subunit of protein phosphatase 4 (PP4) and functions in

the Rad53 circuit to control the deactivation of Rad53 during the recovery from or adaption to DNA damage (SUN *et al.* 2011; FENG *et al.* 2017). Mms21, a potential SUMO E3 ligase, was chosen to check the potential function of Hof1 in the SUMO related DNA repair pathway, and Rad52 was chosen to represent the homologous DNA recombination group (LISBY *et al.* 2001). Siz1 is a potential homolog of ScSiz1 that is a SUMO E3 ligase, functioning separately from Mms21, that helps to compensate for DNA damage in the absence of Nfi1 (TAKAHASHI *et al.* 2006). Rad14, Rad4 and Rad23 were chosen to represent the NER group where Rad14 is part of NER1 complex and Rad4-23 are part of the NER2 complex respectively.

Deletion of either Rad18, Mms21, Mms22 or Rad52 resulted in an MMS sensitive phenotype. The sensitivities of double mutants of Hof1 with Rad18, Mms21, Mms22 or Rad52 to MMS were increased relative to the single mutants (Fig 4.5 A), suggesting an independent role of Hof1 from those proteins in the DNA damage response. Deletion of Rad14 resulted in a slight increase in sensitivity to MMS, while the double deletion of Hof1 and Rad14 also showed an increased sensitivity to MMS relative to the Hof1 deletion strain alone, suggesting Hof1 is separate from Rad14 in response to MMS. Furthermore, deletion of Hof1 in a previously reported Pph3 deletion strain (FENG *et al.* 2013), generated greater sensitivity to MMS than was seen with either single mutant, even at low MMS concentrations (Fig 4.5 A). This synergistic sensitivity implies that Hof1 and Pph3 may also have independent functions. However, in contrast, we found that while deletion of Rad53 resulted in strong sensitivity to MMS (Fig 4.5 B), similar to that of the Hof1 deletion strain, further deletion of Hof1 in the Rad53 deletion strain resulted

in no significant change of sensitivity to MMS (Fig 4.5 D). This result suggests that Rad53 and Hof1 are not acting independently in the response to MMS damage.

The Siz1 single deletion strain showed no visible MMS sensitivity, even at a high concentration of MMS (data not shown). Further deletion of *HOF1* in the Siz1 deletion strain, resulted in an MMS sensitivity similar to that seen with the single *HOF1* deletion mutant (Figure 4.5 C). The Rad23 or Rad4 deletion strains also showed no change in sensitivity to MMS relative to the WT strain. Strikingly however, a double mutant of Hof1 and either Rad23 or Rad4 showed the same lack of sensitivity to MMS as the *RAD23* or *RAD4* deletion strain (Fig 4.5 D). This shows that the MMS sensitivity of the Hof1 mutant was suppressed by loss of Rad23 or Rad4. Taken together, these results suggest that Hof1 shows independent roles from Rad18, Mms21, Mms22, Rad52, Rad14 and Pph3, but appears to be part of the Rad53 checkpoint kinase related circuit, and the importance of this circuit for repair of MMS damage is greatly reduced in a Rad23 or Rad4 mutant strain.

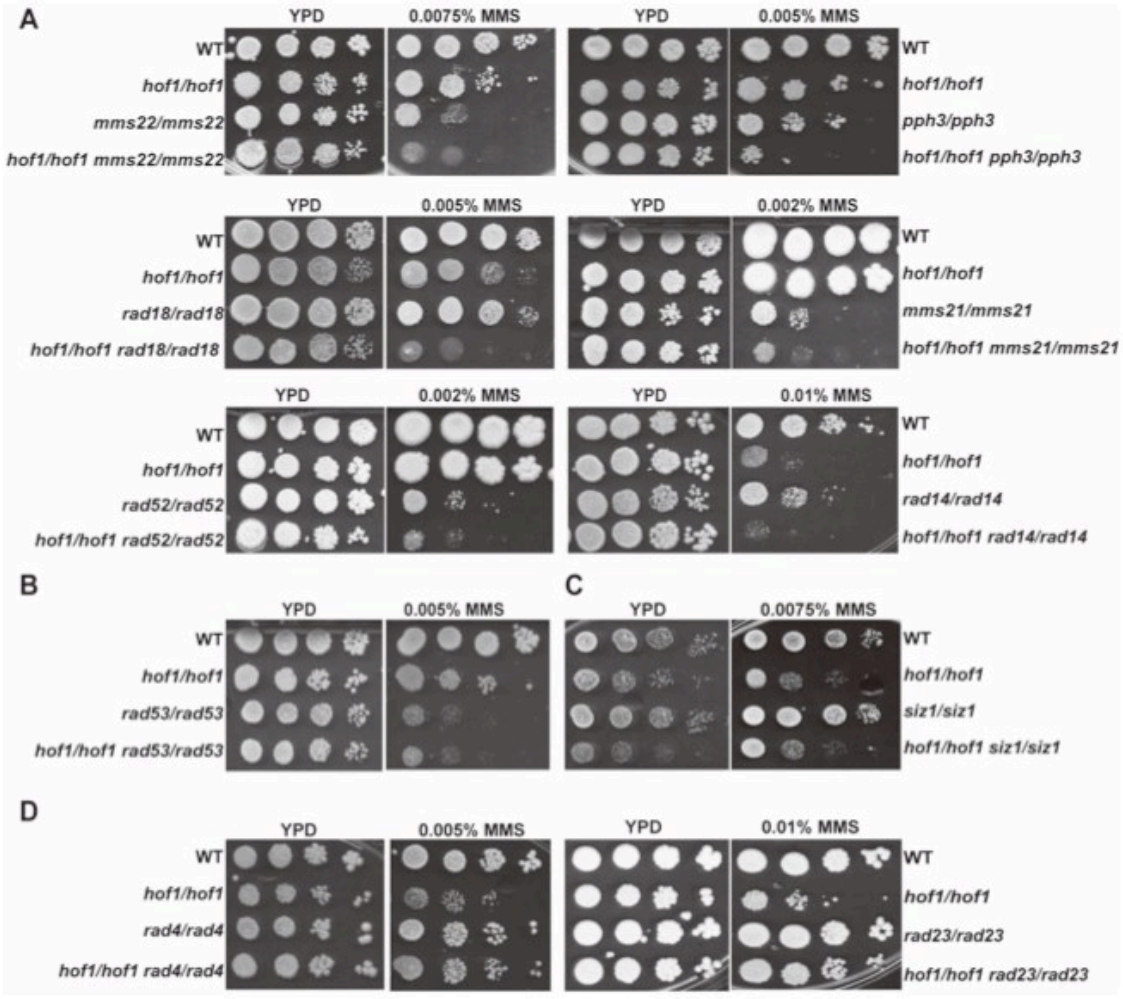


Figure 4.5 Genetic epistasis analysis of *HOF1.* Increased MMS sensitivity was found in the double deletion of Hof1 with Mms22, Pph3, Mms21, Rad18, Rad52 or Rad14 ***(A)***, but not in the double deletion of Hof1 with Rad53 (**B**), or Siz1 (**C**). (**D**) Deletion of Rad23 or Rad4 rescues the MMS sensitivity of the Hof1 deletion strain. All of the single and double deletion strain together with the wild type strain were grown overnight in YPD medium at 30°C, serially diluted and spotted onto a YPD plate or YPD plates containing indicated reagents. Plates were incubated at 30°C for 2-3 days.

