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Mms22p protects *Saccharomyces cerevisiae* from DNA damage induced by topoisomerase II

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ABSTRACT

The cleavage reaction of topoisomerase II, which creates double-stranded DNA breaks, plays a central role in both the cure and initiation of cancer. Therefore, it is important to understand the cellular processes that repair topoisomerase II-generated DNA damage. Using a genome-wide approach with *Saccharomyces cerevisiae*, we found that Δ*mre11*, Δ*xrs2*, Δ*rad50*, Δ*rad51*, Δ*rad52*, Δ*rad54*, Δ*rad55*, Δ*rad57* and Δ*mms22* strains were hypersensitive to etoposide, a drug that specifically increases levels of topoisomerase II-mediated DNA breaks. These results confirm that the single-strand invasion pathway of homologous recombination is the major pathway that repairs topoisomerase II-induced DNA damage in yeast and also indicate an important role for Mms22p. Although Δ*mms22* strains are sensitive to several DNA-damaging agents, little is known about the function of Mms22p. Δ*mms22* cultures accumulate in G_2/M, and display an abnormal cell cycle response to topoisomerase II-mediated DNA damage. *MMS22* appears to function outside of the single-strand invasion pathway, but levels of etoposide-induced homologous recombination in Δ*mms22* cells are lower than wild-type. *MMS22* is epistatic with *RTT101* and *RTT107*, genes that encode its protein binding partners. Finally, consistent with a role in DNA processes, Mms22p localizes to discrete nuclear foci, even in the absence of etoposide or its binding partners.

INTRODUCTION

In order for an organism to survive, it must be able to withstand an array of challenges that damage its genetic material. It has long been known that environmental events can trigger the loss of bases, the formation of DNA adducts or the generation of DNA strand breaks (1–4). More recently, it has become clear that many normal cellular processes also have the capacity to destabilize the genome (5). For example, lipid peroxidation products and radicals generated by oxidative phosphorylation damage bases and induce DNA strand breaks. In addition, enzymes involved in DNA replication and recombination can incorporate incorrect bases, or create chromosomal translocations, insertions or deletions.

Of the enzymes involved in ongoing DNA processes, topoisomerase II potentially is the most lethal (6–13). This essential enzyme is required to remove knots and tangles from the genome (10,14,15). It acts by passing an intact DNA double helix through a transient double-stranded break that it generates in a separate segment of DNA (6–8,11,16). In order to maintain the integrity of the genome during the DNA strand passage event, topoisomerase II forms covalent bonds between active site tyrosyl residues and the newly created 5′-termini of the cleaved DNA (17–19). These covalent protein–DNA complexes are referred to as cleavage complexes. If a cleavage complex is encountered by a DNA tracking system such as a polymerase or a helicase, the ensuing collision converts this transient protein–DNA complex (by a process that is not yet fully understood) to a permanent DNA strand break (20–24). Since topoisomerase II cleavage complexes normally are fleeting catalytic intermediates and are present in low concentrations, they are tolerated by the cell. However, conditions that increase either the concentration or lifetime of these complexes convert topoisomerase II into a potent cellular toxin that fragments the genome (8,25).

The potentially lethal nature of topoisomerase II has been exploited to treat a number of human cancers. Drugs such as etoposide target the enzyme and kill cells by dramatically increasing physiological levels of cleavage complexes (26,27). Because of their mechanism of action, these anticancer agents are known as topoisomerase II poisons to distinguish them from drugs that act by inhibiting the overall catalytic activity of the enzyme (6).

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Despite the importance of topoisomerase II in cancer chemotherapy, there is mounting evidence that the enzyme triggers chromosomal breaks that result in specific leukemias. A small proportion of patients who receive topoisomerase II-targeted drugs as part of their treatment subsequently develop secondary leukemias with characteristic breakpoints in the *MLL* gene at chromosomal band 11q23 (13,28–30). Infant and adult leukemias that display 11q23 rearrangements also have been correlated to exposure to naturally occurring or environmental topoisomerase II poisons (13,29,31–33).

Because the type II enzyme plays an important role in both the cure and the generation of cancer, it is important to understand the processes by which cells protect themselves from topoisomerase II-mediated DNA damage. A previous study utilized *Saccharomyces cerevisiae* as a model system to identify the recombination pathways that repair DNA strand breaks that are generated by topoisomerase II (34). Etoposide-induced cytotoxicity and DNA recombination were monitored in a series of mutant strains that were singly deleted for genes in known recombination repair pathways. Results of this work suggested that the single-strand invasion pathway of homologous recombination plays a major role in repairing topoisomerase II-mediated DNA breaks (34).

Because the previous study investigated only known recombination pathways, it is possible that other mechanisms also help to protect cells from the damaging actions of topoisomerase II. Therefore, the repair of topoisomerase II-mediated DNA damage in yeast was reinvestigated using a genome-wide approach. A *S.cerevisiae* haploid deletion library containing ~4800 isogenic strains (35) was screened for hypersensitivity to etoposide.

Results confirm the importance of the single-strand invasion pathway of homologous recombination. In addition, *MMS22* was found to play a significant role in protecting yeast from topoisomerase II-mediated DNA damage. Δ*mms22* strains were ~10-fold hypersensitive to topoisomerase II poisons. Further studies indicate that Mms22p acts outside of the single-strand invasion pathway, and is a nuclear protein that localizes at discrete foci.

MATERIALS AND METHODS

Materials

Etoposide and amsacrine were obtained from Sigma, prepared as 20 mM solutions in 100% DMSO, and stored at room temperature. Growth media were prepared using standard protocols.

