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The contribution of the S-phase checkpoint genes *MEC1* and *SGS1* to genome stability maintenance in *Candida albicans*

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Abstract

Genome rearrangements, a common feature of *Candida albicans* isolates, are often associated with the acquisition of antifungal drug resistance. In *Saccharomyces cerevisiae*, perturbations in the S-phase checkpoints result in the same sort of Gross Chromosomal Rearrangements (GCRs) observed in *C. albicans*. Several proteins are involved in the *S. cerevisiae* cell cycle checkpoints, including Mec1p, a protein kinase of the PIKK (phosphatidyl inositol 3-kinase-like kinase) family and the central player in the DNA damage checkpoint. Sgs1p, the ortholog of *BLM*, the Bloom's syndrome gene, is a RecQ-related DNA helicase; cells from BLM patients are characterized by an increase in genome instability. Yeast strains bearing deletions in *MEC1* or *SGS1* are viable (in contrast to the inviability seen with loss of *MEC1* in *S. cerevisiae*) but the different deletion mutants have significantly different phenotypes. The *mec1Δ/Δ* colonies have a wild-type colony morphology, while the *sgs1Δ/Δ* mutants are slow-growing, producing wrinkled colonies with pseudohyphal-like cells. The *mec1Δ/Δ* mutants are only sensitive to ethylmethane sulfonate (EMS), methylmethane sulfonate (MMS), and hydroxyurea (HU) but the *sgs1Δ/Δ* mutants exhibit a high sensitivity to all DNA-damaging agents tested. In an assay for chromosome 1 integrity, the *mec1Δ/Δ* mutants exhibit an increase in genome instability; no change was observed in the *sgs1Δ/Δ* mutants. Finally, loss of *MEC1* does not affect sensitivity to the antifungal drug fluconazole, while loss of *SGS1* leads to an increased susceptibility to fluconazole. Neither deletion elevated the level of antifungal drug resistance acquisition.

Keywords

MEC1; *SGS1*; Cell Cycle Checkpoint; Antifungal Drug Resistance; Genome Stability

1. Introduction

Various checkpoints act to insure genome integrity during the cell cycle, as genome integrity is constantly challenged by intrinsic errors or external agents. Studies of cancer-predisposition syndromes and sporadic tumors in humans have identified mutations in many DNA damage checkpoint genes, underscoring the importance of the checkpoint response.

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Checkpoints verify whether the processes at each phase of the cell cycle have been accurately completed before progressing into the next phase. Checkpoints exist to monitor the readiness of G1 cells to enter S phase, while the intra-S checkpoint is activated by replication-induced damage. A G2 checkpoint ensures that cells are ready to enter mitosis, during which a spindle assembly checkpoint verifies that the chromosomes are properly aligned at the mitotic plate before sister chromatid separation. In addition to these cell cycle phase-specific checkpoints, a DNA damage checkpoint is activated in response to any kind of DNA damage during any cell cycle phase.

DNA replication defects are the major source of spontaneous genomic instability in the cell. Cells have evolved S-phase checkpoints as a defense against replication-based instability. Two different checkpoints act during DNA replication: the replication checkpoint and the intra-S checkpoint. The replication checkpoint functions in S phase in response to blocked DNA replication, causing cell-cycle arrest and suppressing late replication origin firing. The intra-S checkpoint activates in response to DNA damage occurring during S phase, blocking initiation and elongation of DNA replication by inhibiting polα/primase activity and by modifying the ssDNA-binding protein RPA. The role of these checkpoints in maintaining genome stability is illustrated by the elevated rates of gross chromosomal rearrangements (GCRs) in S-phase checkpoint mutants (Myung et al., 2001). In comparison, defects in the G1 and G2 DNA damage checkpoints and the spindle checkpoint do not cause increased GCR rates (Myung et al., 2001).

Mec1p is a central component of the DNA damage checkpoint. *MEC1* is the structural homolog of the human *ATR* gene, which is related to the *ATM* gene that is mutated in patients with the cancer-prone syndrome ataxia telangiectasia and to the Schizosaccharomyces pombe *RAD3* checkpoint gene. Mutations in *MEC1* affect the G1, S-phase and G2 checkpoints (Kato and Ogawa, 1994). It is a highly conserved protein kinase of the PIKK (phosphatidyl inositol 3-kinase-like kinase) family, and acts as a transducer kinase responsible for activation of the signal-transduction cascade (Elledge, 1996; Foiani et al., 2000). Mec1p is part of a sensor mechanism that detects DNA damage in the form of single-stranded DNA (ssDNA) (Zou and Elledge, 2003), usually a consequence of stalled replication forks (reviewed in (Branzei and Foiani, 2009; Branzei and Foiani, 2010). Mec1p binds and phosphorylates the ssDNA-binding protein RPA (Kim and Brill, 2003), and also phosphorylates and activates the effector kinases Rad53p and Chk1p (Longhese et al., 2003), resulting in the phosphorylation of key effectors of the DNA damage response. Their activation results in cell cycle delay or arrest, transcriptional up-regulation of DNA repair genes, and stabilization of replication forks. Although Mec1p and Rad53p are not essential in mammals, previous studies have shown that these two kinases are essential for viability in *S. cerevisiae* (Giaever et al., 2002). A study by Shi *et al.* demonstrated that, unlike *S. cerevisiae*, *C. albicans rad53Δ/Δ* cells are viable (Shi et al., 2007).

