Mms21: A Putative SUMO E3 Ligase in *Candida albicans* That Negatively Regulates Invasiveness and Filamentation, and Is Required for the Genotoxic and Cellular Stress Response

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ABSTRACT 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 Gene Replacement And Conditional Expression 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 small ubiquitin-like modifier (SUMO) E3 ligase Mms21, and all other tested members of the sumoylation pathway, were both nonessential and capable of triggering filamentation upon repression, suggesting an important role for sumoylation in controlling filamentation in *C. albicans*. We have investigated Mms21 in detail. Both Mms21 nulls (*mms21*Δ/Δ) and SP [Siz/Pias (protein inhibitor of activated signal transducer and activator of transcription)] domain (SUMO E3 ligase domain)-deleted mutants displayed invasiveness, filamentation, and abnormal nuclear segregation; filament formation occurred even in the absence of the hyphal transcription factor Efg1. Transcriptional analysis of *mms21*Δ/Δ showed an increase in expression from two- to eightfold above that of the wild-type for hyphal-specific genes, 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, hydroxyurea, hydrogen peroxide, and UV radiation, suggesting that the protein is important for genotoxic stress responses. In addition, the *mms21*Δ/Δ mutant displayed sensitivity to cell wall and thermal stresses, and to different antifungal drugs. All these findings suggest that Mms21 plays important roles in cellular differentiation, DNA damage and cellular stress responses, and in response to antifungal drugs.

KEYWORDS *Candida albicans*; filamentation; Mms21; stress response; sumoylation

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 its ability 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), gastrointestinally induced transition (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

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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).

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 if we are 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 Gene Replacement And Conditional Expression (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 invasiveness and filamentation. Intriguingly, the genes encoding the small ubiquitin-like modifier (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 nonessential 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 responses, cellular differentiation, and protein transport (Seeler and Dejean 2003; Wohlschlegel *et al.* 2004; Zhou *et al.* 2004). The steps in the sumoylation process 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 a thioester bond between Uba2 and SUMO. The E1 enzymes then transfer activated SUMO to the E2-conjugating enzyme (Ubc9) forming a thioester bond between the 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 (where Ψ is a hydrophobic amino acid and 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 is carried out by specific SUMO E3 ligases (Meulmeester *et al.* 2008). Mms21 in *S. cerevisiae*, and its ortholog Nse2 in both humans and *Schizosaccharomyces pombe*, is an E3 ligase containing the so-called Siz/PIAS (SP)-RING domain that is exclusively found in SUMO E3s. Mms21 has important roles in post-translational modification, homologous recombination, DNA repair, and the maintenance of chromosomal integrity and telomeric length (McDonald *et al.* 2003; Pebernard *et al.* 2004; Andrews *et al.* 2005; Potts and Yu 2005, 2007; Zhao and Blobel 2005; 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 nonessential filamentation repressors. In *S. cerevisiae*, the Smc5/6 complex is required for nucleolar exclusion of Rad52 foci to prevent rDNA hyperrecombination (Torres-Rosell *et al.* 2007). In *C. albicans*, deletion of Rad52 causes filamentation and cell cycle-related defects with increased hyphal-specific gene (HSG) expression that is independent of Efg1 (Andaluz *et al.* 2006). The deletion of the SUMO protein Smt3 also results 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 and Whiteway 2005; Bachewich *et al.* 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.* 2015). 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 on the process the yeast–filamentous switching control, and to investigate the importance of Mms21 in response to different genotoxic and cellular stresses.

Materials and Methods

Strains and culture conditions

Strains used in this study are described in Supplemental Material, Table S2. For general growth and maintenance, strains were cultured in fresh YPD medium (1% w/v yeast extract, 2% w/v Bacto peptone, 2% w/v dextrose, and 80 mg/liter uridine with the addition of 2% w/v agar for solid medium) at 30°. 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.

GRACE library and invasive assay

The GRACE library is a collection of ~2500 conditional mutants created in the CaSS1 strain background for 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 *Escherichia 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, and permanent loss of the transactivation module can be identified by growing them on 5-FOA-containing medium to detect loss of the *URA3*-linked module.

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°. 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° for 4 days, followed by 10 sec washing of each colony with a stream of water. The colonies that stayed on the plates after washing were scored as invasive and those that were washed away were considered noninvasive. Images of plates and colonies were scanned at 600 dots per inch (dpi) using an Epson Perfection v500 photo scanner.

Deletion of the MMS21 and Mms21 SP domains

Oligonucleotides and plasmids used in this study are shown in Table S3. The two alleles of *MMS21* were deleted in the SN148 strain (wild-type) background by a standard gene deletion method using two-step PCR disruption (Dignard *et al.* 2007). The first allele was replaced with *HIS1* and the second allele with *ARG4* from the pFA-HIS1 and pFA-ARG4 plasmids, respectively (Gola *et al.* 2003). Since both plasmids have a common DNA backbone, oligonucleotides Amj37Fw and Amj38Rv (Table S3) were used to prepare the cassettes for the deletion of the *MMS21* gene. The *HIS1* marker was transformed into the parent strain SN148 using the standard lithium acetate method. The correct insertion of *HIS1* was confirmed by PCR analysis using genomic DNA from positive colonies and the resulting strain was named *MMS21/mms21*Δ. The second allele was deleted with *ARG4* in strain *MMS21/mms21*Δ using same transformation method, and was confirmed by PCR to generate the *mms21*Δ/Δ or null *mms21* mutant.

Deletion of the Mms21 SP domain in strain *MMS21/mms21*Δ was performed using plasmid pFa-TAP-Arg4 (Lavoie *et al.* 2008) using a similar strategy. The SP domain was deleted from strain *MMS21/mms21*Δ with a PCR-generated cassette, using oligonucleotides Amj73Fw and Amj72Rv, and subsequently confirmed by PCR. Western analysis of the mutant protein using anti-TAP antibodies was carried out to confirm the correct sizes.

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 1465-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 *E. coli* strain DH5α. 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 successful *MMS21* integration into the null *mms21* mutant was confirmed.

Microscopic analysis

For cell morphology, overnight cultures grown in YPD at 30° were subjected to phase differential interference contrast (DIC) microscopy. For nuclear segregation analysis, DAPI (Sigma [Sigma Chemical], St. Louis, MO) staining of live cells was performed without permeabilization. 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, and 1.59% w/v complete amino acids mixture, pH 7.0) and incubated for 3.5–6 hr (until successful completion of the first cellular division of both wild-type and mutant strains) followed by the addition of 2 μg/ml DAPI into each tube. Calcoflour staining (2 μg/ml) was performed using a similar strategy. Cells were examined by DIC at 63× and fluorescent microscopy at ×100 magnification using a Leica DM 6000 microscope mounted with a Hamamatsu Orca R2 CCD camera.