Yeast strains and plasmids

Other than the initial screen for etoposide sensitivity (see following section), all cellular studies employed *S.cerevisiae* strains that carried the JN362acc background (MATa *ura3-52 leu2 trp1 his7 ade1-2 ISE2 can1 cyh2*) (26,36) (Table 1). For homologous recombination assays, a JN362acc strain containing the *top2S740W* allele in place of the *TOP2* gene was used (34). Deletion mutants were generated using one-step gene replacement (37) and were confirmed by PCR of genomic DNA. Genomic DNA was prepared using a MasterPure Yeast DNA Purification Kit (Epicentre). *MMS22* was cloned using PCR primers ~250 bp upstream and downstream of the coding region. The clone was then inserted via SacI/KpnI sites into the multiple cloning site of vector pRS416 to create the vector pMMS22. The recombination reporter plasmid YCpHR has been described previously (34,38).

Table 1. *Saccharomyces cerevisiae* strains

Strain[a]	Genotype	Strain origin
JN362acc	MATa *ura3-52 leu2 trp1 his7 ade1-2 ISE2 can1 cyh2*	keda, derived from Nitiss *et al.* (26,36)
MS001	*top2S740W*	Sabourin *et al.* (34)
MS111c	*rad52::TRP*	Sabourin *et al.* (34)
EB001	*mms22::KAN*	This study
EB002	*rad54::TRP*	This study
EB003	*mms22::KAN rad54::TRP*	This study
EB004	*top2S740W mms22::KAN*	This study
EB005	*rtt101::KAN*	This study
EB006	*rtt107::KAN*	This study
EB007	*mms22::HYG rtt101::KAN*	This study
EB008	*mms22::TRP rtt107::KAN*	This study
EB009	*rtt101::KAN rtt107::HYG*	This study
EB010	*mms22::TRP rtt101::KAN rtt107::HYG*	This study

[a]All strains are isogenic to JN362acc except where noted.

Etoposide-sensitivity screen

A MATa (BY4741) haploid *S.cerevisiae* deletion library generated by the *Saccharomyces* Gene Deletion Project (35) was screened for sensitivity to etoposide. Strains in the library were thawed and plated onto YPD medium containing drug solvent (DMSO) or 1 mM etoposide. Plates were incubated at 30°C and drug sensitivity was determined by cell density. Strains that displayed high sensitivity to etoposide were confirmed by spotting serial dilutions to medium containing DMSO or 1 mM etoposide.

Drug cytotoxicity assays

JN362acc yeast strains (~$1–2 \times 10^6$ cells/ml) were incubated in YPD or selective medium (to maintain plasmids) with 0–200 μM etoposide or 0–150 μM amsacrine for 8 or 24 h. Cells were plated in triplicate to corresponding medium solidified with 1.5% Bacto-agar and incubated at 30°C for 3–4 days to visualize colonies. Drug sensitivity was monitored by counting surviving colonies. For plate assays, cells were spotted in 10-fold serial dilutions to media containing DMSO or the indicated topoisomerase II poison.

FACS analysis of yeast

Wild-type and Δ*mms22* strains were grown in the presence of DMSO or 50 μM etoposide for 6 h. Cells were fixed with 100% ethanol, resuspended in 50 mM sodium citrate (pH 7.0) containing 0.08 mg/ml RNase A and incubated for 1 h at 50°C. Proteinase K (0.25 mg/ml) was added and the mixture was incubated for 1 h at 50°C. Cells were stained with 1 μM Sytox Green (Molecular Probes) in 1 ml of 50 mM sodium citrate (pH 7.0) for 1 h in the dark at room temperature. DNA content was measured on a Becton Dickinson FACScan.

Determination of homologous recombination frequency

Homologous recombination frequency was determined as previously described (34). Briefly, strains transformed with

YCpHR (recombination reporter plasmid) were grown overnight and diluted to 2×10^6 cells/ml. Cultures were grown for 5 h in the presence of DMSO or 50–200 μM etoposide, and dilutions were plated in triplicate on SC-URA/ARG medium to assess total cell viability or on SC-URA/ARG + 60 μg/ml canavanine for selection of the recombined plasmid. Recombined plasmids were analyzed by growing single colonies from SC-URA/ARG + canavanine plates to confluency. To confirm that canavanine resistance resulted from a homologous recombination event, plasmids were rescued into *Escherichia coli* using the EZ Yeast Plasmid Prep Kit (Geno Technology, Inc.). The resulting *E.coli* transformants were isolated, plasmid DNA was purified and plasmids were digested with PstI.

GFP-Mms22p localization

An N-terminal GFP-Mms22p fusion was constructed by cloning PCR-amplified *MMS22* into the pGFP-N-FUS vector at SmaI/XhoI sites (39). The resulting fusion protein was expressed under the control of the *MET25* promoter, therefore cells were grown in SC-URA/MET medium to maintain the plasmid and to induce the promoter. Δ*mms22*, Δ*mms22* Δ*rtt101*, Δ*mms22* Δ*rtt107* and Δ*mms22* Δ*rtt101* Δ*rtt107* strains were transformed with pGFP-N-FUS or pGFP-MMS22. Cells were grown overnight and examined for fluorescence through a GFP optimized filter (Chroma Technology) on an Olympus BX60 microscope equipped with a Photometrics Quantix digital camera. DNA was visualized using Hoechst stain.

RESULTS

Double-stranded breaks in the genetic material are repaired primarily by DNA recombination pathways. The most common pathways used by the budding yeast, *S.cerevisiae*, are depicted in Figure 1 (40–42). The initial processing of double-stranded DNA breaks generally relies on the Rad50p/Mre11p/Xrs2p complex to generate single-stranded ends at the site of the break (40–43). Following this processing, the DNA can be shuttled into three well-characterized recombination pathways (40–42). The break can be repaired by the single-strand invasion pathway of homologous recombination. This pathway, which utilizes Rad51p/52p/54p/55p/57p as well as the replication machinery, is capable of repairing the initial double-stranded DNA break in an error-free manner. Alternatively, the break can be repaired by the single-strand annealing pathway of homologous recombination. This pathway is dependent on the presence of direct repeats (or closely related sequences) proximal to and flanking the initial break site. It relies on Rad52p and the Rad1p/Rad10p endonuclease. Single-strand annealing is not an error-free pathway and deletes one of the repeated sequences, as well as the genetic information that is located between them. Finally, the break can be rejoined by the nonhomologous end-joining pathway (40,41,44,45). This pathway utilizes Ku70p/Ku80p and Lig4p, and results in the loss of sequences proximal to the original DNA break. If multiple breaks are present in the genome, nonhomologous end-joining can lead to the formation of chromosomal rearrangements or translocations. In general, homologous recombination pathways are considerably more active than nonhomologous end-joining in *S.cerevisiae*.