Sgs1p is a fork-associated helicase that acts to prevent illegitimate recombination at stalled forks during the intra-S checkpoint (Fabre et al., 2002; Frei and Gasser, 2000). It is a member of the RecQ family of DNA helicases, which have been shown to be involved in maintaining genome stability by regulating stalled replication forks (Barbour and Xiao, 2003; Fabre et al., 2002). Three of the five human RecQ helicases are encoded by the *BLM*, *WRN* and *RECQ4* genes (Ellis et al., 1995; Kitao et al., 1999; Yu et al., 1996); defects in these genes have been implicated in heritable diseases associated with genomic instability and a predisposition to cancer (Ellis et al., 1995; Kitao et al., 1999; Yu et al., 1996). Unicellular organisms usually express a single RecQ helicase homolog. In *S. cerevisiae* the only RecQ helicase is encoded by *SGS1*, which is an ortholog of the human *BLM* gene (Gangloff et al., 1994). Several studies have shown that Sgs1p functions in the intra-S checkpoint as a sensor for damage during replication in combination with Rad53p (Frei and

Gasser, 2000). Sgs1p is involved in a template-switching form of repair, where it acts as a Holliday junction resolvase in combination with Top3p (Gangloff et al., 1994) to resolve hemicatenane structures that arise during the repair process (Mankouri and Hickson, 2007), reviewed in (Branzei and Foiani, 2010).

Candida albicans exhibits significant karyotypic variability, both in clinical isolates and in strains maintained in the laboratory (Janbon et al., 1998; Magee, 1993; Magee et al., 1992). Drug resistance has been linked to karyotypic variability – change in chromosome number or structure can allow cells to become resistant to fluconazole, for example (Legrand et al., 2004; Perepnikhatka et al., 1999; Selmecki et al., 2006; Selmecki et al., 2008). In addition, cells bearing deletions in double-strand break repair (DSBR) genes exhibit an elevated level of chromosome instability, and also an increased frequency of antifungal drug resistance acquisition (Legrand et al., 2007).

Based on our prior data linking DSB repair and antifungal drug resistance in *Candida albicans* (Legrand et al., 2007), we examined the role of the checkpoints that most commonly monitor DSB formation. To this end, we constructed *C. albicans* strains with deletions of both copies of *MEC1* or *SGS1*. We found that these two genes are not essential for cell viability in *C. albicans*. We characterized the DNA repair activities of the deletion mutants using a series of phenotypic assays, including sensitivity to oxidizing agents, alkylating agents and ultraviolet (UV) light. We also investigated genome stability in the deletion mutants by monitoring events at the *GAL1* locus on chromosome 1 (Ch1), using a *GAL1/gal1::URA3* reporter construct (Legrand et al., 2007). Finally, we monitored antifungal drug sensitivity and ability to acquire resistance to the antifungal drug treatment.

2. Materials and Methods

2.1 Strains and media

The yeast strains used in this study are described in Table 1. *C. albicans* and *S. cerevisiae* strains were maintained on YEPD media (1% Yeast Extract, 2% Peptone, 2% Dextrose) supplemented with 20mg/L uridine at 30°c. Construction of the parental strain DKCa39 was described previously (Legrand et al., 2007). For each mutant, at least two independent isolates were constructed and analyzed.

2.2 Gene disruption and reintegration of C. albicans wild-type genes

To construct homozygous mutant strains, both alleles were deleted in strain DKCa39 using a PCR-based cassette method, using methods previously described (Legrand et al., 2007). The first allele was replaced with the *Candida dubliniensis HIS1* marker and the second allele was replaced with the *C. dubliniensis ARG4* marker. The cassettes were amplified with the same set of primers since the *HIS1* and *ARG4* markers have been cloned into the same location in the same vector (Noble and Johnson, 2005). Transformations used a variation of the standard LiAc transformation protocol (Legrand et al., 2007). Transformants initially were screened by colony PCR with primers positioned within the marker sequence and outside the integration site. Positive transformants were cultured for DNA extraction and both boundaries of the integration site were verified by PCR. The *SGS1* deletion removed nucleotides 80 to 3386 of the 3570 bp gene. The full-length deletion of *MEC1* removed nucleotides 80 to 6899 of the 6978 bp gene, while the partial internal deletion removed nucleotides 6000 to 6899, eliminating the putative kinase domain. We were unsuccessful in constructing reintegration strains using the NAT1-FLP cassette (Shen et al., 2005). The large size of the *MEC1* gene hindered efforts to assemble a wild-type full-length construct, while the homozygous *SGS1* deletion strain did not transform efficiently and all potential reintegrants that were obtained had integrated randomly in the genome rather than at the

native *SGS1* locus. To offset the lack of reintegrant strains, we examined at least two independent homozygous diploid mutants for each gene.

2.3 Oligonucleotides

The following primers were used in the construction of the deletion strains. Bold letters correspond to vector sequences of the plasmids containing *C. dubliniensis HIS1* or *ARG4* genes. All sequences are 5′ to 3′.

CaMEC1-KO-F:

ATGACGTCGAATCAATCAATAAGTACTACTGAACTACTACAGTTTTTAACAG
ATATAGAAACCAATATCGACGATCATG**GCATCAAGCTTGGTACCGAGC**

CaMEC1-partialKO-F:

ACTTAAATTCCAAGCACAAGGGGTTTTCACGTTCTTCTTCAATTTCATTCGAT
GGGTTTGATGATAATGTGAATATTTT**GCATCAAGCTTGGTACCGAGC**

CaMEC1-KO-R:

CTACATATAAGCTGCCCAACCTGCATACATTTGTGATAATCTCTCTAATGAT
GTAGCTTCTTGAATCAACACATCTACT**CCCTCTAGATGCATGCTCGAG**

CaSGS1-disrupt-F:

ATGATAAATAATTTGCAAGAACAAGTGGCTTGGGTGAAACGAACAAACCCT
CATATAACACCACAGGCCGTAATAGATT**GCATCAAGCTTGGTACCGAGC**

CaSGS1-disrupt-R:

CTTGGTATAGCTTGATTGAGTAGAACTTTTACTGGGCTTTGGCTGTGATGCC
GCCCGTAATGTATCCAATATCTCTTGA**CCCTCTAGATGCATGCTCGAG**

2.4 Phenotype assays

Growth rate determination was done using previously described protocols (Legrand et al., 2007). Doubling times were determined in two independent experiments using the following formula: Doubling time= ln2 x (t / (ln$b$ – ln$a$) where t is the time period in hours, a is the OD_{600} at the beginning of the time period and b is the OD_{600} at the end of the time period. The data are presented as means +/− one standard deviation.