Stress phenotypes

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

Microarray profiling

The *mms21*Δ/Δ and SN148 cultures were grown in YPD overnight at 30°, diluted to OD^{600} of 0.1 in YPD at 30°, grown to an OD^{600} of 0.8–1.2, then harvested and stored at −80° until RNA extraction. Total RNA isolation and cDNA preparation were performed as previously described (Tebbji *et al.* 2014). *C. albicans* whole-genome arrays were obtained from the National Research Council Canada Biotechnology Research Institute and were hybridized in a hybridization chamber at 42° 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.

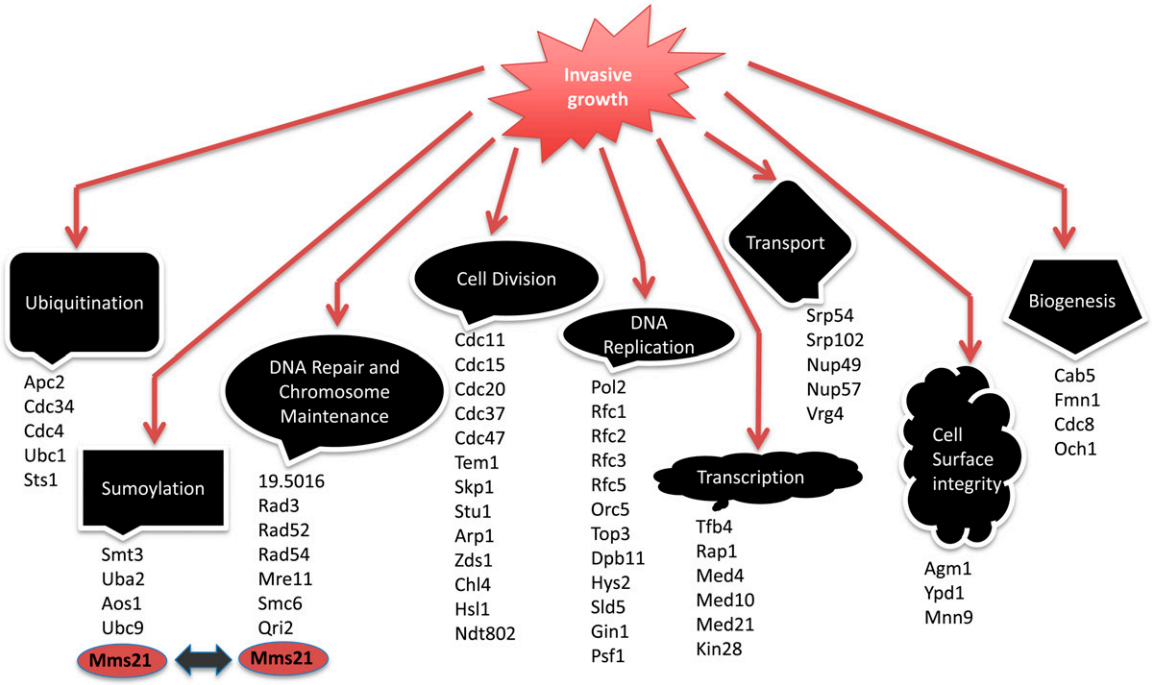


Figure 1 Invasive mutants collection from GRACE screening. A total of 69 mutants were scored invasive from GRACE library screening of which 60 mutants are shown here with their respective cellular processes. GRACE, Gene Replacement And Conditional Expression.

RNA-sequencing

The *mms21*Δ/Δ and SN148 cultures were grown in YPD overnight at 30°, diluted to OD^{600} of 0.1 in YPD at 30°, and then grown to an OD^{600} of 0.8–1.2 on a 220-rpm shaker. Total RNA was extracted using the QIAGEN (Valencia, CA) RNeasy minikit protocol, and RNA quality and quantity were determined using an Agilent bioanalyzer. Paired-end sequencing (150 bp) 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 preprocessed 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 (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 the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) (Edgar *et al.* 2002).

Data availability

Strains and plasmids are available upon request. Table S1 contains 69 invasive strains recovered from GRACE library screening. Strains used in this study are listed in Table S2. Plasmids and primers used with their respective sequences are listed in Table S3. Table S4 contains 373 hits from transcription profiling, while Table S5 represents RNA-seq up- and down-regulated genes need to change in figshare portal too. Figure S1 contains efg1 mms21 double-deletion filamentation. Transcription profiling data are available at GEO with the accession number: GSE116544. RNA-sequencing (RNA-seq) data are accessible through GEO Series accession number: GSE121210. Supplemental material available at Figshare: https://doi.org/10.6084/m9.figshare.7385114.

Results

Large-scale invasive growth screening in C. 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) YPD agar at 30° for 96 hr, followed by a 10-sec 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 2357 single mutants in the GRACE collection that were invading the agar medium during tetracycline repression, suggesting that those genes code for functions that inherently repress invasiveness and filamentation (Table S1). The genes observed included several transcription regulators and, overall, were implicated in a variety of cellular processes including cell division, chromosome