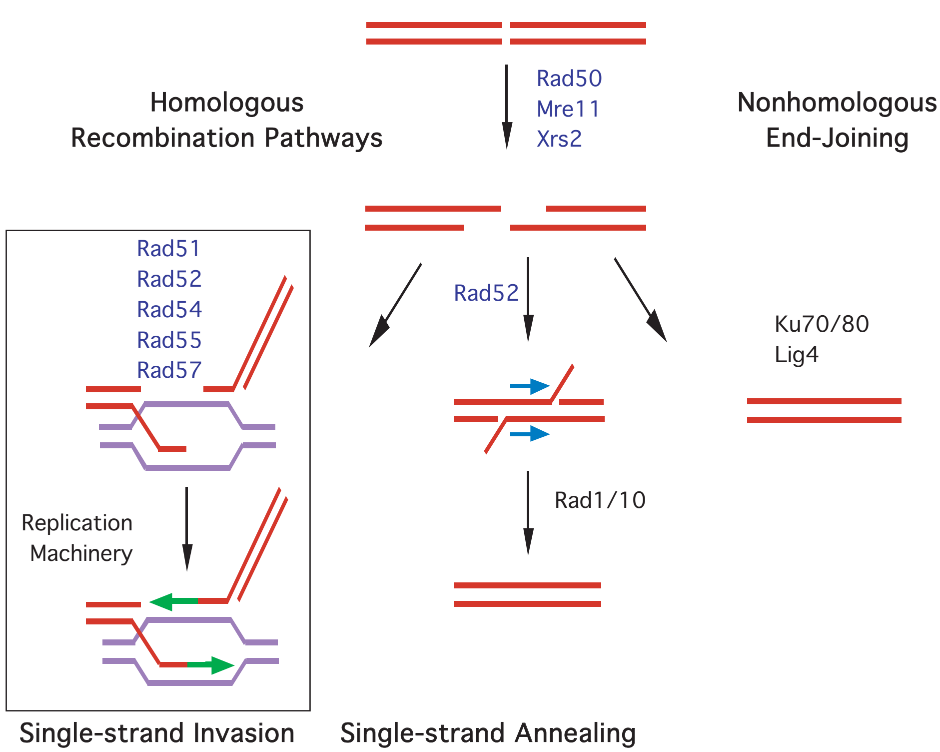


Figure 1. Pathways used to repair double-stranded DNA breaks in *S.cerevisiae*. Components of the pathways that play integral roles in homologous recombination and nonhomologous end-joining are shown. A previous study that used deletion mutants in these pathways suggested that the single-strand invasion pathway of homologous recombination is primarily responsible for repairing topoisomerase II-generated DNA breaks that are stabilized by etoposide (34).

Screen for etoposide-sensitive yeast strains

A previous study analyzed cytotoxicity and recombination in a series of strains that carried single deletions of genes involved in each of the above pathways (34). Based on results with the topoisomerase II poison, etoposide, this work concluded that topoisomerase II-generated double-stranded DNA breaks are repaired primarily by the single-strand invasion pathway of homologous recombination. The non-homologous end-joining pathway also is triggered by topoisomerase II-mediated DNA cleavage, but due to its reduced presence in yeast, does not contribute significantly to cell survival (34).

Since that study examined only known repair pathways, it is possible that other unidentified mechanisms help to protect cells from the damaging actions of topoisomerase II. Therefore, a genome-wide approach was used to further investigate the repair of topoisomerase II-mediated DNA damage in yeast. To this end, a *S.cerevisiae* haploid deletion library of ~4800 strains (35) was screened for sensitivity to topoisomerase II-generated DNA breaks. These breaks were induced by exposing yeast cultures to the topoisomerase II poison etoposide (34). This drug is specific for the type II enzyme and kills cells by dramatically increasing levels of topoisomerase II-mediated DNA breaks (26,27).

Deletion strains were plated onto medium containing either 1 mM etoposide or drug solvent (DMSO). The high concentration of etoposide was required because normal laboratory yeast strains display poor drug uptake. Since the loss of *RAD52* dramatically increases cytotoxicity to topoisomerase II poisons by 2–3 orders of magnitude (34,36), a Δ*rad52* deletion strain was used as a positive control for drug hypersensitivity.

Strains that displayed an etoposide sensitivity that approached that of Δ*rad52* were streaked onto plates containing 1 mM etoposide to re-examine cell growth. Deletion strains that conferred drug hypersensitivity in this second screen were spotted in serial dilutions onto medium containing the topoisomerase II poison (Figure 2). On the basis of these criteria, nine strains that were at least 10-fold hypersensitive to etoposide were identified. The first eight were Δ*mre11*, Δ*xrs2*, Δ*rad50*, Δ*rad51*, Δ*rad52*, Δ*rad54*, Δ*rad55* and Δ*rad57*. Every one of these genes encodes a protein required for the single-strand invasion pathway of homologous recombination (40–42). In contrast, no strains with deletions in any gene specific for the single-strand annealing or nonhomologous end-joining pathways (other than *RAD52* which also is required for single-strand invasion) were identified. These findings confirm the results of Sabourin *et al.* (34), and establish the single-strand invasion pathway of homologous recombination as the major pathway whereby *S.cerevisiae* cells repair topoisomerase II-generated DNA damage.