Colony and cell morphologies were analyzed as previously described (Legrand et al., 2007). Cells were examined using a Nikon E600 microscope to determine cell morphology. Pictures of colonies were obtained with a Nikon CoolPIX900 camera attached to a Zeiss Stemi DRC microscope.

Antifungal drug resistance was monitored using E-test strips (AB Biodisk) on casitone+uri agar plates (0.5% Yeast Extract, 1% Sodium Citrate, 0.9% Bacto Casitone, 2% Glucose, 2% agar, 20mg/L Uridine), as previously described (Legrand et al., 2007).

2.5 DNA damaging agent sensitivity

All assays were conducted as previously described (Legrand et al., 2007). For sensitivity to oxidizing agents, YEPD+uri plates containing 4mM H_2O_2, 0.1mM Menadione or 2mM tetrabutyl hydrogen peroxide (TBHP), or 5ml YEPD+uri+4mM H_2O_2 liquid culture media were used. For ultraviolet radiation (UV) sensitivity, appropriate dilutions of cells on plates were irradiated with 3.2μW/cm^2 UV light for five seconds. For alkylating agent sensitivity, cells were placed on YEPD+uri plates containing 100μM Camptothecin, 0.03% ethylmethane sulfonate (EMS) or 0.01% methylmethane sulfonate (MMS) and incubated at

30°C for 24h. To examine sensitivity to hydroxyurea (HU), serial dilutions of cells were placed on YEPD+20mM hydroxyurea plates and scored for growth after two days' incubation at 30°C.

2.6 Chromosome 1 integrity

All protocols were performed as previously described (Legrand et al., 2007). 100,000 cells were plated on minimal + 2-deoxygalactose (2-DG) and minimal + 5-fluoroorotic acid (5-FOA) plates. Dilutions were also plated on YEPD+uri plates to confirm cell counts. The number of colonies on each plate was recorded on day 2 for the YEPD+uri plates and on day 3 for the 2-DG and 5-FOA plates. To characterize the spectrum of alterations leading to 2-DG and 5-FOA resistance, twenty 2-DG^R and twenty 5-FOA^R colonies were selected, genomic DNA was extracted, and screened by PCR to assess the presence/absence of the *GAL1* and *URA3* genes. The oligonucleotides CaGAL1+474-F, CaGAL1-256-R and CaURA3+386-R were used in the same PCR mix (Legrand et al., 2007). If the *GAL1* or *URA3* genes were still present in 2-DG^R cells or 5-FOA^R cells, respectively, the PCR product was sequenced, while if they were absent Single Nucleotide Polymorphism (SNP) analysis was used to determine the extent of the loss. SNPs 1322–2294 and F12n4 are located on chromosome 1, flanking the *GAL1*/*URA3* locus; both SNP sequences contain a restriction site polymorphism (Fig. 3). The regions containing the SNPs were amplified by PCR using the oligonucleotides AF-1322-2294-F/R and XU-F12n4-F/R (Legrand et al., 2007). The PCR product was digested with either *Bcc*I or *Hpa*II respectively and analyzed by gel electrophoresis, to determine the presence or absence of the restriction site.

3. Results

3.1 Sequence analysis

A BLAST search (TBLASTN) of the *C. albicans* database (http://www.candidagenome.org/cgi-bin/nph-blast) was performed using the *S. cerevisiae MEC1* and *SGS1* protein sequences as query. For both genes, a single homolog was detected. In pairwise BLAST comparisons, the matches have e-values of $2.5e^{-221}$ (*MEC1* – 26% amino acid identity) and $8.6e^{-196}$ (*SGS1* – 31% identity), suggesting that the *C. albicans* genes are likely to be orthologs of the *S. cerevisiae* DNA repair genes.

While Single Nucleotide Polymorphisms (SNPs) were not detected in the *SGS1* sequence (orf19.5335), the genome database revealed extensive heterozygosity between the two 6978bp *MEC1* alleles, encoded by orf19.1283 and orf19.8870. Fifty SNPs occur in the two alleles; nine alter the protein sequence. Because the sequences reported in the *C. albicans* database are from the wild-type strain SC5314, we determined the sequence of each *MEC1* allele in SN76, our parental strain (Noble and Johnson, 2005). The *MEC1* locus is found on chromosome 5 (Ch5), which also bears the heterozygous mating type locus (*MTL*). Previous studies have shown that homozygosis of the *MTL* locus can be induced upon growth on sorbose as sole source of carbon (Janbon et al., 1998; Magee and Magee, 2000). Following growth on sorbose, we identified *MTLa/a* and *MTLα/α* derivatives of our parental strain, and sequenced the *MEC1* locus in each derivative. Eight of the nine protein sequence altering SNPs reported in SC5314 are present in SN76 (Table 2). To determine if these variants have differing functions, we monitored sensitivity to DNA-damaging agents and Ch1 integrity in *MTLa/a* and *MTLα/α* derivatives of SN76. No observable phenotypic differences were detected (data not shown), indicating that the varying *MEC1* alleles (or other variant sequences on Ch5) do not possess differing functions.

3.2 Gene disruptions

To determine the role of *MEC1* and *SGS1* in the biology of *C. albicans*, we constructed disruptions of both genes. We were able to readily construct homozygous deletion mutants of both genes, demonstrating that neither *MEC1* nor *SGS1* are essential for viability of *C. albicans* cells. In addition to disrupting the entire ORF, we also constructed a partial disruption of the *CaMEC1* gene, removing the putative kinase domain. Previous studies demonstrated that two amino acids (D2224 and D2243) located in the Mec1p kinase domain are required for cell viability and for proper DNA damage response in *S. cerevisiae* (Paciotti et al., 2001). In *C. albicans*, these two residues are D2177 and D2196 (based on protein sequence alignment). This partial deletion mutant (a 900bp region encompassing these two amino acids) exhibited phenotypes identical to the complete deletion mutant.