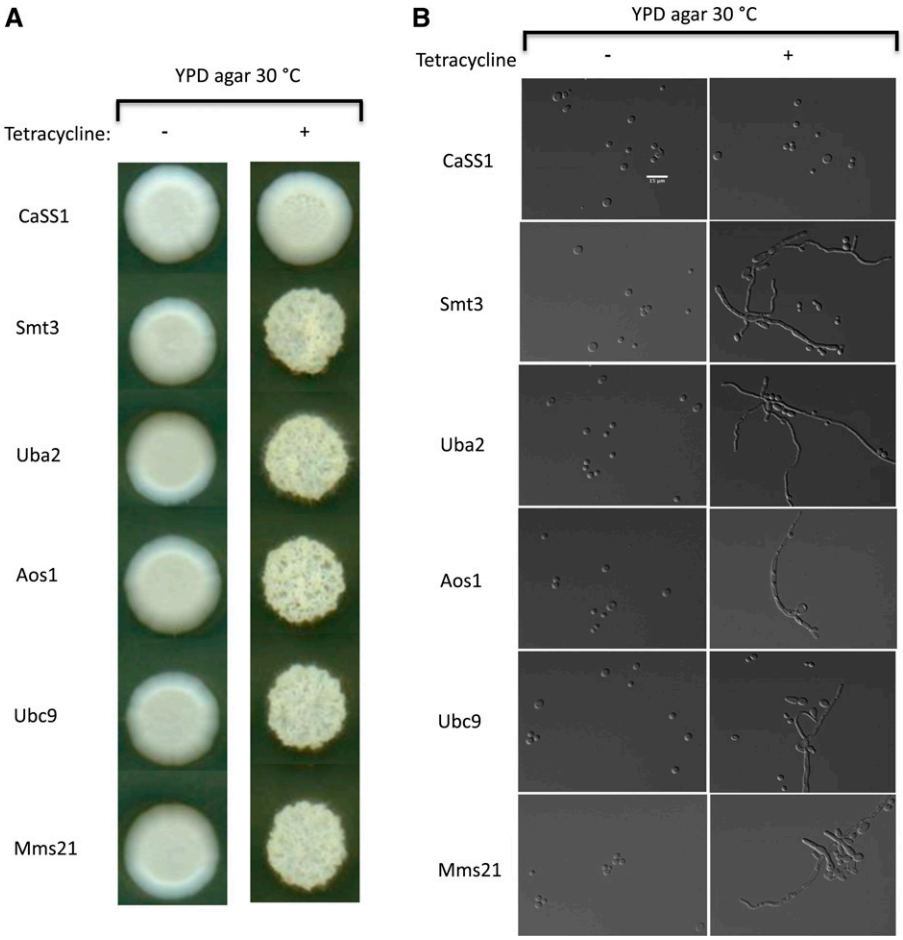


Figure 2 Members of the SUMO pathway negatively regulate filamentation. (A) Putative members of the sumoylation pathway in *C. albicans* were grown at 30° on YPD agar with and without 100 μg/ml tetracycline for conditional repression. Each mutant repression caused fuzzy colonies except for the wild-type CaSS1. (B) Representative DIC images of repressed and nonrepressed SUMO mutants at ×63 magnifications using a Leica DM 6000 microscope; mutant cells displayed filamentous (hyphal and pseudohyphal) morphology, while the wild-type CaSS1 showed normal yeast morphology. Bar, 15 μm. SUMO, small ubiquitin-like modifier.

maintenance, DNA repair, transport, ubiquitination, and sumoylation (Figure 1).

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 the control of filamentation that include transcription regulators, genes involved in cellular transport and biogenesis, and some members of the ubiquitination and sumoylation pathways (Table S1).

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 (Figure 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 (Figure 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.

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

Figure 3 T-COFFEE (tree based consistency objective function For alignment evaluation) structural protein alignment (Expresso) of Mms21/Nse2 SP [Siz/Pias (protein inhibitor of activated signal transducer and activator of transcription)] domain among different organisms. The arrows indicate residue CXH/CX_4C required for Zn^{2+} binding. The main score (91–95) is the total consistency value. Red regions represent conserved residues while asterisks are used to label residues that are identical in the consensus sequence. Color scheme of "BAD AVG GOOD" represents level of consistency between final alignment and the library used by T-COFFEE. A score of nine (dark red regions) represents consistency score of strong alignment.

predicted to start at S^{192} and run until L^{239}. As shown in Figure 3, the SP domain is highly conserved among different organisms when we performed a T-COFFEE (tree based consistency objective function For alignment evaluation) structural protein alignment (Armougom *et al.* 2006; Di Tommaso *et al.* 2011). This domain contains the motif CXH/CX_4C, which is required for zinc ion (Zn^{2+}) binding to create protein stability and is important for E3 ligase function (Yunus and Lima 2009).

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 our GRACE screening that inactivation of Mms21 derepressed invasiveness, suggesting that 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 nonessential protein in *C. albicans* required to repress invasiveness and filamentation, we generated a complete knockout 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* 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 grew slowly in liquid YPD medium and formed small but highly invasive wrinkled colonies on YPD agar under standard yeast growing conditions (Figure 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 nonessential protein in *C. albicans*, but a key player in morphogenesis control.

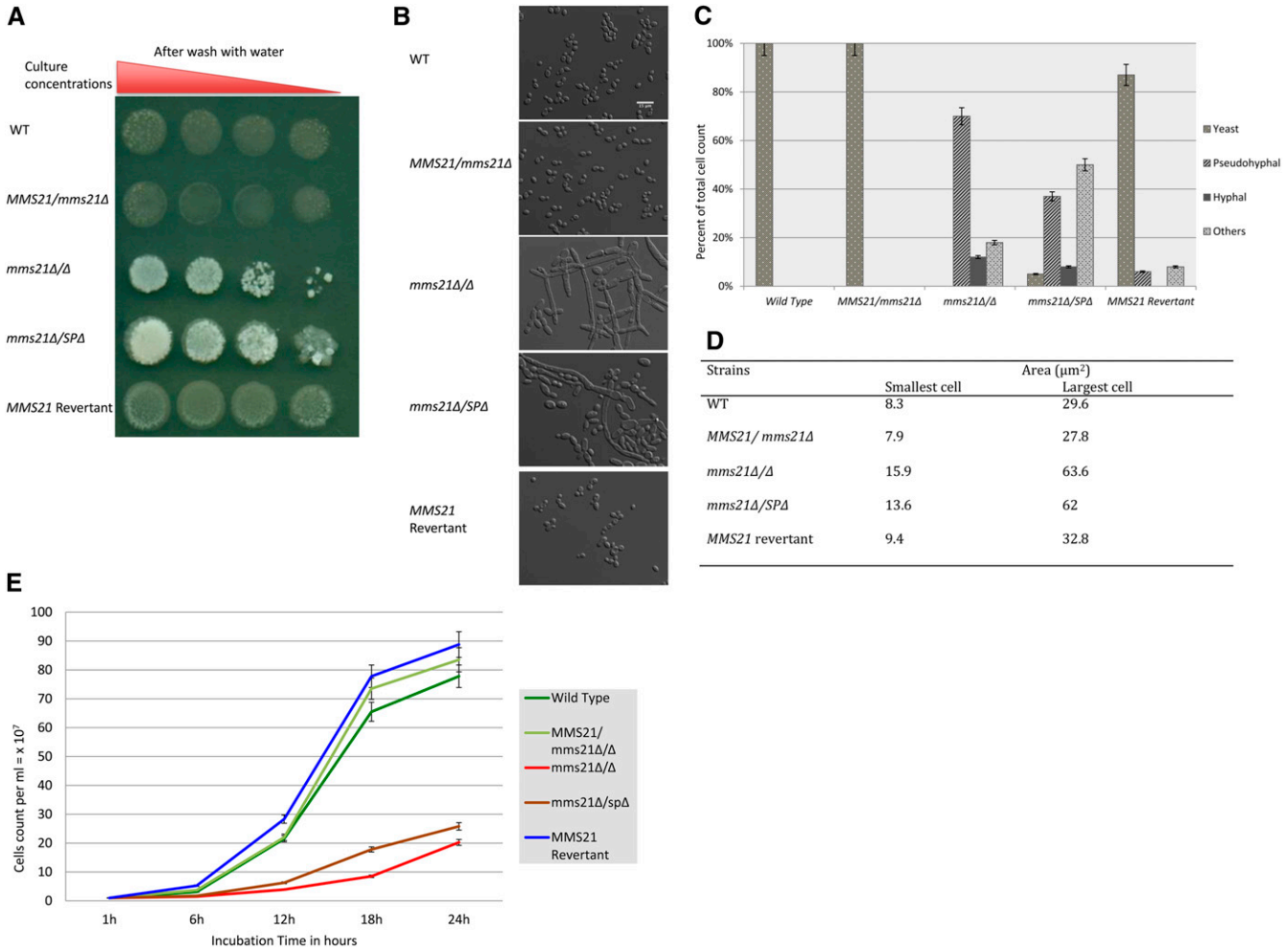
Mms21 SP domain-deleted mutants are also invasive