4.3.5 Hof1 a Putative Checkpoint Related Co-factor in The Rad53 Circuit

According to the above genetic interaction data, Hof1 appears to be connected to the Rad53 related circuit. In *C. albicans*, Rad53 is a main checkpoint kinase; it is

phosphorylated and becomes active in response to DNA damage, and is dephosphorylated and inactivated after the DNA damage is repaired (YAO *et al.* 2017). Because our genetic interaction assay suggested that Hof1 and Rad53 function in the same pathway, we checked the behavior of Rad53 in a strain with a deletion of *HOF1*. As shown in Figure 4.6 A, in response to MMS, Rad53 was phosphorylated in both wild type and *HOF1* deletion strains. After removal of MMS, Rad53 could be dephosphorylated in *HOF1* deletion cells, similar to the situation in wild type cells. Therefore, deletion of Hof1 has no apparent impact on the post-translational modification of Rad53.

We next asked whether Rad53 modulates the behavior of Hof1. Because the level of the Hof1 protein is down-regulated in response to MMS, we asked whether this down-regulation was influenced by Rad53. We checked the level of Hof1 in the *RAD53* deletion strain by Western blot. As shown in Figure 4.6 B, deletion of *RAD53* had no impact on the level of Hof1 in normal cells. When treated with MMS, the level of Hof1 decreased in wild type strain as expected. In contrast, however, in the *RAD53* deletion cells the amount of Hof1 was maintained at the same level as in untreated cells. Thus, deletion of *Rad53* blocked the reduction of Hof1 protein levels normally seen in response to MMS treatment.

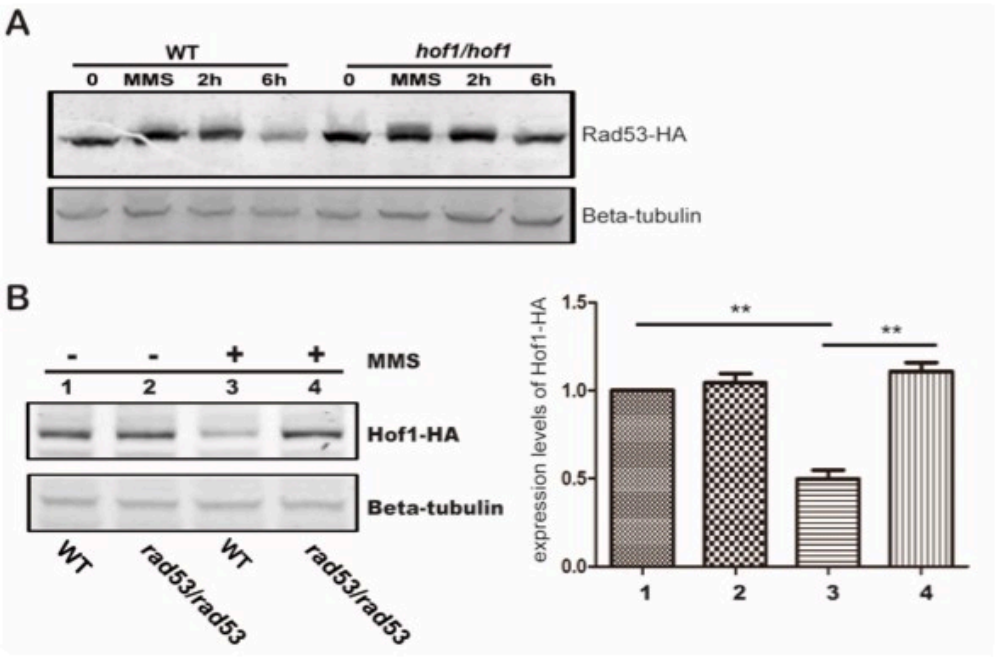


Figure 4.6 Down regulation of Hof1 depends on checkpoint kinase Rad53 (**A**) The wild type strain (WT) and the *HOF1* deletion strain, carrying a Rad53-HA tag were incubated in YPD medium containing 0.02 % MMS for 90 mins, collected by centrifugation, suspended in YPD medium without MMS, and further incubated for indicated times (2 H and 6 H) at 30°C. Cells were harvested for protein extraction and Western blot analysis with anti-HA antibody. Untreated cells (0) were included as controls. Beta tubulin was used as a loading control. (**B**) The expression of Hof1-HA in the wild type (WT) and the *RAD53* deletion strain were tested by Western blot. Cells were grown in YPD to log phase, treated with 0.02 % MMS for 90 mins before harvested for protein extraction. An anti-HA antibody was used for checking the expression of Hof1 by Western blot. Beta tubulin was used as a loading control. The expression of Hof1-HA was normalized according to the expression in WT cells without treatment.

4.3.6 Rad23 Rescues The MMS Sensitivity of The Hof1 Deletion

Through the genetic interaction assay, we observed that the *RAD23* deletion could rescue the sensitivity to MMS that resulted from deleting Hof1 (Fig 4.5 D). To confirm this phenotype, we treated these mutant cells with MMS for 2 hours and tested the survival ratio (Fig 4.7 A). As previously found, deletion of *HOF1* decreased the

survival rate after MMS treatment, deletion of *RAD23* increased the survival rate after MMS treatment, and the *HOF1 RAD23* double deletion strain had a similar resistance to MMS as did the *RAD23* deletion strain. This result suggests loss of Rad23 can rescue the damage sensitivity caused by loss of Hof1.

To establish if this rescue is specific for MMS or not, we also checked the phenotype of these mutants to other genotoxic stresses. We found that loss of Rad23 also rescued the damage sensitivity to HU caused by loss of Hof1. However, for UV treatment, the double deletion of Hof1 and Rad23 showed nearly the same sensitivity as did the Rad23 single deletion (Fig 4.7 B). Similar results were found for Hof1 and the Rad4 double deletion (data not shown). Since Hof1 is a cytokinesis related protein, we asked the question of whether loss of Rad23 could rescue the observed defects in cell division. We found that the double deletion of Hof1 and Rad23 showed similar cellular and colony morphology to that of the Hof1 deletion strain, not only in liquid YPD medium, but also on solid YPD medium (Fig 4.7 C). Thus the ability of the *rad23* mutation to suppress the phenotype of the *hof1* mutant is limited to the DNA damage sensitivity phenotype, and does not extend to the cytokinesis defect.

In *S. cerevisiae*, Rad23 is critical for nucleotide excision repair and is part of a ubiquitin/proteasome pathway (REED AND GILLETTE 2007), and decreased proteasome function enables increased global DNA repair (LOMMEL *et al.* 2000). Since Rad53 is a main checkpoint kinase in response to DNA damage, we tested the expression of Rad53 by Western blot. As shown in Figure 4. 7 D, an increased expression of Rad53 was found in *RAD23* deletion cells, but not in *HOF1* deletion cells. Furthermore, in *HOF1 RAD23* double deletion cells, an increased level of Rad53 was found. Thus, rescuing of the Hof1 deletion strain by deleting Rad23 could be caused by the increased repair efficiency, potentially depending on checkpoint kinase Rad53.