3.3 Phenotypic analysis

Both the full-length deletion and internal deletion *mec1Δ/Δ* mutants, as well as the *sgs1Δ/Δ* mutants, exhibit a slow growth phenotype. The doubling times are presented in Table 3. The colony and cell morphologies of the *sgs1Δ/Δ* mutants differ from wild-type, unlike the *mec1Δ/Δ* mutants. The *sgs1Δ/Δ* mutants exhibit a wrinkly colony phenotype on YEPD+uri agar at 30°C. In YEPD+uri broth, the wild-type and *mec1Δ/Δ* cultures contain only yeast cells whereas the *sgs1Δ/Δ* cultures contain many elongated pseudohyphal-like cells in addition to yeast cells (Fig. 1). This phenotype is similar to one that has been previously described for DNA repair mutants (Ciudad et al., 2004; Legrand et al., 2007). The reduction in growth rate in the *MEC1* mutants is not due to excessive cell death – viable counts of the parental and *mec1* mutants were comparable, and colonies of the mutant strains did not exhibit the ragged-edge colony morphology that is indicative of ongoing cell death. Loss of *SGS1* activity interfered with successful integration of a wild-type copy of the *SGS1* gene at the native locus. Transformation frequency was reduced, and the few potential transformants obtained did not integrate the wild-type copy at the native locus as determined by PCR.

3.4 Sensitivity to DNA-damaging agents

In *S. cerevisiae*, Mec1p and Sgs1p are involved in DNA damage sensing and signal transduction cascades in response to DNA damage, resulting in cell cycle arrest to allow DNA repair. We tested the response of the *mec1Δ/Δ* and *sgs1Δ/Δ* mutants to several DNA-damaging agents, including the oxidizing agent tetrabutyl hydrogen peroxide (TBHP), ultraviolet (UV) radiation, and alkylating agents, as well as compounds known to induce double-strand breaks (EMS, MMS and Camptothecin) and interfere with replication (HU). The *sgs1Δ/Δ* mutants show a high sensitivity to all DNA-damaging agents while the *mec1Δ/Δ* mutants are only sensitive to EMS, MMS and HU (Fig. 2a–d).

3.5 Chromosome instability assays

Mec1p and Sgs1p are genome integrity checkpoint proteins. In order to investigate their roles in maintenance of genome stability in *C. albicans*, we monitored Ch1 integrity in the deletion mutants using a *GAL1/URA3* marker system (Legrand et al., 2007). The diploid parental strain used to construct the checkpoint mutants has one copy of the *GAL1* locus on chromosome 1 replaced with the *URA3* gene. Loss of the *GAL1* gene (due to point mutation, gene conversion, segmental/total chromosome loss or break induced replication) renders the cells resistant to 2-deoxygalactose (2-DG), while loss of *URA3* confers resistance to 5-fluoroorotic acid (5-FOA).

Initially, the frequency of appearance of 2-DG^R and 5-FOA^R colonies was compared in the parental strain and the mutant strains, by growing 100,000 cells on 2-DG and 5-FOA. This first assay showed an increase in the frequency of appearance of 2-DG^R and 5-FOA^R

colonies in the *mec1Δ/Δ* mutants as compared to the parental strain, indicating an increase in Ch1 instability (Table 4). There was a 68-fold and 11-fold average increase for the rate of appearance of 2-DG^R and 5-FOA^R colonies, respectively. The *sgs1Δ/Δ* strain did not exhibit any change in genome stability by this assay (Table 4).

We further investigated the mechanism by which the cells were acquiring 2-DG or 5-FOA resistance in the parental strain and mutant strains. PCR across the *GAL1*/*URA3* locus using primers in flanking unique DNA was used to determine the presence or absence of each gene. We next performed SNP analysis on those isolates in which the gene was not detected by PCR, using restriction site polymorphisms present in the 1S region (1322/2294 SNP) and 1J region (F12n4 SNP) of chromosome 1 (Fig. 3). Loss Of Heterozygosity (LOH) of both SNPs would suggest loss of the whole chromosome, loss of only 1322/2294 heterozygosity suggests that a large portion of the left arm of chromosome 1 had undergone a LOH event, while continued heterozygosity at both SNPs would suggest a localized gene conversion event. Characterization of the 2-DG^R and 5-FOA^R colonies in the *mec1Δ/Δ* and *sgs1Δ/Δ* mutants showed that 100% of the 2-DG^R and 5-FOA^R colonies in the parental strain and the deletion mutants lost either the *GAL1* or *URA3* genes. SNP typing showed LOH only for the left arm of chromosome 1 (Fig. 3), not the complete chromosome, indicating that the affected area was larger than is typical for a simple gene conversion event, but not as large as the whole chromosome. These events may be due to either crossover events, some form of break-induced replication (BIR), or chromosomal truncations (as recently demonstrated in *rad52*Δ/Δ strains (Andaluz et al., 2011)).

3.6 Antifungal drug sensitivity

Genome rearrangements are often observed in clinical isolates that have acquired resistance to the antifungal drug fluconazole. To determine if Mec1p or Sgs1p play a role in acquisition of drug resistance, we tested the sensitivity of the *mec1Δ/Δ* and *sgs1Δ/Δ* mutants to fluconazole using an E-test assay. The MICs of the *mec1Δ/Δ* and *sgs1Δ/Δ* mutants were determined after 48h exposure to fluconazole E-test strips. The *mec1Δ/Δ* mutants have a MIC of 0.75ug/ml, identical to the parental strain, whereas the *sgs1Δ/Δ* mutants are more sensitive to fluconazole, with a MIC of 0.19ug/ml (Fig. 4).

Prior work from our group demonstrated that strains bearing mutations in mismatch repair (MMR) or double-strand break repair (DSBR) genes give rise to fluconazole-resistant isolates at a much higher frequency than the parental strain (Legrand et al., 2007). These resistant colonies grow in the cleared inhibition ellipse halo surrounding the E-test strip. In this study, we observed no resistant colonies in the *sgs1*Δ/Δ and *mec*Δ/Δ strains (data not shown), indicating that the increased DNA damage occurring in the *sgs1*Δ/Δ strains and the increased genome instability observed in the *mec1*Δ/Δ strains are not leading to DNA alterations that provide fluconazole resistance to the cells.