Our GRACE screening 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 tag and an *ARG4* selectable marker. The *mms21Δ/SP*Δ mutant was confirmed by both PCR and western blot analysis. These *mms21Δ/SP*Δ mutants also formed highly invasive and slow-growing small wrinkled colonies on YPD agar plates under yeast growth conditions (Figure 4A), suggesting that Mms21-mediated sumoylation plays a major role in *C. albicans* invasive growth control.

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 causes 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/SP*Δ cells displayed abnormal phenotypes at 30° when cultured in liquid YPD. Individual mutant cells were morphologically aberrant, ranging from enlarged and elongated yeast-like cells to pseudohyphal or hyphal cells (Figure 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° (Figure 4C). The *mms21Δ/SP*Δ mutants, on the other hand, were 8% hyphal, 37% pseudohyphal, 50% abnormal, and 5% yeast-type. In the wild-type 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 to 63.6 μm^2 in size, while *mms21Δ/SP*Δ yeast-like cells were in the range of 13.6–62 μm^2 (Figure 4D). 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 to 32.8 μm^2. Thus, either Mms21 deletion or its SP domain's 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-hr incubation period by counting the number of cells using a hemocytometer 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/SP*Δ mutants reached 25×10^7 cells/ml while



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 4 Colony and cell morphologies of *MMS21* mutants. (A) A 1:10 serial dilution of overnight culture of mutants was spotted on to YPD agar and then incubated at 30° for 4 days. Individual colonies were washed with a stream of water for 10 sec; *mms21*Δ/Δ and Mms21 SP-deleted mutants were found to be invasive. (B) DIC images of mutants grown under yeast conditions at ×63 magnification; Bar, 15 μm. The *mms21*Δ/Δ and *mms21*Δ/*SP*Δ mutants have displayed psuedohyphae, hyphae, and others (large cells nearly identical to gut, gray, or opaque cells), while the *MMS21* revertant strain recovered the WT yeast phenotype. (C) A clustered column graph showing the percentage of different cell morphologies displayed by mutants. The *mms21*Δ/Δ and *mms21*Δ/*SP*Δ mutants have increased filamentous phenotypes. (D) A table of the cell-size area of the log-phase growing strains measured in the DAPI channel using ImageJ Fiji 1.0. (E) A graph showing cell counts of mutants and WT cultures in YPD at 30° for 24 hr. The initial cell count was started at 1×10^7 cells/ml. Both *mms21* mutants doubled their first cell population at the 8-hr mark, while the revertant and the WT doubled every 2 hr. Error bars are based on the SD from two biological replicates of each data point reading taken every 6 hr. SP, Siz-/Pias (protein inhibitor of activated signal transducer and activator of transcription) domain; WT, wild-type.

the *mms21*Δ/Δ mutants acquired cellular densities of only 20×10^7 cells/ml after 24-hr incubation at 30° in liquid YPD. The wild-type and the revertant strains reached much higher cell densities, near or above 80×10^7 cells/ml (Figure 4E). Furthermore, measuring cell densities using a spectrophotometer (OD 600 nm) revealed that the wild-type strain reached an OD of 1 from 0.1 within 6 hr of incubation under yeast growth conditions, while *mms21*Δ/Δ mutants took ~14 hr to acquire an OD of 1. These data highlight the impact of a structural and enzymatically functional Mms21 on the growth of *C. albicans*.

In *S. cerevisiae*, the SUMO E3 ligase activity encoded by *MMS21* is essential for nuclear integrity and the 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 Mms21 null and its SP domain deletion on nuclear segregation, cells ($n = 200$ for each) of the mutant and wild-type strains were stained with DAPI and observed under a microscope. Interestingly, both null and SP-deleted mutants showed variable nuclei within a high proportion of cells (Figure 5A). In the case of the wild-type strain, 92% ($n = 62$) of large-budded cells have two nuclei, one in each 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