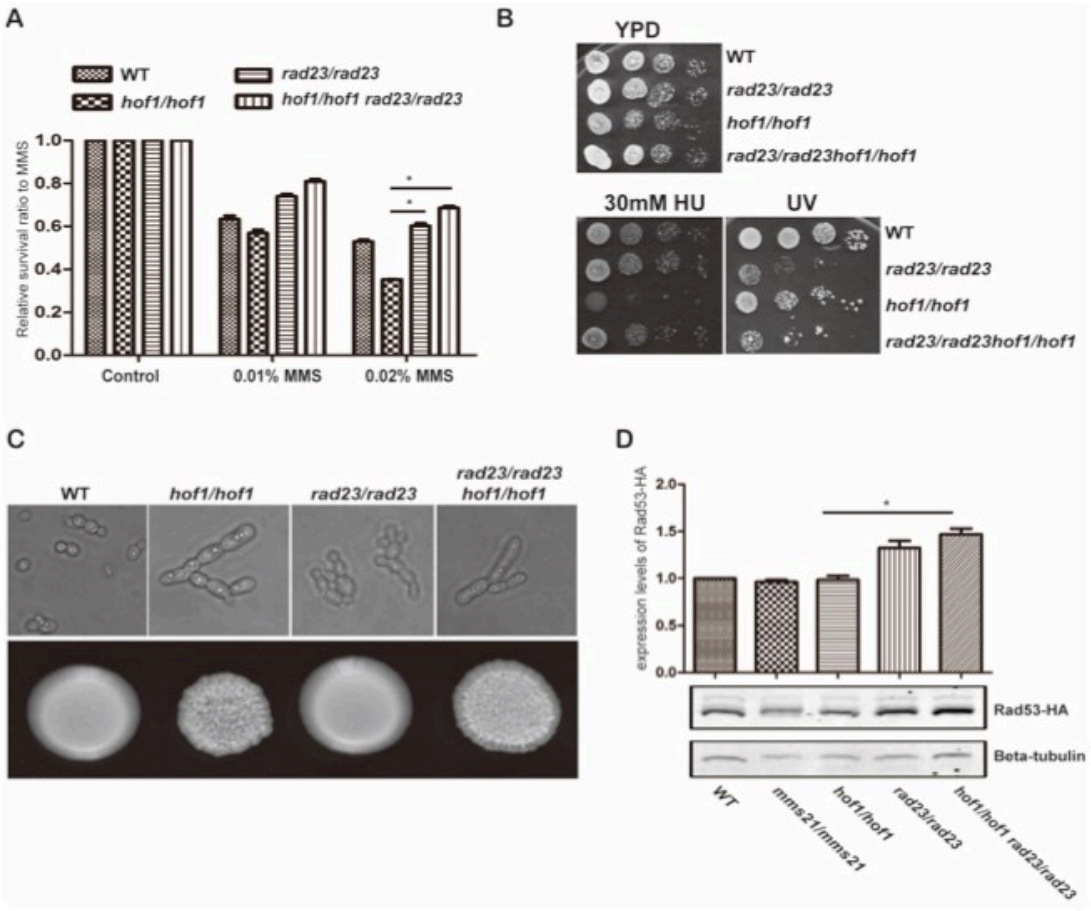


Figure 4.7 Rad23 deletion rescues the MMS sensitivity of the Hof1 deletion. (**A**) The

wild-type (WT) , the *HOF1* deletion strain, the *RAD23* deletion strain and the *HOF1 RAD23* double deletion strain were incubated in liquid YPD medium to log phase and then treated with MMS for 2 hours before spotting on to YPD plates with suitable dilutions. The relative MMS sensitivity was normalized by comparing the numbers of colonies on YPD with or without treatment of MMS. (**B**) The wild-type (WT), the *HOF1* deletion strain, the *RAD23* deletion strain and the *HOF1 RAD23* were used to check the sensitivity to HU and UV. (**C**) The wild-type (WT), the *HOF1* deletion strain, the *RAD23* deletion strain and the *HOF1 RAD23* were grown in liquid YPD or on Solid YPD plates. Cell morphologies were shown. (**D**) The wild-type (WT) strain, the *HOF1* deletion strain, the *MMS21* deletion strain, the *RAD23* deletion strain and the *HOF1 RAD23* double deletion strain, carrying a Rad53-HA tag were incubated in liquid YPD medium to log phase and harvested for protein extraction. Expression of Rad53-HA was checked by Western blot analysis with anti-HA antibody as before. Beta tubulin was used as a loading control. The level of Rad53 is normalized according to the expression in WT cells.

4.4 Discussion

We have screened the GRACE library for MMS sensitive strains and characterized the potential function of Hof1 in DNA damage response. Deletion of Hof1 renders cells sensitive to MMS and causes genome instability; this is correlated with partial activation of the checkpoint kinase Rad53. Hof1 is down regulated in a Rad53 dependent manner in response to MMS, and genetic interaction analysis shows that Hof1 is involved in the Rad53 pathway and its importance is reduced in a Rad23 or Rad4 mutant strain.

Hof1 has been well characterized for its interaction with septins and actins during the formation of a septum, and for its role in cell division during cytokinesis in *S. cerevisiae* (VALLEN *et al.* 2000; BLONDEL *et al.* 2005). A similar role of Hof1 in cytokinesis was reported in *C. albicans* (LI *et al.* 2006). In this study, we confirmed the defect in cell division caused by deleting *HOF1*, but also investigated the MMS sensitivity of the Hof1 deletion strain. Since cytokinesis is a late event during cell cycle, typically occurring

after DNA replication, it is possible that the function of Hof1 in response to DNA damage is separate from its role in cytokinesis. We have found that Hof1 is down regulated in response to MMS, HU, H_2O_2 and rapamycin, while a significant up regulation of Hof1 was found in response to Congo red and fluconazole. The differential level of the Hof1 protein in response to various stresses may support a model of Hof1 as a multi-functional protein that may have separate roles in response to gentoxic stress from cytokinesis. Furthermore, in this study, we reported Rad23 plays a negative role in response to MMS induced DNA damage. Deletion of Rad23 rescued the MMS sensitivity of the *HOF1* deletion, but not the cell morphology defect. The double deletion of *RAD23 HOF1* showed an elongated cell morphology and cell separation defects, which is similar to Hof1 deletion strains, but not Rad23 deletion strains. Thus, the role of Hof1 in genotoxic stress including MMS induced DNA damage could be a role independent from cytokinesis.

During the evolution of eukaryotic organisms, a critical DNA repair system was formed to monitor genome integrity and repair spontaneous mutations. In the case of DNA damage, numerous DNA checkpoint kinases, like Rad53 in budding yeast, are activated to promote the expression of downstream genes to repair damaged genome DNA. In this study, deletion of Hof1 causes sensitivity to MMS as well to other genotoxic stresses, suggesting a positive role of Hof1 in the DNA damage response. However, during the repair phase, the cells also need to suppress the expression of some genes to facilitate the repair process. In *S. cerevisiae, HST3* and *HST4* are down regulated in case of MMS induced DNA damage (MAAS *et al.* 2006), allowing H3 K56 acetylation to persist.