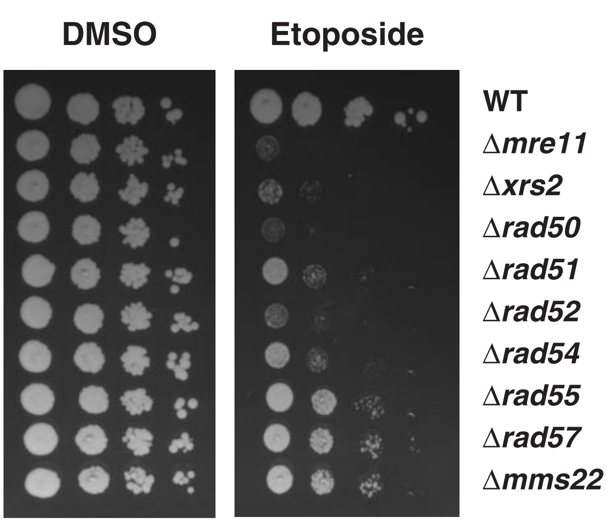


Figure 2. Genes involved in protecting cells from etoposide-induced DNA damage. A *S.cerevisiae* haploid deletion library (~4800 strains) was screened for sensitivity to etoposide. Wild-type (WT, BY4741) and indicated deletion strains were plated in serial dilution onto YPD medium containing drug solvent (DMSO) or 1 mM etoposide.

In addition to the above deletions, Δ*mms22* conferred hypersensitivity to etoposide. Mms22p is a protein of unknown function that is believed to be involved in DNA repair (www.incyte.com) (46,47). Strains that are deleted for *MMS22* are hypersensitive to a variety of DNA damaging agents, including methyl methanesulfonate, hydroxyurea, bleomycin, ultraviolet- and ionizing-irradiation, and camptothecin (46,48,49). The present study represents the first report that deletion of *MMS22* also confers hypersensitivity to topoisomerase II-mediated DNA strand breaks.

Hypersensitivity of Δ*mms22* to topoisomerase II-mediated DNA breaks

As discussed above, most *S.cerevisiae* strains display low permeability to topoisomerase II-targeted drugs. Therefore, to further analyze the effects of Mms22p on the sensitivity of cells to topoisomerase II-mediated DNA damage, the *MMS22* deletion was re-created in the JN362acc background. This parental strain contains the *ISE2* permeability mutation, which allows facile drug uptake, and has been used for numerous studies of agents that enhance topoisomerase II-mediated DNA cleavage (34,36,50,51).

Cytotoxicity assays were carried out in the presence of two topoisomerase II poisons, etoposide and amsacrine. As seen in Figure 3, Δ*mms22* cells were ~10-fold hypersensitive to both topoisomerase II poisons. These results are as compared to Δ*rad52* cells, which were >100-fold hypersensitive.

To confirm that the drug hypersensitivity of Δ*mms22* cells resulted from the lack of Mms22p, the *MMS22* gene under the control of its endogenous promoter was cloned in a plasmid vector (pMMS22). While a Δ*mms22* strain that carried the empty vector was hypersensitive to etoposide, a Δ*mms22* strain that carried pMMS22 displayed wild-type sensitivity (Figure 4). These data confirm the initial deletion screen and demonstrate that Mms22p protects cells from DNA damage generated by topoisomerase II.

Effects of Mms22p on cell cycle distribution in the presence of topoisomerase II-mediated DNA damage

A high proportion of Δ*mms22* populations exist as large-budded cells in the absence of environmental insults (46). Therefore, to further analyze the effects of Mms22p on cell cycle distribution, FACS analysis was performed on asynchronous wild-type and Δ*mms22* cells in the absence or presence of etoposide. As shown in Figure 5, Δ*mms22* cells displayed a phenotype distinct from that of wild-type cells. Even in the absence of etoposide, the proportion of Δ*mms22*

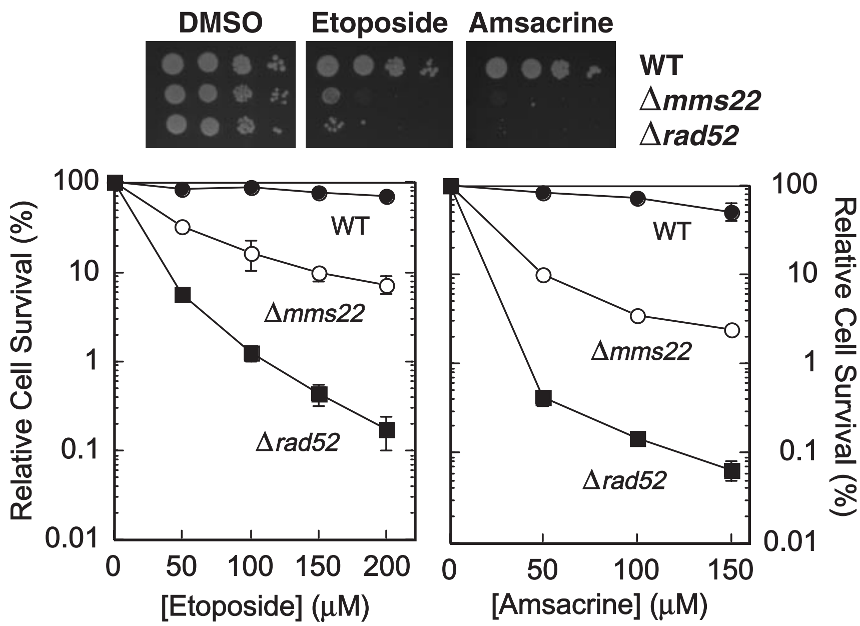


Figure 3. Δ*mms22* cells are hypersensitive to etoposide and amsacrine. The sensitivity of Δ*mms22* to topoisomerase II poisons was tested. Serial dilutions of wild-type (WT), Δ*mms22* and Δ*rad52* cultures were plated onto YPD medium containing DMSO or 100 μM drug (top). Cytotoxicity assays were performed using the indicated strains. Cells were exposed to etoposide (bottom, left panel) or amsacrine (bottom, right panel) for 8 h in liquid culture. Error bars represent the SD values of at least three independent experiments.