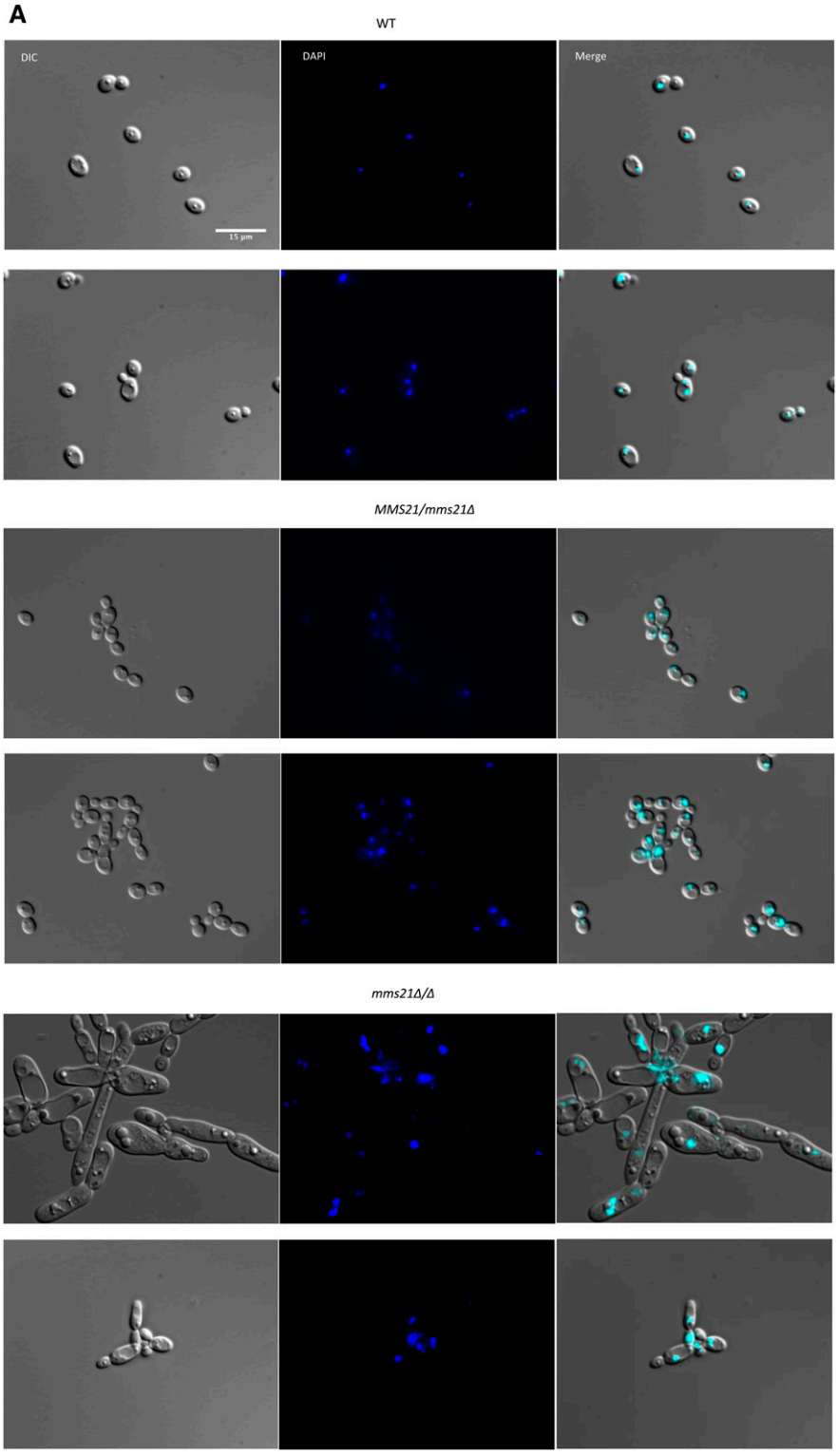


Figure 5 Staining of the *MMS21* mutants with DAPI and calcoflour. Cultures were allowed to grow for 3.5 hr for WT and revertant strains, and 6 hr for mutants under yeast growth conditions, and then stained with 2 μg/ml DAPI or 2 μg/ml calcoflour. Individual cells were examined under ×100 magnification using a Leica DM 6000 microscope, Bar, 15 μm. Only (more or less) yeast-looking cells of mutants were considered for better comparison to the WT. (A) WT cells and strains with one WT allele of *MMS21* showed normal nuclear segregation. The *mms21*Δ/Δ and *mms21*Δ/*SP*Δ mutants displayed considerable variation in the number of nuclei present in the individual cells. (B) Calcoflour-stained cells where WT cells display even chitin distributions and prominent septa, while both *mms21* mutants have abnormal chitin composition and inconspicuous septa. Reinsertion of Mms21 into the null mutant resulted in highly noticeable septa. WT, wild-type.

and 91% ($n = 66$) for small-budded cells, respectively. Both these strains showed a normal pattern of yeast cell morphogenesis where large-budded cells with two nuclei were in late M phase, while small-budded cells were in late G2 phase of the cell cycle (Berman 2006). Interestingly, both null and SP-deleted mutants showed variable defects in nuclear segregation, with large-budded cells containing only one nucleus either in the mother or in the bud cell, or nonbudded 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 irregularly shaped nuclei (Figure 5A). These abnormal nuclear