In our study, we found a similar down regulation of *HOF1* in response to MMS, suggesting a negative role of Hof1 during MMS induced DNA damage. Furthermore, the suppression of Hof1 is checkpoint related, because deletion of the main checkpoint kinase Rad53 blocked the reduction of Hof1. In response to MMS, Rad53 is activated while Hof1 is suppressed. However, we did not see a continuous reduction of Hof1 when treated with MMS for 3 hours. After MMS was washed out for 2 hours, the Rad53 still exists in a phosphorylated form while the Hof1 level recovers to the normal level (data not shown). These results suggest that the altered level of Hof1 is transient during DNA damage. Here, we propose that Hof1 plays a dual function in the DNA damage response, which is useful for DNA damage signal transduction but may be inhibited to facilitate the repair signal multiplication. Once the repair machinery is fully activated, the Hof1 recovers to its normal level.

We have also found that deletion of Rad23, a NER component, could rescue the MMS sensitivity of Hof1 deletion strains. In *S. cerevisiae*, Rad23, which links the DNA repair pathway to the ubiquitin/proteasome pathway, is not only a NER component, but also a negative regulator of multi-ubiquitin chain assembly which regulates protein turnover. According to the current results, Rad23 is a suppressor of DNA damage repair caused by MMS. Consistently, an increased protein level of Rad53 was found in both Rad23 deletion and Hof1 Rad23 double deletion strains. Here, we propose that Rad23 plays a negative role in response to MMS induced DNA damage. In normal cells, DNA repair related genes, including Rad53, are suppressed by Rad23 to a low level to ensure normal cell cycle progression. In the case of DNA damage, deletion of *RAD23* increases

the level of those DNA repair proteins needed to correct the damaged DNA. Although we cannot exclude the function of Rad23 in regulation other DNA repair genes, current results show a negative regulation of Rad23 in DNA repair pathway is, at least partially, relied on checkpoint kinase.

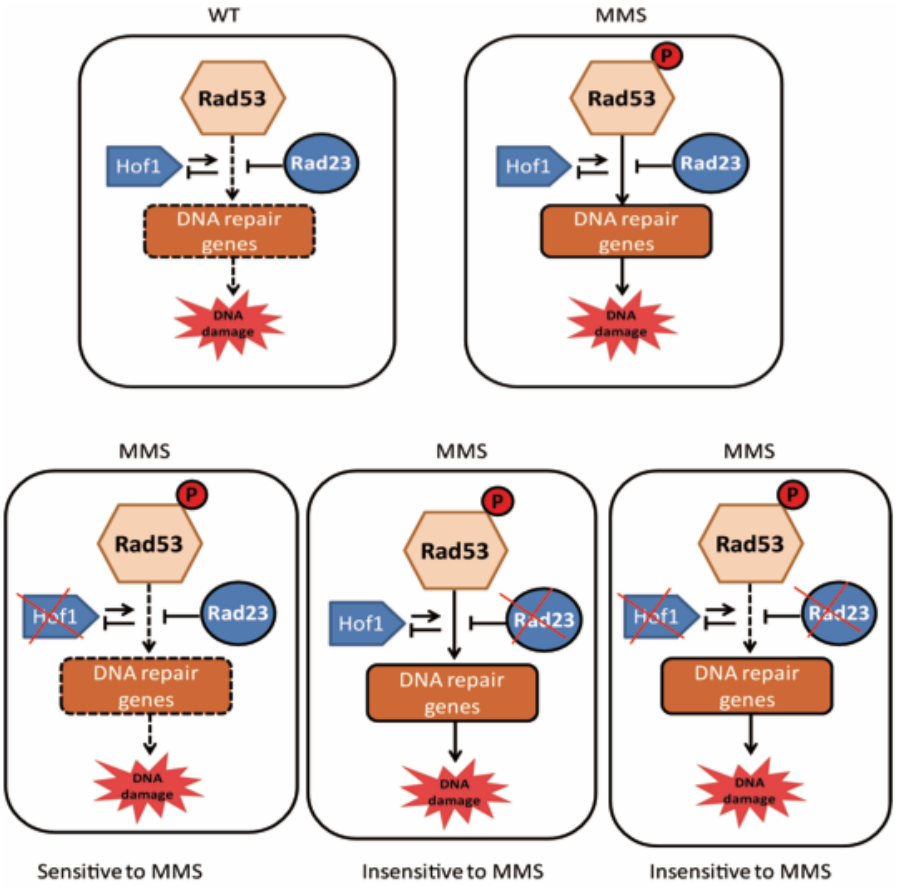


Figure 4.8 Potential regulating model of Hof1 and Rad23 as well as Rad53 in *C. albicans*. Arrows represent activation, while bars represent inhibition. Dashed lines represent defects in activation. Dashed DNA repair genes box represents inactive form.

To better understand the regulating mechanism found in this study, we provide a possible model of the control process. In normal cells, Rad23 acts as a negative regulator in protein stability, and Hof1 is a co-factor in DNA damage signal transduction

that is repressed once the repair machinery is activated. Deletion of Hof1 inhibits or partly inhibits the activation of repair genes, so the cell cannot repair the damaged DNA properly, resulting in MMS sensitivity. Deletion of Rad23 promotes the stability of repair genes, which is beneficial for cells to deal with the damaged DNA, resulting in insensitivity to MMS. In Hof1 and Rad23 double deletion strains, because of removal of Rad23, the repair genes are stable enough to deal with damaged DNA. The loss of Hof1 may be compensated by over expression of other co-factors because of the increased level of Rad53 in the Rad23 Hof1 double deletion strain, resulting in insensitivity to MMS.

Chapter 5: Conclusions and Future Work

The yeast-filamentous transition is critical for *C. albicans* virulence (JACOBSEN *et al.* 2012). Filamentous growth or invasiveness is a highly complex process as evident from its control circuits; at least three different cellular pathways - the MAPK, cAMP/PKA, and Hog1 pathways, are connected to filamentation under different environmental conditions (HUANG 2012). We have screened the GRACE library (ROEMER *et al.* 2003) and have identified genes that upon repression cause invasiveness. These genes have been implicated in a variety of cellular processes such as ubiquitination, sumoylation, DNA replication and damage repair, chromosome maintenance and transcription. Analysis suggests that not only individual genes but the coordination of their common functions that may be affecting invasiveness in *C. albicans*.