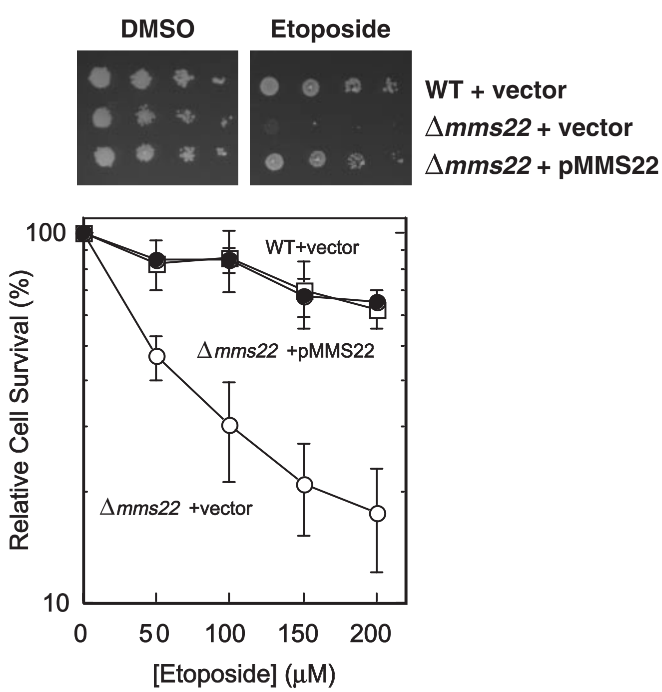
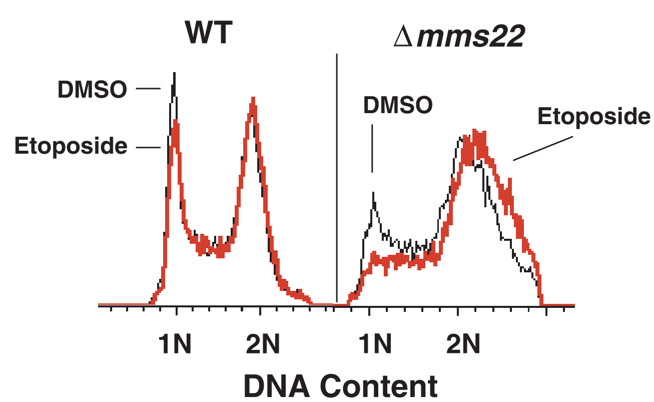


Figure 4. Expression of plasmid-encoded *MMS22* rescues the Δ*mms22* drug hypersensitive phenotype. *MMS22* was cloned, along with its endogenous promoter, into pRS416 to generate pMMS22. Serial dilutions of the wild-type strain carrying pRS416 (empty vector) as well as the Δ*mms22* strain carrying either pRS416 or pMMS22 were plated onto SC-URA medium containing DMSO or 100 μM etoposide (top). Cytotoxicity assays were performed using the indicated strains (bottom). Cells were exposed to etoposide for 8 h in liquid culture. Error bars represent the SD values of at least three independent experiments.



	WT		Δ*mms22*	
	DMSO	Etop	DMSO	Etop
G_0/G_1	23.1	19.8	14.1	3.8
S	35.4	35.6	30.1	32.2
G_2/M	41.5	44.6	55.8	64.0

Figure 5. Δ*mms22* cells accumulate in G_2/M. Asynchronous wild-type and Δ*mms22* cells were grown for 6 h in the presence of DMSO (black line) or 50 μM etoposide (red line). Peaks representing haploid (1 N) and diploid (2 N) DNA contents are indicated (top). The percent of cultures in G_0/G_1, S or G_2/M phase are shown (bottom). Cells were analyzed with Sytox Green as the DNA stain. Results are representative of three independent experiments.

cells in G_0/G_1 was only 60% of that observed for wild-type cells. In addition, a significantly higher proportion of the mutant cells was in G_2/M. The accumulation of cells in G_2/M suggests that Mms22p plays a role in allowing cells to cope with endogenous damage in their genetic material.

The addition of 50 µM etoposide to cultures had little effect on the cell cycle distribution of the parental strain. However, there was a substantial decrease of cells in G_0/G_1 and an increase in G_2/M cells in the Δ*mms22* strain. These findings provide additional evidence that Δ*mms22* cells display an abnormal response to increased levels of topoisomerase II-generated DNA breaks.

Mms22p does not appear to be part of the single-strand invasion pathway of homologous recombination

To determine whether Mms22p plays a role in the single-strand invasion pathway or is part of a separate pathway that repairs topoisomerase II-mediated DNA breaks, a Δ*mms22* Δ*rad54* double mutant was constructed. Rad54p is a member of the Swi2p/Snf2p superfamily of DNA-dependent ATPases and is involved in joint-molecule formation during single-strand invasion (40–42) (see Figure 1). Deletion of *RAD54* sensitizes yeast cultures to etoposide to a greater extent than observed with a Δ*mms22* strain (Figure 6). As determined by serial dilution plate assays, the Δ*mms22* Δ*rad54* double mutant was 1–2 orders of magnitude more sensitive to etoposide than either single deletion mutant. Furthermore, the Δ*mms22* Δ*rad54* double mutant was ∼3-fold more sensitive than the Δ*rad54* strain in liquid culture cytotoxicity assays following a 24-h drug exposure (Figure 6). These results indicate that *MMS22* is not epistatic to *RAD54* and suggest that Mms22p acts outside of the single-strand invasion pathway of homologous recombination. A similar conclusion recently was drawn by Araki *et al*. (47), based on the sensitivity of a Δ*mms22* Δ*rad51* double mutant to methyl methanesulfonate. Taken together, these findings imply that Mms22p represents part of a novel pathway that plays an important role in the cellular response to topoisomerase II-generated DNA damage.