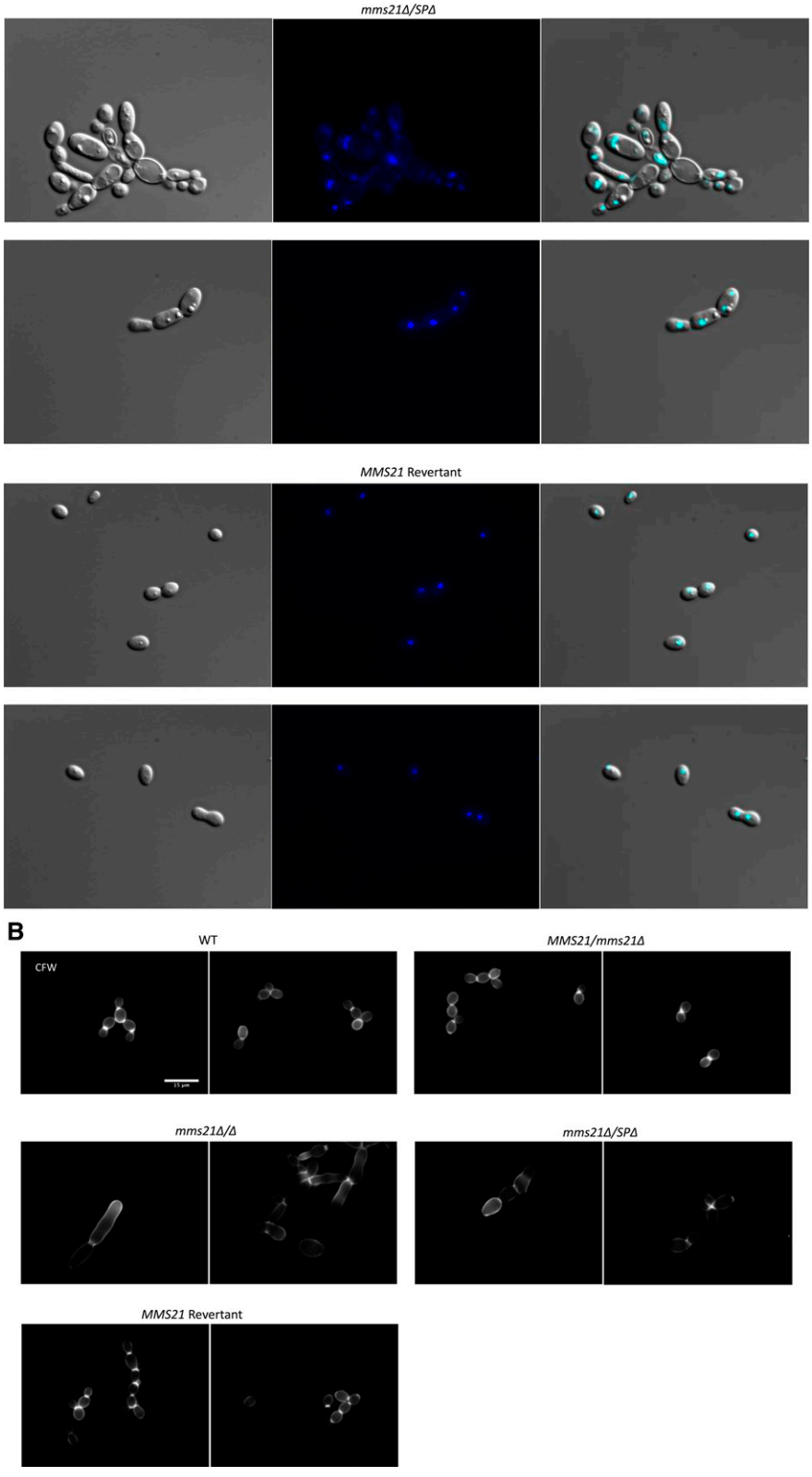


Figure 5 continued.

variations were 68% (n = 136) in null and 47% (n = 96) in SP-deleted mutant cells, respectively, indicating that cells might be in late S/G2 arrest or have a defective M cycle, perhaps due to a failure in the DNA repair machinery causing abnormal nuclear content and a respective increase in cell size.

Septal ring formation is central to faithful nuclear segregation between mother and daughter cells (Berman 2006).

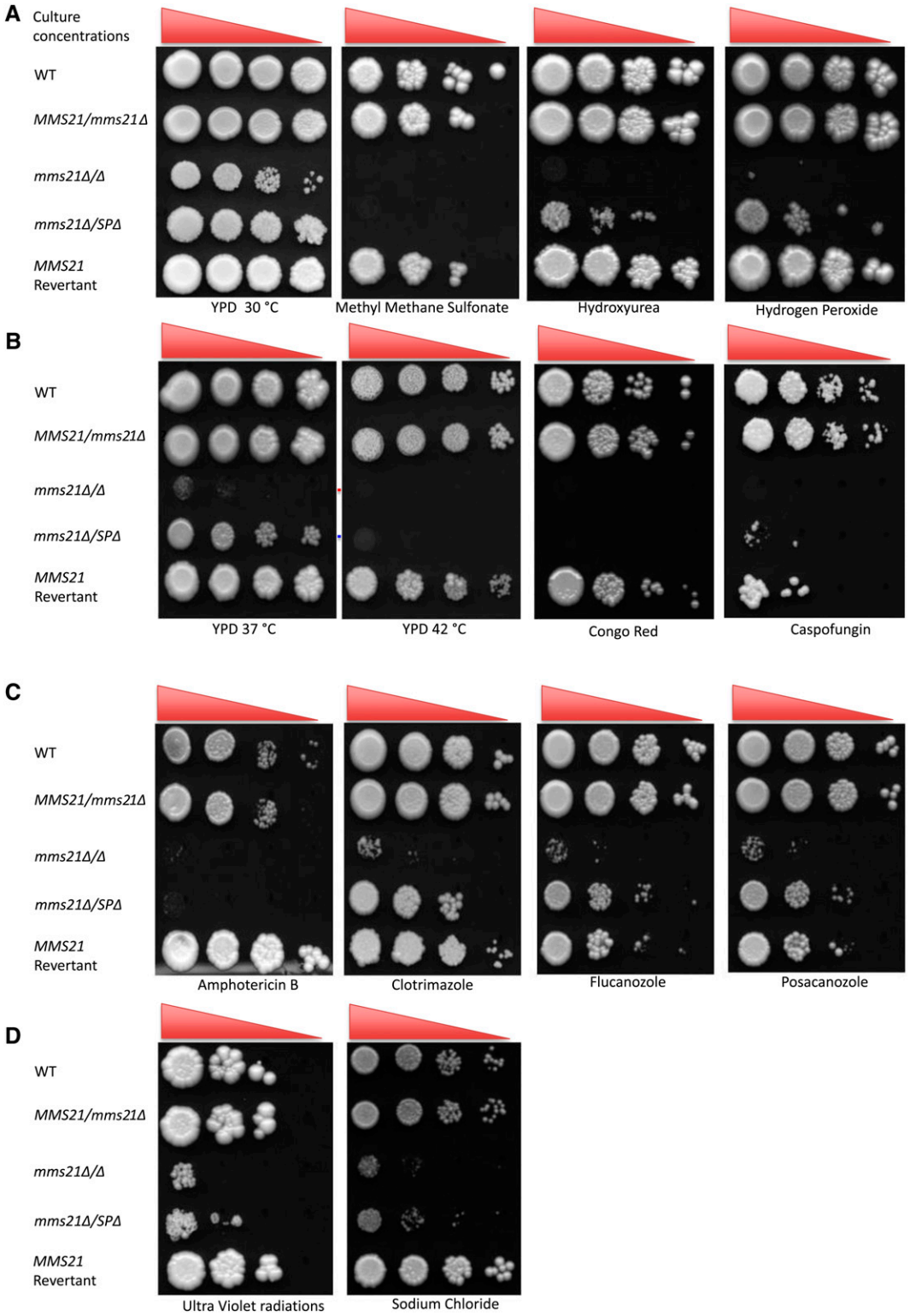


Figure 6 Genotoxic and cellular stress assay. A 1:10 serial dilution of overnight cultures grown in yeast growth conditions were spotted (highest cell concentrations being 1×10^6 cells/ml and lowest being 1×10^3 cells/ml) onto YPD agar plates containing different chemicals and were incubated at 30° for 4 days except for caspofungin (7 days). (A) strains were subjected to genotoxic stress of MMS (0.01% v/v), hydroxyurea (10 mM), and hydrogen peroxide (5 mM). (B) Strains response to heat (37 and 42°) and cell wall stress caused by Congo red (150 μg/ml) and caspofungin (0.75 μg/ml). (C) Determination of resistance to different cell membrane-damaging drugs: amphotericin B (1 μg/ml), clotrimazole (0.5 μg/ml), flucanozole (10 μg/ml), and posacanozole (10 μg/ml). (D) sensitivity to UV radiation and NaCl (1 M). Experiment was repeated three times for each sample for consistent results. WT, wild-type.