We have characterized, via phenotypic and genetic analysis, Candida Mms21, a protein involved in negative regulation of filamentation. We have found that Mms21 and all members of the sumoylation pathway were both non-essential and induced filamentation upon repression. This suggests that the SUMO pathway in *C. albicans*, unlike in its close relative *S. cerevisiae*, is dispensable. Smt3, the unique yeast SUMO protein, has previously been found non-essential in *C. albicans* (LEACH *et al.* 2011). However deletion of Mms21 resulted in serious cellular and growth consequences such as increased cell size, irregular shaped cells and slower cell division. The mutant cells showed abnormal nuclear segregation and lacked conspicuous septa. Similar cellular defects had been reported previously in *C. albicans* for the deletion of the DNA repair protein Rad52 (ANDALUZ *et al.* 2006) or for the deletion of SUMO-encoding Smt3 (LEACH

et al. 2011). In *S. cerevisiae*, Mms21 is part of the DNA repair Smc5/6 complex, and mutations in this complex result in G2/M phase cell arrest (MENOLFI *et al.* 2015). Because an unperturbed Smc5/6 complex is required to regulate Rad52 (TORRES-ROSELL *et al.* 2007) it is possible that these cellular defects are a consequence of G2/M phase cell arrest. Thus both roles of Mms21 i.e. its Smc5/6 complex stability and SUMO E3 ligase activity are required for normal cell division progression.

Our microarray and RNA seq analysis have found key players of filamentation and virulence to be highly up-regulated in cells deleted for *MMS21*. Most of these HSGs are tightly regulated by signals including some activators and repressors. Efg1 is one such activator of HSGs and it acts downstream in the cAMP/PKA pathway (STOLDT *et al.* 1997; SHARKEY *et al.* 1999; SOHN *et al.* 2003). We have found that a Mms21 deleted strain formed filaments in the absence of Efg1. The HSGs in Mms21 deleted strains were transcriptionally induced without increased expression of Efg1 but we did find Ume6, another activator of HSGs, to be highly up regulated. Therefore, deletion of Mms21 caused increased expression of Ume6, which in turn activates HSGs to form hyphae.

Mms21 mutants were highly sensitive to genotoxic and cellular stress agents including cell membrane and cell wall damaging agents highlighting the requirement of a functional *MMS21* by *C. albicans* to cope with these stresses. Congo red and caspofungin are two important chemicals that target glucan and chitin synthase enzymes thus affecting the chitin content of the cell wall, which ultimately compromises cell integrity. In the absence of the Mms21 protein or its SUMO E3 ligase domain, *C. albicans* cells became sensitive to these two chemicals. As chitin synthase proteins are

required to cope with cell wall stress caused by these chemicals (WALKER *et al.* 2008), these proteins are candidate targets of Mms21 mediated sumoylation involved in coping with cell wall stresses. Our results were also consistent with the observations that Smt3 inactivation might affect the Pkc1 pathway and sumoylation targets critical for the cell wall stress response in *C. albicans* (LEACH *et al.* 2011). Moreover, *mms21Δ/Δ* and *mms21Δ/SPΔ* were both sensitive to 42°C, therefore, there is the possibility of sumoylation of heat shock proteins via Mms21 mediated sumoylation (LEACH *et al.* 2011).

In Chapter 3, we have screened 69 invasive GRACE shut off *C. albicans* strains as *MTL a/α* for mating. We found both GRACE shut off and Mms21 deleted strains to mate as *MTL a/α*. We observed that this mating showed by *mms21Δ/Δ* mutants was carbon source and temperature dependant as only those mutant strains that were growing on GlcNAc and at 25°C temperature were able to mate. This suggests that these mutant strains did follow the norms of white-opaque switching and mating. Mms21 deleted cells displayed a 5 % increase in white to opaque switching as compared to the WT when grown on GlcNAc medium at 25°C. This result was consistent with previous findings that an increase in genotoxic stress or defects in DNA repair machinery cause an increase white to opaque switching (ALBY AND BENNETT 2009).

We have proposed that a homozygosis event at the *MTL* locus incurred in *mms21* deleted strains prior to white-opaque switching and then mating. Since Mms21 is involved in DNA repair and the genotoxic stress response, absence of Mms21 is resulting in increased recombination events. This was evident from an increased loss of

the *URA3* locus in *mms21Δ/Δ* strains when grown on 5-FOA medium. Furthermore, *mms21Δ/Δ a/α* cells were able to mate with 3315*αα* testers so we proposed that this is because of the increase homozygosis at *MTLa* in null *mms21* mutants. Our PCR analysis of sorbose positive colonies have confirmed that *MMS21* deletion in *C. albicans* has indeed resulted in increased homozygosis at *MTLa.* The exact mechanism of how this loss of *MTLα* (increase homozygosis at *MTLa*) happen is unknown and requires further analysis. One reason could be that slow growing strains, as in the case of *mms21Δ/Δ*, prefer to lose *MTLα* more than *MTLa*. To confirm this, WT *MTL a/α* strains will be subjected to MMS and HU stress prior to plating on sorbose medium and then PCR will be used for confirmation of LOH.

We have also found Mms21 deleted cells to negatively regulate biofilm formation. Surprisingly, we found that eliminating Mms21 SUMO E3 ligase function increased biofilm formation more than the full length Mms21 protein deletion. This could be due to Mms21 mediated sumoylation repressing genes involved in biofilm formation. However, the extremely poor growth of null *mms21* strains could also be the contributing factor of lower biofilm formation than seen in the SP deleted mutant. A detailed biofilm analysis, using deletion of Ubc9 (only SUMO E2 conjugating enzyme) and Siz1 (another SUMO E3 ligase), will clear up the picture regarding the role of sumoylation, or Mms21 specifically, in biofilm formation.

In Chapter 4, we screened the GRACE library for MMS sensitive strains and found 56 strains in the GRACE collection that were sensitive to MMS upon repression. Among those potential DNA damage related genes, we chose a cytokinesis related gene, *HOF1*,

for further study. We deleted the *HOF1* gene in the SN148 background and found the anticipated defect in cell division, suggesting the function of Hof1 in cytokinesis is conserved between *C. albicans* and *S. cerevisiae*. Deletion of Hof1 also rendered cells sensitive to genotoxic stress, including MMS. As well, deletion of Hof1 caused strong genome instability that partially activates checkpoint kinase Rad53. Through domain analysis, we found that Hof1 has 2 domains: an N-terminal F-BAR domain and a C-terminal SH3 domain. Deletion of the SH3 domain has no impact on cell morphology and sensitivity to MMS, while deletion of the F-BAR domain caused similar cell morphology and sensitivity to MMS as the Hof1 deletion strain. Through Western blotting we found deletion of the N-terminal F-BAR domain resulted in dysfunction in Hof1. We also found Hof1 was down-regulated in response to MMS or HU, but up regulated in response to Congo red.

To check the potential function of Hof1 in the DNA damage pathway, genetic interaction research was performed. The results showed that Hof1 is independent from Rad52, Mms22, Mms21, Rad14, Rad18 and Pph3, but involved in Rad53 related pathway. Furthermore, the sensitivity to MMS upon Hof1 deletion, could be rescued in a Rad23 or Rad4 mutant strain. We also found that deletion of Rad53 blocks the down regulation of Hof1 in response to MMS, while deleting Hof1 has no impact on the activation or deactivation of Rad53, suggesting that Hof1 is regulated by Rad53 in response to DNA damage.