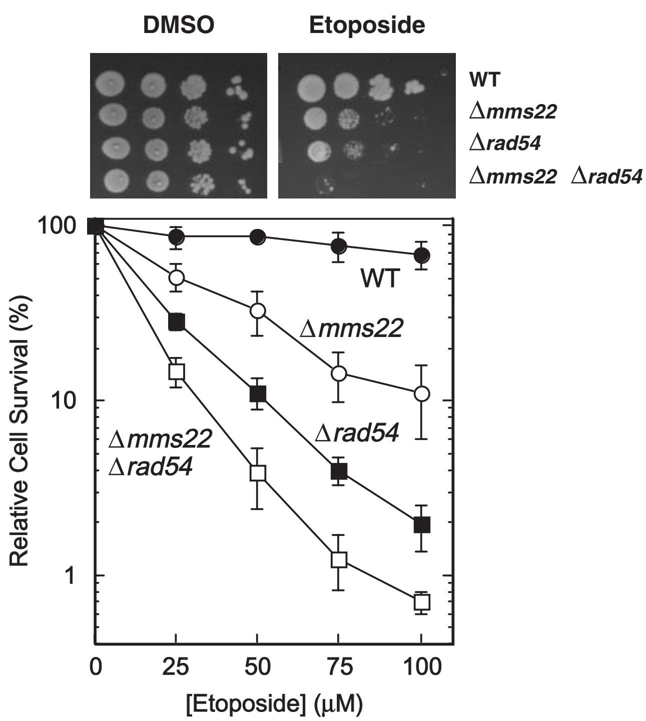


Figure 6. *MMS22* is not epistatic to *RAD54*. A Δ*mms22* Δ*rad54* double mutant was constructed to determine whether Mms22p is involved in the single-strand invasion pathway of homologous recombination. Wild-type, Δ*mms22*, Δ*rad54* and Δ*mms22* Δ*rad54* double mutant cells were plated in serial dilution onto YPD medium containing DMSO or 50 µM etoposide (top). Cytotoxicity assays were performed using the indicated strains (bottom). Cells were exposed to etoposide for 24 h in liquid culture. Error bars represent the SD values of at least three independent experiments.

Etoposide-induced homologous recombination is lower in Δ*mms22* cells

Since the single-strand invasion pathway of homologous recombination appears to be the major pathway by which topoisomerase II-mediated DNA strand breaks are repaired, the effects of deletion mutants on this process were characterized (Figure 7). A plasmid-based homologous recombination reporter system was employed for these studies (34,38). In this system, yeast strains are transformed with YCpHR, a plasmid that contains the canavanine sensitivity gene, *CAN1*, flanked on either side by a copy of the *LEU2* gene. Homologous recombination between the two *LEU2* genes results in the deletion of *CAN1*. Since the chromosomal allele of *CAN1* is disrupted in the parental yeast strain, recombination is scored by the ability of cells to grow in the presence of canavanine.

Yeast strains employed for these recombination studies all harbored the mutant *top2S740W* yeast topoisomerase II allele in place of the wild-type *TOP2* gene. The S740W point mutation, which has been well characterized, confers increased etoposide sensitivity due to the formation of a more stable drug-induced DNA cleavage complex (52). Inclusion of this hypersensitive topoisomerase II allele promotes a greater cellular response to etoposide and increases the levels of homologous recombination observed with the reporter plasmid (34).

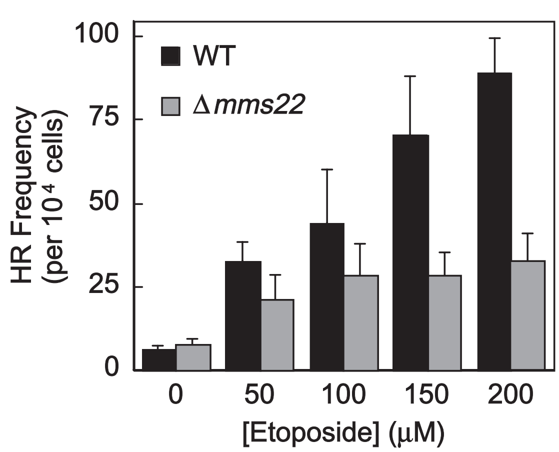


Figure 7. Etoposide-induced homologous recombination is lower in the Δ*mms22* strain. A plasmid-based (YCpHR) reporter assay (34,38) was used to assess levels of homologous recombination in yeast. A strain expressing allelic *top2S740W* was utilized in these studies, since this mutant topoisomerase II is hypersensitive to etoposide. Wild-type and Δ*mms22* cells containing the *top2S740W* allele were exposed to etoposide for 5 h. Error bars represent the SD values of four independent experiments.

In the absence of etoposide, the frequency of homologous recombination in Δ*mms22* cells was similar to that of the parental *MMS22* strain (Figure 7). However, significant differences were observed in the presence of the topoisomerase II poison. Recombination frequencies in the *MMS22* strain rose ~14-fold following exposure to 200 μM etoposide. In contrast, frequencies in the Δ*mms22* strain rose only 4-fold over the same drug range.

To verify that canavanine resistance arose from a homologous recombination event on YCpHR rather than a microdeletion or point mutation in *CAN1*, plasmids were rescued from Δ*mms22* colonies and analyzed by restriction enzyme digestion (not shown). In all cases, the loss of canavanine sensitivity was accompanied by a deletion of ~6 kb. This length corresponds to the size of the predicted *CAN1* fragment that would be lost following homologous recombination between the two *LEU2* genes.

Even though Mms22p does not appear to play a direct role in the single-strand invasion pathway, these results strongly suggest that the loss of this protein impairs the ability of yeast cells to repair topoisomerase II-mediated DNA damage via homologous recombination.