4. Discussion

C. albicans possesses single homologs of *MEC1* and *SGS1*. For *MEC1*, the sequences of the two diploid alleles exhibit an elevated amount of heterozygosity, which could lead to the expression of two Mec1 proteins with slightly different functions (Table 2). To investigate this hypothesis, we examined sensitivity to DNA-damaging agents and changes in Ch1 integrity in *MTLa/a* and *MTLα/α* derivatives, as *MEC1* is located on chromosome 5. No phenotypic differences were uncovered, indicating that the *MEC1* alleles (or other variable Ch5 sequences) do not differ in function. However, it is possible that the difference might only be relevant *in vivo*, in response to host factors or specific treatments. The CaSgs1p sequence revealed strong sequence conservation with other RecQ helicases within a central

helicase region of 350–400 amino acids. No amino acid differences were detected between the two *SGS1* alleles.

MEC1 is not essential for viability in *C. albicans*, as we were able to generate *mec1Δ/Δ* null mutants, in contrast with the essential role of *MEC1* in *S. cerevisiae* (Giaever et al., 2002). Several studies have shown that the lethality of the *mec1Δ/Δ* mutants in *S. cerevisiae* can be rescued by increasing the dNTP pool, which can be achieved by overexpression of the ribonucleotide reductase (RNR) genes (Desany et al., 1998), by deletion of the RNR inhibitor *SML1* (Zhao et al., 2001; Zhao et al., 2000; Zhao et al., 1998) or by removal of the RNR transcriptional repressor *CRT1* (Huang et al., 1998). Wild-type ScMec1p helps to maintain a high level of deoxyribonucleotides during DNA replication and in response to DNA damage by removal of the ribonucleotide reductase inhibitor Sml1p. A TBLASTN search of the *C. albicans* database (www.candidagenome.org/cgi-bin/compute/blast-sgd.pl) using the *S. cerevisiae* Sml1 protein sequence detects a single weak potential homolog, orf19.5642, with an evalue of 1.1e-05. If this is a functional homolog of the *S. cerevisiae SML1* protein, it apparently does not interact with CaMec1p in the same manner as the *S. cerevisiae* Sml1p interacts with ScMec1p; this could explain why *MEC1* is not essential in *C. albicans*. While *MEC1* homologs have been shown to be essential for cell viability in *Aspergillus nidulans*, *S. cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster*, the human *MEC1* homolog *ATM* is not essential for viability (reviewed in Schultz et al., 2000), indicating that the *C. albicans MEC1* system may be closer functionally to the human system than other model organisms.

The diploid *sgs1Δ/Δ* mutants exhibit a slow growth phenotype. In *S. cerevisiae*, several studies have shown that *sgs1* mutants divide with wild-type kinetics (Gangloff et al., 2000; Lu et al., 1996) in haploid cells, although a study by Deutschbauer *et al.* demonstrated that homozygous deletions of *ScSGS1* in diploid cells cause a slow growth phenotype (Deutschbauer et al., 2005). The differing phenotypes suggest a ploidy effect that might be explained by the role that Sgs1p plays in resolving structures during homologous recombination. These observations agree with the slow growth phenotype exhibited by our *sgs1*-deficient cells in the diploid *C. albicans*. Finally, the *sgs1Δ/Δ* mutants produce wrinkled colonies with a high proportion of pseudohyphal-like cells. Previous studies have shown that genotoxic stresses (treatments that impair genome integrity) trigger polarized growth in *C. albicans* and other fungi (Enserink et al., 2006; Malavazi et al., 2006; Shi et al., 2007). The cell morphology phenotype observed in *sgs1Δ/Δ* mutants suggests that the absence of Sgs1p leads to DNA lesions that mimic genotoxic stress and trigger polarized growth.

The *mec1Δ/Δ* mutants also exhibited a slow growth phenotype (Table 3). As Mec1p acts as a checkpoint, triggering cell cycle arrest if any damage is detected, one might expect the *mec1Δ/Δ* mutants to exhibit wild-type or shorter doubling times due to the loss of this inhibiting activity. The longer doubling times we observed in *mec1Δ/Δ* mutants could be explained by an increase in the proportion of dead cells, resulting from a lack of DNA repair and a failure to arrest the cell cycle in the absence of Mec1p. However, the *mec1Δ/Δ* mutants produce smooth colonies that look identical to wild-type colonies, whereas strains with high levels of cell death often form colonies with ragged edges. Also, we did not observe significant differences in viable counts of parental wild-type and *mec1* mutant strains. Finally, when *mec1*Δ/Δ cells are examined microscopically, the majority resemble yeast cells, with a small fraction appearing to be pseudohyphal. The low frequency of pseudohyphal-like cells in *mec1Δ/Δ* cultures suggests that the DNA lesions occurring in this background do not constitute a genotoxic stress, or that Mec1p plays a central role in the pathway that triggers polarized growth in response to genotoxic stress. Loss of recombination and DNA damage proteins has been linked to excess filamentation by a

number of research groups previously (Andaluz et al., 2006; Enserink et al., 2006; Legrand et al., 2007; Malavazi et al., 2006; Shi et al., 2007); our data provide support for the hypothesis that the *MEC1* DNA damage checkpoint is involved in filamentation.

As shown in Figure 2a–d, the *sgs1Δ/Δ* mutants show a high sensitivity to TBHP, UV light and DNA-break inducing agents including camptothecin, EMS and MMS. The *sgs1* strains also show extreme sensitivity to HU (Figure 2d), as reported for deletions of *S. cerevisiae SGS1* (Miyajima et al., 2000; Onoda et al., 2000; Saffi et al., 2000; Yamagata et al., 1998). These observations suggest that any kind of DNA damage is detrimental in *sgs1Δ/Δ* cells. In contrast, the *mec1Δ/Δ* mutants are very sensitive to EMS, MMS and HU, but resistant to camptothecin. The alkylating agents MMS and EMS modify DNA by adding methyl or alkyl groups to bases, while camptothecin inhibits topoisomerase I after DNA cleavage, trapping the enzyme while it is covalently attached to the DNA. Although the direct DNA lesions caused by EMS/MMS or camptothecin are different (alkylated base vs DNA nicks), it is assumed that a common indirect consequence of camptothecin, EMS, MMS or HU treatment is DNA double-strand breaks. However, the comparison of the *mec1Δ/Δ* and *sgs1Δ/Δ* data demonstrate differences in the way cells behave in response to each treatment, implying that the DNA repair intermediates leading to double-strand breaks are different in HU-, EMS- or MMS-treated cells as compared to the ones generated in camptothecin-treated cells.