Deletion of Rad23 or Rad4 rescued the sensitivity of the Hof1 deletion to MMS, but also to HU. However, for UV treatment, the double mutants of Hof1 and Rad23 or

Rad4 showed the similar phenotypes as Rad23 or Rad4 single gene mutants, suggesting that the Hof1 function in response to UV is covered by Rad23 or Rad4. Furthermore, a significant increase of Rad53 level was found in Hof1 and Rad23 deletion strains, suggesting rescuing of the Hof1 deletion strain by deleting Rad23 could be caused by the increased repair efficiency, potentially depending on checkpoint kinase Rad53.

In general, Hof1 is a checkpoint related co-factor in response to DNA damage and the importance of Hof1 is reduced in a Rad23 or Rad4 mutant strain. In this study, we did not find the direct targets of Hof1 in response to DNA damage. In future, protein interaction strategies and RNA seq could be used to explore the direct regulating targets and mechanism of Hof1 in DNA damage pathway.

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Appendices

Appendix 1 *Candida albicans* Strains Used in Chapter 2 and 3

Strain	Parent	Mating type	Description	Source
GRACE Library (CaSS1)	CAI4	*a/α*	*his3::hisG/his3::hisG leu2::tetRGAL4AD-URA3/LEU2*	Roemer *et al*., (2003) Molecular Microbiology, **50**, 167-181
SN148	SN76	*a/α*	*arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434*	Noble *et al*., (2005) Eukaryotic Cell, **4**, 298-309
SN148	SN76	*aa*	*arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434*	Lab Stock
MMS21/mms21Δ	SN148	*a/α*	*mms21::HIS1/MMS21; arg4/arg4; leu2/leu2; ura3 imm434/ura3 imm434*	This study
MMS21/mms21Δ	SN148	*a/α*	*mms21::URA3/MMS21; his1Δ/his1Δ arg4Δ/arg4Δ leu2Δ /leu2Δ*	This study
mms21Δ/Δ	SN148	*a/α*	*mms21::HIS1/mms21::ARG4; leu2/leu2; ura3 imm434/ura3 imm434*	This study
mms21Δ/Δ	SN148	*a/α*	*mms21::URA3/mms21::ARG4; his1Δ/his1Δ leu2Δ /leu2Δ*	This study
mms21Δ/Δ	SN148	*aa*	*mms21::HIS1/mms21::ARG4; leu2/leu2; ura3 imm434/ura3 imm434*	This study
mms21Δ/SPΔ	SN148	*a/α*	*mms21::HIS1/SP::ARG4; leu2/leu2; ura3 imm434/ura3 imm434*	This study
Mms21 Rev	SN148	*a/α*	*mms21::HIS1/mms21::ARG4; leu2/leu2; ura3 imm434/ura3 imm434*	This study
efg1Δ/Δ	SN148	*a/α*	*efg1::LEU2/efg1::URA3; his1/his1; arg4/arg4*	Regan *et al.,* (2017) Molecular Microbiology, **105**, 810-824
3315	A505	*αα*	*trp1Δ/trp1Δ lys2Δ/lys2Δ*	P.T. Magee
3745	A505	*aa*	*trp1Δ/trp1Δ lys2Δ/lys2Δ*	P.T. Magee

Appendix 2 Oligonucleotides and Plasmids Used in Chapter 2 and 3

Name	Sequence
Amj37Fw	TTAGAATTGAGATTCAGTTGCTGGTAAATTATTTAAGCATTAATTTATCACCAAG AACAATAGCACGGGAAACAAAACAAAACAAAACGGAAAACTGAAGCTTCGTAC GCTGCAGGTC
Amj38Rv	AATAAAGAATACCTCAGAAGATTTGGAAAATCCTCTCCACTCTTCGACACTTCAA ATGAATAGGCAAAAAGGTTACCGTGCCTGTTTTTATACTAATCTGATATCATCGA TGAATTCGAG
Amj39Fw	AGGTGAAGTTATTACCACTCGTGG
Amj40Rv	TACTAGTTATGCAGTGGCTCTTCC
Amj32HisFw	GAGCGGTGCCGATATACAGTTTAG
Amj31HisRv	ACACAAGACATATTCCTGAGCCGC
Amj30ArgFw	CCAATCTCCATTAGGATCTGGTGC
Amj29ArgRv	GTGGCATCAATGAAGAACCAGTAG
Amj73Fw	ACCATTTCTGATTCAACAATAACGAAACAAGGGTATCAATTTTTGAAACAGGTTT TGTTTCTGTTTAAAAACCCTGAAGATGCTATACCCGATGAAACAGAAGGTCGAC GGATCCCCGGGTT
Amj72Rv	GTTCTGAATAAAGAATACCTCAGAAGATTTGGAAAATCCTCTCCACTCTTCGACA CTTCAAATGAATAGGCAAAAAGGTTACCGTGCCTGTTTTTATACTAATCGATGAA TTCGAGCTCGTT
Amj74Fw	GCTCCAGATGAGGTATTACC
AmjHindIIIFw	GCAGCGGCGAAGCTTAGCATTGAGTAGGGTATATAA
AmjXhoIRv	TGCTGCTGCCTCGAGCAAAGAAGTAATCGCAATTCA
AmjsqFw1	AGCATTGAGTAGGGTATATAA
AmjsqRv1	ATAATTTACCAGCAACTGAAT
AmjsqFw2	TGGCAGAATATTATATTGATT
AmjsqRv2	ACTCCCAACTTTGCATATTCT
AmjsqFw3	TTCTTCTTCATTTGAATACTC
AmjsqRv3	TCAAACAACAGTCTCCATATT
Amj68UraFw	TATTGCCCAACGTGATATGGGTGG
Amj69UraRv	TGAGGCATGAGTTTCTGCTCTCTC

Amj77UraRv	ATAACCCTCTTGGCTCTTGG
*MTLα1*Fw	TTGAAGCGTGAGAGGCAGGAG
*MTLα1*Rv	GTTTGGGTTCCTTCTTTCTCATTC
*MTLα2*Fw	TTCGAGTACATTCTGGTCGC
*MTLα2*Rv	TGTAAACATCCTCAATTGTACCCG

Plasmid Name	**Description**	**Reference**
pFA-URA3	Disruption cassette	Gola *et al.,* (2003) Yeast **20**, 1339-1347
pFA-ARG4	Disruption cassette	Gola *et al.,* (2003) Yeast **20**, 1339-1347
pFA-HIS1	Disruption cassette	Gola *et al.,* (2003) Yeast **20**, 1339-1347
pFA-TAP ARG4	Disruption cassette and/or epitope tagging	Lavoie *et al.*, (2008) BMC Genomics **9**, 578
pFA-GFP ARG4	Disruption cassette and/or GFP tagging	Gola *et al.,* (2003) Yeast **20**, 1339-1347
CIp10	Complementation plasmid	Murad *et al.*, (2000) Yeast **16**, 325-327

Appendix 3 Mating competency in uracil auxotrophs recovered from 5-FOA media showing genes that displayed mating, markers and genotypes they retained from parental GRACE strains