Δ*rtt101* and Δ*rtt107* strains are hypersensitive to topoisomerase II-mediated DNA breaks

A high-throughput study that utilized mass spectrometry to characterize protein complexes in *S.cerevisiae* identified Rtt101p and Rtt107p as binding partners of Mms22p (53). Both of these proteins appear to be involved in the regulation of Ty1 transposition (54). In addition, Rtt101p displays ubiquitin ligase activity (55), and Rtt107p has been identified as a phosphorylation target of Mec1p and is believed to play a role in the resumption of DNA synthesis following genomic damage (56). To determine whether *MMS22* is epistatic to *RTT101* or *RTT107*, a series of deletion mutants was constructed in the JN362acc background and tested for sensitivity to etoposide (Figure 8). The singly deleted Δ*rtt101* and Δ*rtt107* strains were ~2-fold more sensitive to the topoisomerase II poison than was the parental wild-type strain, while the Δ*rtt101* Δ*rtt107* double mutant was ~3-fold hypersensitive. The sensitivity of the Δ*mms22* Δ*rtt101* and Δ*mms22* Δ*rtt107* double mutants, as well as the Δ*mms22* Δ*rtt101* Δ*rtt107* triple mutant was similar to or less than that of Δ*mms22* alone. These results suggest that *MMS22* is epistatic to *RTT101* and *RTT107*, and that the protein products of these three genes act within the same pathway to repair topoisomerase II-generated DNA damage.

Mms22p localizes to the nucleus at discrete foci

If Mms22p is involved in DNA repair processes, it would be expected to localize in the nuclei of yeast cells. Therefore, to analyze the cellular localization of Mms22p, a *GFP-MMS22* hybrid gene construct was created using the pGFP-N-FUS vector system. The construct was designed to generate an N-terminal GFP-Mms22 fusion protein that was expressed under the control of the *MET25* promoter. As determined by serial dilution plate assays, GFP-Mms22p is functional (Figure 9). The etoposide sensitivity of Δ*mms22* cells that harbored pGFP-MMS22 was comparable to that of wild-type yeast (i.e. *MMS22*) that carried the pGFP-N-FUS.

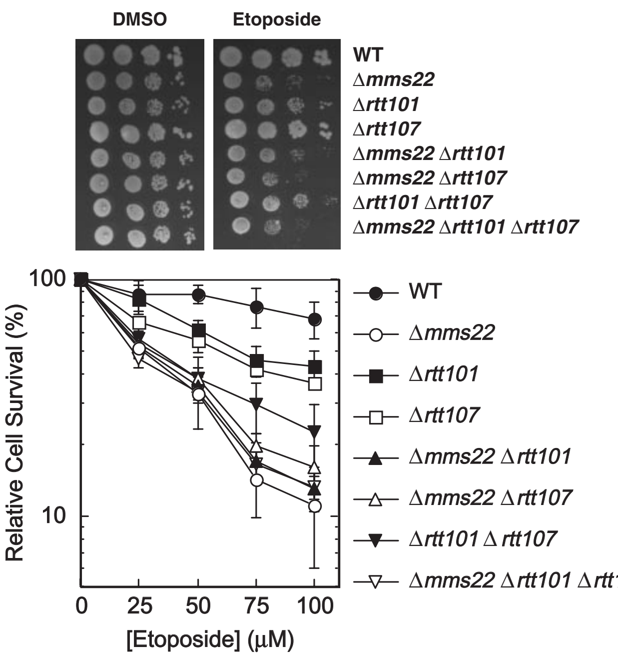


Figure 8. *MMS22* is epistatic to *RTT101* and *RTT107*. Wild-type (WT); Δ*mms22*, Δ*rtt101* and Δ*rtt107* single mutant; Δ*mms22* Δ*rtt101*, Δ*mms22* Δ*rtt107* and Δ*rtt101* Δ*rtt107* double mutant; and Δ*mms22* Δ*rtt101* Δ*rtt107* triple mutant cells were plated in serial dilution onto YPD medium containing DMSO or 50 μM etoposide (top). Cytotoxicity assays were performed using the indicated strains (bottom). Cells were exposed to etoposide for 24 h in liquid culture. Error bars represent the SD values of at least three independent experiments.

In contrast, Δ*mms22* cells that harbored the pGFP-N-FUS vector were hypersensitive to the drug.

Localization studies utilized Δ*mms22* cells that carried either the pGFP-N-FUS vector or pGFP-MMS22 that expressed the fusion construct. While GFP alone distributed throughout the cell (with the exception of the vacuole), the GFP-Mms22p fusion protein localized to the nucleus in discrete foci (Figure 9). Similar results were observed in the presence of etoposide (not shown).

Δ*mms22* Δ*rtt101*, Δ*mms22* Δ*rtt107* and Δ*mms22* Δ*rtt101* Δ*rtt107* cells also were transformed with pGFP-N-FUS or pGFP-MMS22. The localization of GFP and GFP-Mms22p in these cells was similar to that described above (not shown). These results imply that the localization of Mms22p to nuclear foci does not require either of its binding partners, Rtt101p or Rtt107p.

DISCUSSION

Although drugs that increase levels of topoisomerase II-mediated DNA cleavage are front-line therapy for a variety of human malignancies, considerable evidence suggests that these same scission events can trigger the chromosomal breaks that initiate specific leukemias (13,28–30). Despite the central role that the type II enzyme plays in curing and causing cancer, the cellular pathways by which topoisomerase II-generated DNA breaks are processed and repaired are not fully understood. Therefore, budding yeast was used as a model genetic system to address this important issue.