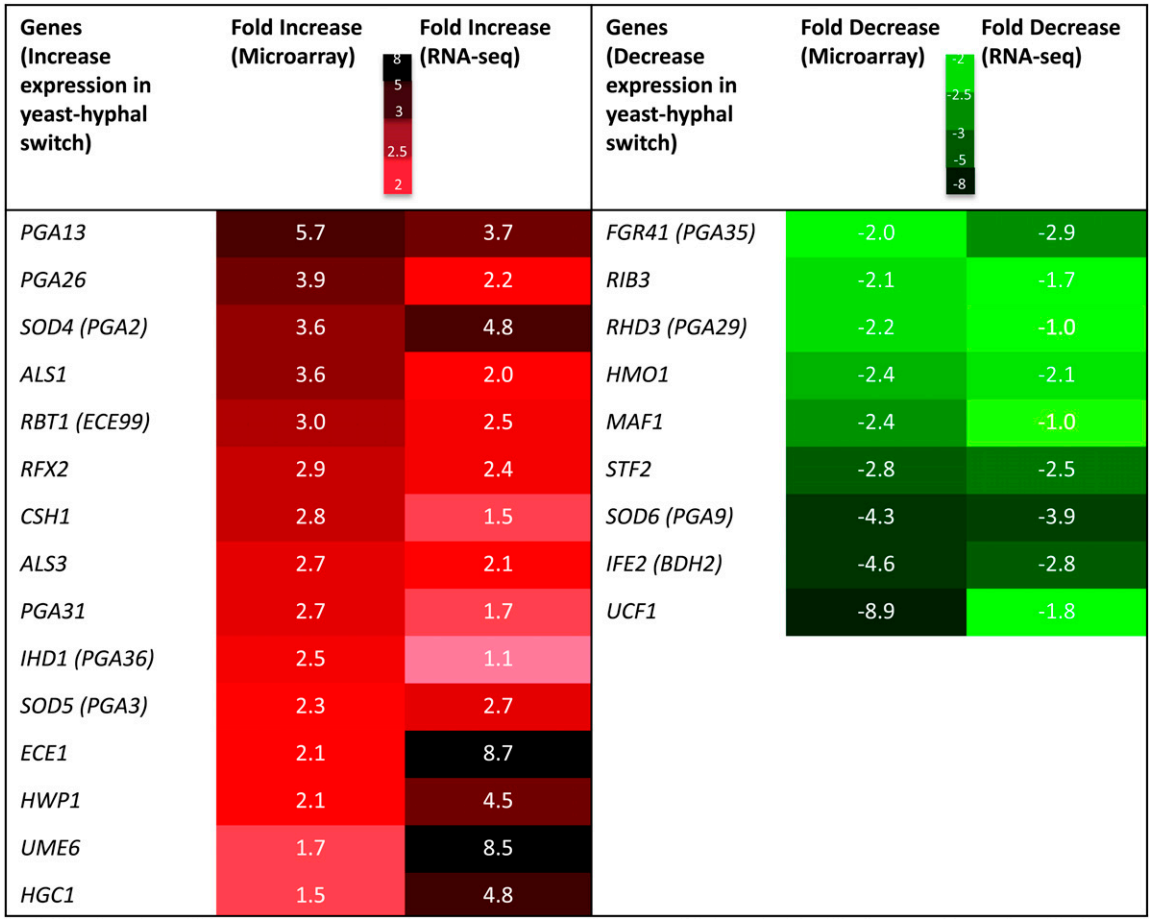


Figure 7 A list of genes that are highly significant for hyphal development and virulence, and their fold increase and decrease in expression in *mms21* null against wild-type cells using microarray and RNA-sequencing analysis (RNA-seq). Color coding is used to highlight an increase or decrease in gene expression. Dark red regions represent highly increased gene expression and dark green regions represent highly decreased gene expression.

We tested all of our strains for the chitin composition of the cell wall and septa using calcofluor staining. All of the wild-type cells (n = 214) showed 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 was not as prominent and distinct as in wild-type cells (Figure 5B). The null mutants had 46% (n = 201) while SP-deleted cells had 40% (n = 194) of the cells showing chitin and septal abnormalities, suggesting that septa and chitin deposition were perturbed in the mutants rather than occurring at the start of nuclear and cellular division. Overall, the modifications in chitin deposition and in nuclear structure are not simply representative of the enhanced level of filamentation, but are likely a direct consequence of loss of *MMS21* function, as filamenting wild-type cells do not show the abnormalities seen in the *mms21* mutants. 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 that Mms21 is required for normal cellular physiology in *C. albicans*.

Mms21 is required for the genotoxic and cellular stress responses

MMS21 was first identified as a gene in *S. cerevisiae* whose inactivation conferred sensitivity to MMS, X-rays, and UV 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 Mms21 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–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 stress.

We assayed the response of *mms21Δ/Δ* and *mms21Δ/SPΔ* cells to genotoxic stress-causing agents including the alkylating agent MMS, the DNA replication-stalling chemical HU, the oxidative stress agent hydrogen peroxide, and thymine dimer-causing UV radiation (Figure 6, A and D). Both *mms21* null and SP-deleted mutants were highly sensitive to MMS and UV, showing that the loss of the Mms21 protein or its putative SUMO SP domain function blocks cells from recovering from MMS- and UV-induced cellular stress. The null mutant, but not the SP-deleted mutant, never recovered from HU- and hydrogen peroxide-induced stresses, highlighting the importance of Mms21 for a potential structural role in maintaining the Smc5/6 complex as 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°, consistent with Smt3 being implicated in sumoylating heat shock proteins in *C. albicans*. As well as not growing at 42°, cells lacking both alleles of Mms21 showed extremely poor growth at 37° (Figure 6B). The fact that *mms21Δ/MMS21* and the Mms21 revertant grew normally at 37° implies that at least one fully functional allele of Mms21 gene may be required for *C. albicans* to thrive under normal human body temperature.