Deletion of *MEC1* gives rise to an increased frequency of 2-DG^R or 5-FOA^R colonies (with average increases of 68-fold and 11-fold, respectively) compared to the parental strain, using the *GAL1/URA3* assay system on chromosome 1. These increases are the largest seen in this assay for any of the mutants that we have tested to date; previously, mutations in the *RAD50* and *MRE11* double-strand break repair genes exhibited the largest increases, with changes of 11x and 16x for 2-DG resistance and 8x for 5-FOA resistance respectively (Legrand et al., 2007; Legrand et al., 2008). A more detailed analysis of the *mec1*Δ/Δ 2-DG^R or 5-FOA^R cells revealed that loss of *GAL1* or *URA3* function is the result of Loss Of Heterozygosity (LOH) events. We observed a similar pattern of LOH events in the 2-DG^R and 5-FOA^R colonies from the parental strain, but at a much lower frequency (Table 4). This result leads to the possibility that the events that might generate an LOH event are usually detected and repaired in the *MEC1* parental strain, but loss of the *MEC1*-dependent checkpoint greatly increases either their incidence, or decreases the likelihood of repair. We favor the latter interpretation. We previously identified a similar effect in the *GAL1*/*URA3* assay in *MRE11* and *RAD50* mutants (Legrand et al., 2007), in which deletion of either gene led to an increase in overall LOH frequency, without an alteration in the spectrum of changes detected. One model that is consistent with our results and the known activities of these proteins is that the *MEC1*-dependent pathway mediates repair, through the *RAD50* and *MRE11*-dependent DSBR pathway, of DNA damage that can generate LOH events. However, the higher level observed in the *mec1* mutant relative to the *rad50*Δ/Δ or *mre11*Δ/Δ mutants indicates that there is a second repair activity that is still active in the DSBR mutants, and is affected by the loss of *MEC1*.

The *GAL1*/*URA3* assay in the wild-type parental strain consistently shows a bias towards events that lead to a loss of *GAL1* (Table 4). This bias is maintained in the *MEC1* and *SGS1* mutants, and was previously seen in other mutants as well (e.g. *rad50*Δ/Δ, *mre11*Δ/Δ, *rad2*Δ/Δ and *ntg1*Δ/Δ mutants (Legrand et al., 2007; Legrand et al., 2008)). One explanation for this bias would be an excess of double-strand breaks forming on the *URA3*-bearing homolog, as these DSBs would be repaired using the *GAL1* chromosome as the template. In keeping with this interpretation, we note that the *S. cerevisiae URA3* gene exhibits meiotic DSB formation when placed in an ectopic location (Kearney et al., 2001).

No change in genome stability was detected in the *GAL1*/*URA3* assay with *sgs1Δ/Δ* mutants. Other researchers have suggested that the *S. cerevisiae* intra-S checkpoint might be divided into two branches that function in parallel (Myung and Kolodner, 2002), with one branch dependent on Rad24p and the other dependent on Sgs1p. This redundancy could explain our *sgs1Δ/Δ* results in *C. albicans* - defects in only one branch might cause little or no increase in the GCR rate. Alternatively, it is possible that the genomic events occurring in *sgs1Δ/Δ* cells that would lead to acquisition of 2-DG or 5-FOA resistance are lethal in these cells.

E-test assays showed that loss of Sgs1p leads to an increased susceptibility to the antifungal drug fluconazole, while the *mec1Δ/Δ* mutants do not appear to have any alteration in their overall sensitivity to fluconazole. Surprisingly, we have not detected any increase in the appearance of drug resistant colonies in the *mec1*Δ/Δ or *sgs1*Δ/Δ mutants. Prior studies with DNA repair mutants showed a correlation between elevated levels of genome instability, DNA double-strand break repair defects, and increased acquisition of antifungal drug resistance (Legrand et al., 2007; Legrand et al., 2008). The *MEC1* and *SGS1* data suggest that either the genome changes leading to acquisition of drug resistance might still be properly repaired in *mec1*Δ/Δ and *sgs1*Δ/Δ cells, or that they could be too detrimental to survival and trigger cell death. While the slow-growth phenotype exhibited by both mutants supports the latter idea, the wild-type viability and the smooth colony morphology of the *mec1*Δ/Δ strains are not consistent with this interpretation. Alternatively, as the *MEC1* protein monitors events that occur during the cell cycle, it is possible that events that generate antifungal resistance arise in cells that are not actively going through a cell cycle, and thus are not normally affected by the *MEC1* checkpoint. No matter the explanation, our data clearly indicate that the connections between genome stability, DNA damage repair, and antifungal drug resistance are complex in nature, and correlations between these phenotypes need to be carefully evaluated.