Gene	Markers	Mating competency	*MTL*
RAP1	Ura-, His-, Sat+	Good with 3315α	*a*
QRI2	Ura-, His+, Sat+	Good with 3315α	*a*
MED4	Ura-, His+, Sat+	Good with 3315α	*a*
APC2	Ura-, His-, Sat+	Good with 3745a	*α*
CDC20	Ura-, His+, Sat+	Decent with 3315α	*a/α*
MMS21	Ura-, His+, Sat+	Weak with 3315α	*a/α*
CDC47	Ura-, His+, Sat+	Decent with 3315α	*a/α*
MED21	Ura-, His+, Sat-	Weak with 3315α	*a*
KIN28	Ura-, His+, Sat+	Weak with 3315α	*a/α*
G1N1	Ura-, His-, Sat+	Weak with 3315α	*a/α*

Appendix 4 Strains Used in Chapter 4

Strains	Genotype	Source
SN148	*arg4/arg4 leu2/leu2 his1/his1 ura3::imm434/ura3::imm434 iro1::imm434/iro1::imm43*	Lab stock
JR7	RM1000 *pph3::hisG/pph3::HIS1*	Feng, 2013
CJ1	SN148 *hof1::HIS1/hof1::HIS1*	this study
CJ2	SN148 *::CIP10*	this study
CJ3	CJ1::CIP10	this study
CJ4	CJ1::CIP10-CaHOF1	this study
CJ5	SN148 *hof1Δ22-193aa/hof1Δ22-193aa*	this study
CJ6	SN148 *hof1ΔSH3::HIS1/hof1ΔSH3::HIS1*	this study
CJ7	SN148 *rad23::ARG4/rad23::ARG4*	this study
CJ8	SN148 r*ad18::ARG4/rad18::ARG4*	this study
CJ9	SN148 r*ad14::ARG4/rad14::ARG4*	this study
CJ10	SN148 r*ad4::ARG4/rad4::ARG4*	this study
CJ11	SN148 r*ad52::ARG4/rad52::ARG4*	this study
CJ12	SN148 *mms22::ARG4/mms22::ARG4*	this study
CJ13	SN148 *rad53::HIS1/rad53::HIS1*	
CJ14	SN148 *siz1::ARG4/siz1::ARG4*	this study
CJ15	CJ1*rad23::ARG4/rad23::ARG4*	this study
CJ16	CJ1 r*ad18::ARG4/rad18::ARG4*	this study
CJ17	CJ1 r*ad14::ARG4/rad14::ARG4*	this study
CJ18	CJ1 r*ad4::ARG4/rad4::ARG4*	this study
CJ19	CJ1 r*ad52::ARG4/rad52::ARG4*	this study
CJ20	CJ1 *mms22::ARG4/mms22::ARG4*	this study
CJ21	CJ1 *siz1::ARG4/siz1::ARG4*	this study
CJ22	CJ1 *ubc9::ARG4/ubc9::ARG4*	this study
CJ23	*mms21Δ::URA3/mms21Δ::ARG4 hof1::HIS1/hof1::HIS1*	this study
CJ24	CJ13 *hof1::ARG4/hof1::ARG4*	this study
CJ25	SN148 *hof1::myc-HIS1/hof1::myc-HIS1*	this study
CJ26	SN148 *hof1Δ22-193aa::myc-HIS1/hof1Δ22-193aa::myc-HIS1*	this study
CJ27	SN148 *hof1::HA-HIS1/hof1::HA-HIS1*	this study
CJ28	SN148 *RAD53/RAD53::HA-HIS1*	this study

CJ29	CJ1 *RAD53/RAD53::HA-ARG4*	this study
CJ30	CJ7 *RAD53/RAD53::HA-HIS1*	this study
CJ31	CJ15 *RAD53/RAD53::HA-URA3*	this study
CJ32	CJ13 *hof1::HA-HIS1/hof1::HA-HIS1*	this study

Appendix 5 Primers Used in Chapter 4

Name	Sequence
HOF1-SG-F	ATTTGATAGCCAAATAAACCTGACAG
HOF1-SG-R	AAAACTGTCAGGTTTATTTGGCTATC
HOF1-RE-F	ACCCTATTTGTGAATAACTTTTGGGGTAACTCCCCAGAGAAAAGTTTTGAAATCGTCACATCCAATACCTCGCCAGAAC
HOF1-RE-R	AAGCATATTTGTTGGGGAATGGTAGATCTAATGGCAAGTGTTTTCTGTAAAAAGATGAAACTCACTATAGGGAGACCG
HOF1-TE-F	GCGAGTATCTAATCCACAAC
HOF1-TE-R	GAATGTGGGTCGGAAGAG
HOF1-F	CGGGGTACCGGACAAACAAATGGAACTGG
HOF1-R	CGGGGTACCGAATGTGGGTCGGAAGAG
RAD23-SG-F	AATGGATTAGTTTGGTTATCGTTTTAGAGCTAGAAATAGCAAGTTAAA
RAD23-SG-R	GATAACCAAACTAATCCATTCAAATTAAAAATAGTTTACGCAAGTC
RAD23-RE-F1	TGTGTTGTTGTGCCGTTC
RAD23-RE-R1	GTTCTGGCGAGGTATTGGACCTGTTGTTATTGTGGAAGTC
RAD23-RE-F2	CGGTCTCCCTATAGTGAGGCTTTTCATCCCTGCTTC
RAD23-RE-R2	CGGGGTACCATCTGGTTCAATAAAGGC
RAD23-TE-NEWF	TAGGGGTAGGTTCTGTATTG
RAD23-TE-NEWR	TGACAACTACAGCCAAAACAG
SIZ1-SG-F	GAATTTAAAGACACTTTTCGGTTTTAGAGCTAGAAATAGCAAGTTAAA
SIZ1-SG-R	CGAAAAGTGTCTTTAAATTCCAAATTAAAAATAGTTTACGCAAGTC
SIZ1-RE-F1	GATGTCTGTCGTTCTCTTGA
SIZ1-RE-R1	GTTCTGGCGAGGTATTGGAGTCCCCCTGTAAACGCAT
SIZ1-RE-F2	CGGTCTCCCTATAGTGAGGTAACTCGCCAAGGAAGG
SIZ1-RE-R2	GCCTCTATCAGTTCAGCC
SIZ1-TE-F	ACGCGTCGACGTTGACGAGGCATTGAGAT
SIZ1-TE-R	ACGCGTCGACGATTTACGCCATAGGAAGC
RAD4-SG-F	AGAATCAGGTAATGATACATGTTTTAGAGCTAGAAATAGCAAGTTAAA
RAD4-SG-R	ATGTATCATTACCTGATTCTCAAATTAAAAATAGTTTACGCAAGTC
RAD4-RE-F	CGAAAGCTATTGAATGATGTTAATATAAGAGAAGAACTGCCACTGAGAAAGAAGCGGGGATCCAATACCTCGCCAGAAC
RAD4-RE-R	GTAAACATCTATATTTCCATATTGATTTTTAGGAACAATGCCATCAGTCACGGGTGGAGGCTCACTATAGGGAGACCG
RAD4-TE-F	CAATGAGCAACCAGAGCC
RAD4-TE-R	GGTCTTCCTCCTCTTGTTC
RAD52-SG-F	AATTTGGCTAATGAAATATTGTTTTAGAGCTAGAAATAGCAAGTTAAA
RAD52-SG-R	AATATTTCATTAGCCAAATTCAAATTAAAAATAGTTTACGCAAGTC
RAD52-RE-F	CAGCAACCTCAACCCAACCAACAGAGGCTTCCCTTTCGACCAGACGAGTCACGAGCTCGTCCAATACCTCGCCAGAAC
RAD52-RE-R	CATAGCTGACCACTTTGGTTGGCAGTTTTGGTGTAACAAAAGCTGCTGCATGGTGGGAAACTCACTATAGGGAGACCG
RAD52-TE-NEWF	ACAAACCCTTCCACCCAC
RAD52-TE-NEWR	ATCAACTGCCATAGACTCG
MMS22-SG-F	ACTAAAAAAAAGGTATCCAAGTTTTAGAGCTAGAAATAGCAAGTTAAA
MMS22-SG-R	TTGGATACCTTTTTTTTAGTCAAATTAAAAATAGTTTACGCAAGTC
MMS22-RE-F	TACCTGAAGACCAAGAAAACGAAAATCATTTGCAATCATATTTGAATACCACGAACTCATCCAATACCTCGCCAGAAC
MMS22-RE-R	TGTACCAGTAAAGCAATTGCATGAATCGTTCAAAAAATGAATCATCAGGGATATCAAATCTCACTATAGGGAGACCG
MMS22-TE-F	GACGATTCAGATTCAAACCC
MMS22-TE-R	CCACTTTACATACCAGCGC
RAD18-SG-F	GCCTCATTTATTACGATTATGTTTTAGAGCTAGAAATAGCAAGTTAAA
RAD18-SG-R	ATAATCGTAATAAATGAGGCCAAATTAAAAATAGTTTACGCAAGTC