We also surveyed the effects of different cellular stress-causing agents in Mms21-defective mutants. The cell wall is the first line of defense for most of unicellular organisms, including bacteria and fungi. The cell wall of *C. albicans* contains an estimated 2–6% chitin, which is very important for the rigidity of the cell to resist damaging external 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 (Figure 6B). 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 well to ergosterol biosynthesis inhibitors including fluconazole, clotrimazole, and posaconazole (Figure 6C). Moreover, both mutants also grew slowly under salt stress (Figure 6D). Altogether, these results show that Mms21 plays important roles in *C. albicans* physiology by helping the pathogen to cope with genotoxic and cellular stresses.

Expression profiling of mms21Δ/Δ

Since the null *mms21* mutant has a significant effect 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 < 0.05 and a cutoff of twofold, we have identified 373 genes whose levels of expression were significantly affected in *MMS21* inactivation; 151 genes were up- and 222 were downregulated (Table S4). The majority of the upregulated genes were involved in ribosome and ribonucleoprotein biogenesis, RNA processing, and the response to reactive oxygen species, while many were associated with nucleic acid and heterocycle metabolic processes. Around 15% of the upregulated genes were associated with *C. albicans* filamentation and virulence. Among the downregulated genes were genes involved in oligopeptide transport, hexose transport, the 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 oligopeptide 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 downregulated 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 (Figure 7). Important genes in this list include *PGA13*, *PGA26*, *PGA31*, *ALS1*, *ALS3*, *ECE1*, *HWP1*, *SOD4*, and *SOD5*. The genes for two important glycosyl phosphatidyl inositol) mannoproteins that constitute 30–40% of the cell wall by weight (Pga13 and Pga26) were highly upregulated 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 that are highly important for fungal adherence to the host cells, the 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 upregulated in the *mms21* null strain and this gene is also upregulated 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.

We followed up on our microarray profiling by performing whole-genome transcriptome sequencing (RNA-seq) analysis of *mms21Δ/Δ* and wild-type cells under yeast growth conditions. By using a *P*-value of < 0.05 and fold cutoff of 2.0, we found 188 up- and 97 downregulated genes (Table S5). Analysis using the Candida Genome Database Gene Ontology Slim Mapper revealed that, among upregulated genes, 21.3% represent 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 responses. Our RNA-seq data were consistent with our microarray data for the aforementioned HSG expression analysis (Figure 7). Furthermore, the RNA-seq analysis revealed Ume6, a true hyphae transcription factor (Zeidler *et al.* 2009), to be more than eightfold upregulated, and Hgc1, a hyphal-specific cyclin (Zheng *et al.* 2004), to be 4.8-fold upregulated.

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

Filamentation in mms21Δ/Δ is independent of Efg1

Efg1 is required for hyphal protein expression under hyphae-inducing conditions (Sohn *et al.* 2003). Intriguingly, our microarray data show no change in the level of expression of this important filamentation transcription factor 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 the filamentation exhibited by the *mms21* null mutant is independent of Efg1, we deleted *MMS21* in an *efg1Δ/Δ* strain as previously described (Regan *et al.* 2017). As shown in Figure S1, the *efg1Δ/Δ* mutant cells were locked in the yeast form, but deleting both alleles of *MMS21* (*efg1Δ/Δ mms21Δ/Δ*) resulted in filament formation as in the *mms21* null cells. Therefore, filamentation caused by the absence of Mms21 is independent of the pathway controlled by Efg1.

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 nonessential in *C. albicans*, which is consistent with the findings that the unique SUMO protein-encoding locus *SMT3* is nonessential 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 two- to sixfold increases in the expression of several genes involved in filamentation and virulence. Previously, a large-scale homozygous mutant study in *C. albicans* had found that *mms21*-deleted mutants showed normal competitive fitness in mice (Noble *et al.* 2010), though they did not detect changes 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 that are 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 pathway, which 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 that is 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 the repressors Nrg1-Tup1 in the null mutant. However, Hgc1 and Ume6 were both upregulated, indicating that these two proteins and the HSGs were transcriptionally induced independently 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 cells 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 arresting 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 affecting 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 the active cyclin-dependent kinase complex would result in pseudohyphae formation (Lew and Reed 1995). Therefore, this increased filamentation in the absence of Mms21 appears to be a result of defects in cell division and the DNA damage repair machinery, as seen for other genes (Bachewich *et al.* 2003; Bachewich and Whiteway 2005). In fact, often the input signals that cause filamentation impose some sort of cell stress, whether these signals are changes in temperature or pH, or use of N-acetylglucosamine 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 irregularly shaped (Figure 4, B and C), and growing at a slow rate when compared to the wild-type. We also observed that many of these mutant cells were multinucleated, suggesting a defect in proper nuclear segregation

(Figure 5A). Similar defects had been previously attributed to 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 contents and inconspicuous septa in these mutant cells (Figure 5B). 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 phase 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 *mms21*Δ/Δ and *mms21*Δ/*SP*Δ mutants showed impaired responses to different genotoxic stress-causing agents (Figure 6, A and D). 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 *mms21*Δ/Δ and *mms21*Δ/*SP*Δ mutants were highly sensitive to both DNA-alkylating MMS and UV radiation, indicating that not only is the whole functional Mms21 domain required to overcome the DNA damage caused by MMS and UV, but also its active C-terminal SP domain. 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 the case of other genotoxic stresses, the null *mms21* mutant cells were more sensitive to the genotoxic stress than the mutation that was 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 the Smc5/6 complex. Compromise of this interaction has been found to be lethal in *S. cerevisiae*, while mutating 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 *mms21*Δ/Δ and *mms21*Δ/*SP*Δ mutants were found to be highly sensitive to a temperature of 42° (Figure 6B). Leach *et al.* (2011) 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 (J. Feng, A. Islam and M. Whiteway, unpublished data). 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 screen, we have found Mms21 to be nonessential 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 (*mms21*Δ/*SP*Δ) mutants, we have found out that the full Mms21 protein and 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 the *C. albicans* response to genotoxic stresses, and thus plays a vital role in preserving genome integrity. Mms21 is also important for the normal response of cells to cell wall, cell membrane, and thermal stresses. Our data show that Mms21 plays important roles in cellular differentiation, and 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.

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