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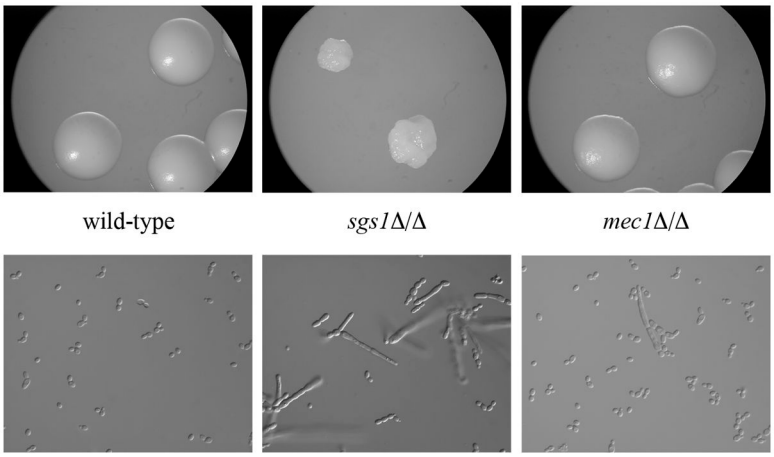

Figure 1. Colony and cellular morphology of parental strain DKCa39 and *mec1*Δ/Δ and *sgs1*Δ/Δ mutants

Top panel: Cells were incubated on YEPD+uri plates at 30°C for 48h. The parental strain (DKCa39) and the *mec1*Δ/Δ strain (DKCa596) have normal colony morphologies; the *sgs1*Δ/Δ mutant (DKCa636) exhibits a rough colony morphology. Bottom panel: Cells from an overnight liquid YEPD+uri culture at 30°C were examined under a Nikon E600 microscope. The majority of the *sgs1*Δ/Δ cells have an abnormal elongated pseudohyphal-like cell shape.

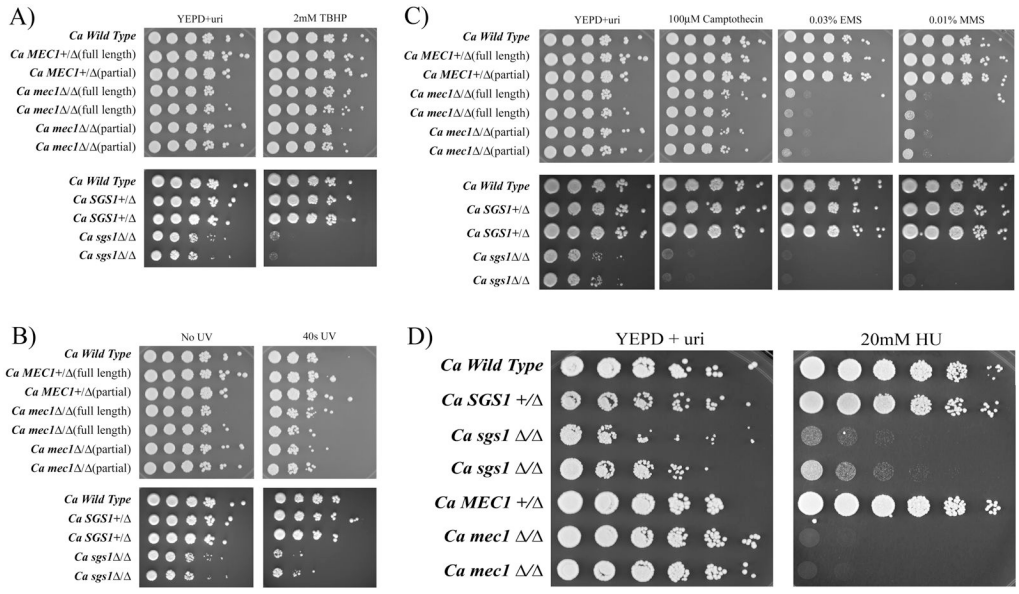

Figure 2.

Phenotypic analysis of *mec1*Δ/Δ and *sgs1*Δ/Δ mutants For all assays, cells from an overnight liquid YEPD+uri culture were serially diluted and spotted onto YEPD+uri plates with or without appropriate compound/condition, and incubated at 30°C for 48h. A) Sensitivity of *mec1*Δ/Δ and *sgs1*Δ/Δ mutants to oxidizing agents. YEPD+uri plates contained 2mM TBHP. B) Sensitivity of *mec1*Δ/Δ and *sgs1*Δ/Δ mutants to UV light. Plates were irradiated with 3.2μW/cm2 UV light for 40s, wrapped in foil and incubated. C) Sensitivity of *mec1*Δ/Δ and *sgs1*Δ/Δ mutants to various alkylating agents and a type 1 topoisomerase inhibitor. YEPD+uri plates contained 100μM camptothecin, 0.03% EMS or 0.01% MMS. D) Sensitivity of *mec1*Δ/Δ and *sgs1*Δ/Δ mutants to hydroxyurea. YEPD+uri plates contained 20mM HU. Only the full-length *mec1*Δ/Δ deletion strain is shown.

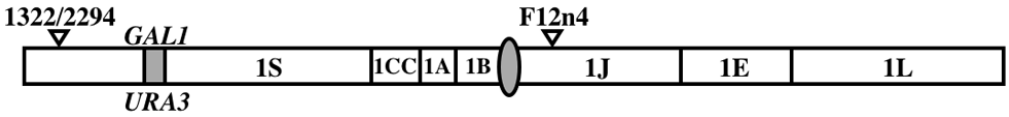

Figure 3. Chromosome 1 SNP locations

This diagram shows the position of the SNPs 1322/2294 and F12n4 relative to the *GAL1* locus. SNP 1322/2294 is located in the middle of a *Bcc*I restriction site; one of the alleles (CC**A**TCA) contains the *Bcc*I site while the other (CC**C**TCA) does not. SNP F12n4 is located in the middle of an *Hpa*II restriction site; one of the alleles (ATC**C**GG) has the *Hpa*II site while the other (ATC**T**GG) lacks it.

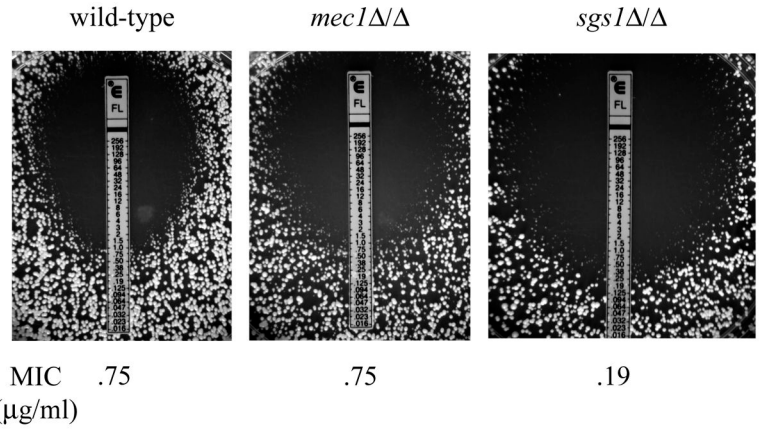

Figure 4. Sensitivity of *mec1*Δ/Δ and *sgs1*Δ/Δ mutants to fluconazole

Fluconazole (FL) E-tests on the parental strain (DKCa39) and the *mec1*Δ/Δ (DKCa596) and *sgs1*Δ/Δ (DKCa635) mutants. The minimum inhibitory concentration (MIC) is given based on the scale of fluconazole concentration printed on the E-test strip (in μg/ml).