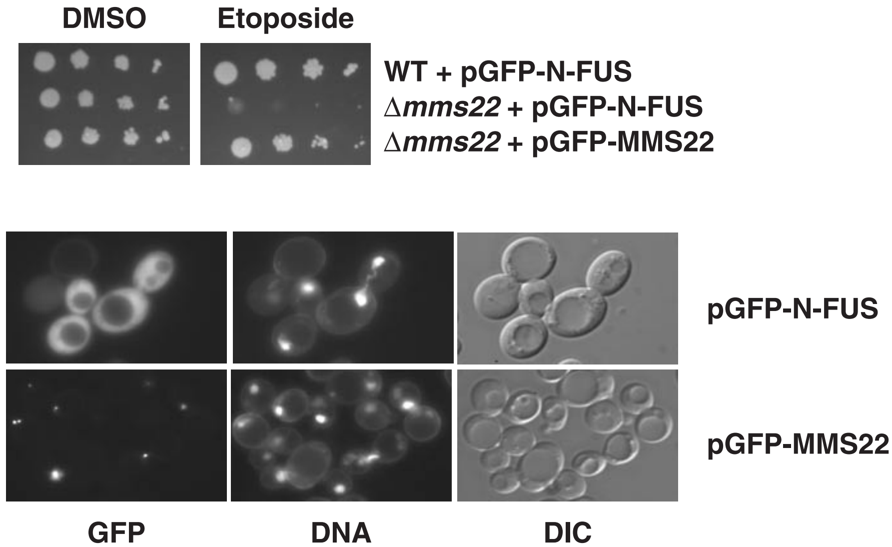


Figure 9. GFP-Mms22p localizes to nuclear foci. Δ*mms22* cells containing a vector (pGFP-N-FUS) that expressed *GFP* or an N-terminal *GFP-MMS22* fusion construct (pGFP-MMS22) were examined for hypersensitivity to etoposide to confirm that the GFP-Mms22p fusion protein was functional. Wild-type (WT) cells carrying pGFP-N-FUS as well as Δ*mms22* cells carrying either pGFP-N-FUS or pGFP-MMS22 were plated in serial dilution onto SC-MET/URA medium containing DMSO or 100 μM etoposide (top). GFP and the GFP-Mms22p fusion protein were visualized in cells by direct fluorescence microscopy. DNA was localized by Hoechst staining. Differential image contrast (DIC) images of the visualized yeast cells are shown for reference.

A previous study that characterized individual deletion mutants in known recombination pathways suggested that the single-strand invasion pathway of homologous recombination plays an important role in the repair of topoisomerase II-mediated DNA damage (34). To broaden the scope of this earlier work, the present study utilized a genome-wide approach in which a *S.cerevisiae* haploid deletion library was tested for sensitivity to the topoisomerase II poison etoposide. Eight of the nine strains that displayed ⩾10-fold hypersensitivity to the drug were deleted for components of single-strand invasion. This finding confirms the importance of this yeast recombination pathway in the repair of topoisomerase II-generated DNA breaks.

The ninth strain that was identified in the screen was deleted for *MMS22*. Δ*mms22* strains display increased sensitivity to a variety of agents that induce DNA adducts or strand breaks, or disrupt DNA replication (46,48,49). The present findings extend the range of *MMS22* to include the repair of DNA damage generated by topoisomerase II.

It is notable that other strains were identified in the screen that displayed mild hypersensitivity to etoposide (i.e. <10-fold). Furthermore, since a haploid deletion library was used for the present work, only non-essential genes could be screened for sensitivity to the topoisomerase II poison. Therefore, it is likely that further analysis of the yeast genome will uncover additional genes that are involved in the cellular response to topoisomerase II-mediated DNA damage.

A high-throughput proteomic study identified Rtt101p and Rtt107p as binding partners of Mms22p (53). Δ*rtt101* and Δ*rtt107* strains are hypersensitive to etoposide, albeit to a lesser degree than Δ*mms22*. Additional cytotoxicity studies indicate that *MMS22* is epistatic with *RTT101* and *RTT107*. These results are consistent with the known physical interaction between the three proteins and suggest that Mms22p, Rtt101p and Rtt107p function in the same DNA repair pathway.

Mms22p is localized to discrete foci within the nucleus, even in the absence of etoposide. Punctate nuclear localization patterns have been observed for other proteins that participate in various damage response pathways, including DNA replication, cell cycle checkpoints and double-stranded DNA break repair (57–63). Rad52p and other DNA repair proteins form DNA repair centers following the induction of DNA damage (57). However, foci are observed in a small percentage of cells even in the absence of induced DNA breaks (58). Proteins such as Sgs1p that are involved in the recovery of arrested replication forks also are observed at discrete nuclear foci in the absence of exogenous DNA damaging agents (60). Thus, Mms22p may be involved in the repair of endogenous DNA damage that accumulates during normal growth. Consistent with this suggestion, Δ*mms22* strains display a high level of G_2/M cells in asynchronous populations.

Results of the present work and a previous genetic study (47) indicate that *MMS22* functions in a pathway that is separate from the single-strand invasion pathway of homologous recombination. If these pathways were completely independent from one another, it might be expected that deletion of *MMS22* would shuttle topoisomerase II-induced DNA damage into the single-strand invasion pathway, thereby increasing levels of homologous recombination. However, this was not the case. Following exposure to etoposide, the increase in homologous recombination frequency in Δ*mms22* cells was several-fold lower than that observed in wild-type cells. This finding implies that the *MMS22* and single-strand invasion pathways, although separate, must be linked.

Mms22p has no sequence homologs in mammalian cells (www.incyte.com). At the present time, it is not known whether this protein is unique to yeast or whether Mms22p has functional homologs in higher organisms. With the exception of a nuclear localization signal, Mms22p contains no known sequence motifs (www.incyte.com). Thus, it is difficult to speculate about the biochemical functions of this protein.

Since Δ*mms22* cells are sensitive to agents that induce a variety of DNA aberrations (46,48,49), it is unlikely that Mms22p is directly involved in processing topoisomerase II from the termini of cleaved DNA. A previous study suggested that Mms22p is involved in repairing aberrant DNA structures that accumulate at replication forks in response to DNA damage (47). In light of our genetic and recombination data, we would further speculate that (i) the presence of these aberrant DNA structures prevents the facile repair of the damage by homologous recombination and other pathways; and (ii) Mms22p, together with its partner proteins, helps to process these aberrant DNA structures and convert them to a form that can proceed into the different repair pathways.

The finding that Mms22p protects cells from topoisomerase II-mediated DNA damage adds a new level of complexity to our knowledge of the downstream pathways that process strand breaks generated by the type II enzyme. It is becoming increasingly obvious that multiple pathways impact the response of cells to topoisomerase II-DNA cleavage complexes. Some repair the damage appropriately, some repair the damage but generate inappropriate chromosomal rearrangements, and some trigger cell death. Understanding the interplay between these pathways provides critical information that helps to dissect the opposing roles of topoisomerase II as a target for cancer chemotherapy and as an agent that initiates leukemic chromosomal translocations in humans.

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