RAD18-RE-F1	GCTGTCGCTACCATCTGTC
RAD18-RE-R1	GTTCTGGCGAGGTATTGGAGCGGACATCTATTATCTCG
RAD18-RE-F2	CGGTCTCCCTATAGTGAGGAAAGAGCACGAAGAGGAC
RAD18-RE-R2	CTGGGGATGGAGATGAGAC
RAD18-TE-F	CGCTCCTTCATCAATCAC
RAD18-TE-R	GCTTGCCCTTGTGAACTG
RAD14-SG-F	AGATCGAACAGAATAGACTAGTTTTAGAGCTAGAAATAGCAAGTTAAA
RAD14-SG-R	TAGTCTATTCTGTTCGATCTCAAATTAAAAATAGTTTACGCAAGTC
RAD14-RE-F1	CTGACCAAACTAACACACG
RAD14-RE-R1	GTTCTGGCGAGGTATTGGATTGTCGTGGTGGTGGAGGTG
RAD14-RE-F2	CGGTCTCCCTATAGTGAGCGAATAGAGAAACCCAACC
RAD14-RE-R2	TCACGGGCGAAGACCAATCA
RAD14-TE-F	GAGTTTGGGTAGTATTTGGC
RAD14-TE-R	CCACTTCTTCTGTCTCAATACC
UBC9-SG-F	CAAGGCTTCAGATGGATCCTGTTTTAGAGCTAGAAATAGCAAGTTAAA
UBC9-SG-R	AGGATCCATCTGAAGCCTTGCAAATTAAAAATAGTTTACGCAAGTC
UBC9-RE-F	TGGTCAAGAAAAAGAGAGAGAAAGGGAAAGAAAGAACGAAAGAAGAAATCGAATGGAATGTCCAATACCTCGCCAGAAC
UBC9-RE-R	ATGTGCCCCAAGAGGAGAGTCCGTGGATGTCGATACCATTGCACACCTTGATCAAGTTGGCTCACTATAGGGAGACCG
UBC9-TE-F	GTGTGGTTTGATGTGTCTG
UBC9-TE-R	CAGGTGAATGTGATGGAATG
HOF1-N-REF	ATGCGAGTATCTAATCCACAACTGTCAAAAACCCTATTTGTGAATAACTTTTGGGGTAACTCCGGATCC
HOF1-N-RER	TCCAATCTCGCTTGTAGATTTCGTTTATTTCTTTGTAATTTACTAATGCCATATGATAGTGGGATCCGGAGTTACCCCAAAAG
HOF1-C-SG-F	ATAGCGATGAAGTTGCCAAAGTTTTAGAGCTAGAAATAGCAAGTTAAA
HOF1-C-SG-R	TTTGGCAACTTCATCGCTATCAAATTAAAAATAGTTTACGCAAGTC
HOF1-C-RE-F	GATCTAAATGCAGGTGGTAATGGAGACGTTAATCAATTTAGACGATCATTGAGAAATGGAGGATCC
HOF1-C-RE-R	TAAATCATCTCCTTCAACAATATAGTTGCCAGGAACTAAGCCAATCATACCTGATGACTGGGATCCTCCATTTCTCAATG
HOF1-NEWC-SGF	ATTTGATAGCGATGAAGTTGCCAAAG
HOF1-NEWC-SGR	AAAACTTTGGCAACTTCATCGCTATC
HOF1-NEWC-RE-F	AAAACACCGTCGCCAACTCGGAAACCACCTTCCGACTATCATTCCAACTATTCAGATGATGGATCC
HOF1-NEWC-RE-R	TTTAAAGTACAATTCGCCCTCAGTTTGAGGCTTGAATGAATATCTTGCAATGGCTTTTGTGGATCCATCATCTGAATAGTTGG
HOF1-NSG-F	TGAAACAACTCAAATAATGTGTTTTAGAGCTAGAAATAGCAAGTTAAA
HOF1-NSG-R	ACATTATTTGAGTTGTTTCACAAATTAAAAATAGTTTACGCAAGTC
HOF1-TAG-SH3F	CCAAATCAACAGTGCCAAGAAGATCAATAAAACTTACCCCAGTCAACAAAAAACCATTTGTTTAGTAAGCTGCAGGTCGACGGATC
RAD53-SG-F	TATCAAAATCCACCAAATATGTTTTAGAGCTAGAAATAGCAAGTTAAA
RAD53-SG-R	ATATTTGGTGGATTTTGATACAAATTAAAAATAGTTTACGCAAGTC
RAD53-TAGF	CAAACCTCAGAAGAAATGAATATTATTCCACTTATTTCGGGTTTAAGTAGTATAAGTTCAGCTGCAGGTCGACGGATC
RAD53-TAGR	AATCAAATATCCGATCCTATCCAAAAACAAATGTTTCAGGACAAGTACATGGGTAAACTACACTATAGGGAGACCGGC
RAD53-TF	TTGCTTAGAGGAGGGGAG
RAD53-TR	ATGTGGAATCTCAACGACC
RAD53-RE-F	ATGGAAGTAACACAACGGACGCAGAGTCAGACACAACCAACACAACAGTCACCGACAACTTCCAATACCTCGCCAGAAC
RAD53-NEW-TF	ACATTCAGTAACAACACCCT