Table 1

Candida albicans strains used in this study

Strain:	Derived From:	Phenotype:	Relevant Genotype [a]
SN76		ura$^-$ his$^-$ arg$^-$	*arg4 / arg4; his1 / his1; ura3::imm^{434} / ura3::imm^{434}; iro1::imm^{434}/ iro1::imm^{434}*
DKCa39	SN76	his$^-$ arg$^-$	*GAL1 / gal1Δ::URA3*
DKCa536[b]	DKCa39	arg$^-$	*mec1Δ::C.d.HIS1 / MEC1*
DKCa543[c]	DKCa39	arg$^-$	*mec1Δ::C.d.HIS1 / MEC1*
DKCa596[b]	DKCa536	prototroph	*mec1Δ::C.d.HIS1 / mec1Δ::C.d.ARG4*
DKCa597[b]	DKCa536	prototroph	*mec1Δ::C.d.HIS1 / mec1Δ::C.d.ARG4*
DKCa598[c]	DKCa543	prototroph	*mec1Δ::C.d.HIS1 / mec1Δ::C.d.ARG4*
DKCa599[c]	DKCa543	prototroph	*mec1Δ::C.d.HIS1 / mec1Δ::C.d.ARG4*
DKCa514	SN76	ura$^-$ his$^-$	*sgs1Δ::C.d.ARG4 / SGS1*
DKCa515	SN76	ura$^-$ his$^-$	*sgs1Δ::C.d.ARG4 / SGS1*
DKCa626	DKCa514	ura$^-$	*sgs1Δ::C.d.HIS1 / sgs1Δ::C.d.ARG4*
DKCa627	DKCa515	ura$^-$	*sgs1Δ::C.d.HIS1 / sgs1Δ::C.d.ARG4*
DKCa635	DKCa626	prototroph	*GAL1 / gal1Δ::URA3*
DKCa636	DKCa627	prototroph	*GAL1 / gal1Δ::URA3*
DKCa637	DKCa627	prototroph	*GAL1 / gal1Δ::URA3*
DKCa638	DKCa627	prototroph	*GAL1 / gal1Δ::URA3*

[a] C.d. = *C. dubliniensis* gene

[b] Full length disruption of the *MEC1* ORF

[c] Partial disruption of the *MEC1* ORF

Table 2

Amino acid changes between the two Mec1p alleles (2326 aa).

Mec1a		Mec1b	
nucleotide	**amino acid**	**nucleotide**	**amino acid**
2407 A	803 Ile	2407 G	803 Val
2743 A	915 Asn	2743 G	915 Asp
3323 T	1108 Leu	3323 A	1108 Gln
3509 C	1170 Thr	3509 T	1170 Ile
3755 A	1252 Tyr	3755 G	1252 Cys
4568 C	1523 Thr	4568 T	1523 Ile
4801 C	1601 Gln	4801 A	1601 Lys
5695 C	1899 Leu	5695 T	1899 Phe

Table 3

Doubling times of the *C. albicans* checkpoint mutants.

Strain	Genotype	Mutation	Doubling time (hr)
DKCa39	WT		1.19±0.06
DKCa536	*mec1Δ/+*	Full-length	1.31±0.04
DKCa543	*mec1Δ/+*	Partial	1.35±0.04
DKCa596	*mec1Δ/Δ*	Full-length	**1.53±0.04**
DKCa597	*mec1Δ/Δ*	Full-length	**1.56±0.08**
DKCa598	*mec1Δ/Δ*	Partial	**1.5±0.07**
DKCa599	*mec1Δ/Δ*	Partial	**1.38±0.1**
DKCa39	WT		1.13±0.04
DKCa635	*sgs1Δ/Δ*	Full-length	**1.83±0.28**
DKCa636	*sgs1Δ/Δ*	Full-length	**1.81±0.36**
DKCa637	*sgs1Δ/Δ*	Full-length	**1.77±0.03**
DKCa638	*sgs1Δ/Δ*	Full-length	**1.72±0.23**

Table 4

Appearance of 2-DG^R and 5-FOA^R colonies in the *C. albicans mec1* and *sgs1* mutants after three days.

		2-DG^R colonies		5-FOA^R colonies:	
Strain	**Mutation**	**Appearance**	**Fold change**	**Appearance**	**Fold change**
DKCa39		1.2×10^{-4}	-	3.7×10^{-5}	-
DKCa596	*mec1*Δ/Δ	9.8×10^{-3}	82×	3.3×10^{-4}	9×
DKCa598	*mec1*Δ/Δ	8.1×10^{-3}	67.5×	3.5×10^{-4}	9×
DKCa599	*mec1*Δ/Δ	6.4×10^{-3}	53×	5.5×10^{-4}	15×
DKCa39		3.8×10^{-4}		2.3×10^{-4}	
DKCa635	*sgs1*Δ/Δ	2.5×10^{-4}	0.7×	1.2×10^{-4}	0.5×
DKCa636	*sgs1*Δ/Δ	1.4×10^{-4}	0.4×	1.4×10^{-5}	0.6×
DKCa637	*sgs1*Δ/Δ	9.3×10^{-5}	0.2×	1.5×10^{-5}	0.7×
DKCa638	*sgs1*Δ/Δ	2.3×10^{-4}	0.6×	8×10^{-4}	3.5×

Each value is the average of at least 2 independent experiments