

Topoisomerase III is required for accurate DNA replication and chromosome segregation in *Schizosaccharomyces pombe*

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ABSTRACT

The deletion of the *top3⁺* gene leads to defective nuclear division and lethality in *Schizosaccharomyces pombe*. This lethality is suppressed by concomitant loss of *rqh1⁺*, the RecQ helicase. Despite extensive investigation, topoisomerase III function and its relationship with RecQ helicase remain poorly understood. We generated *top3* temperature-sensitive (*top3*-ts) mutants and found these to be defective in nuclear division and cytokinesis and to be sensitive to DNA-damaging agents. A temperature shift of *top3*-ts cells to 37°C, or treatment with hydroxyurea at the permissive temperature, caused an increase in 'cut' (cell untimely torn) cells and elevated rates of minichromosome loss. The viability of *top3*-ts cells was decreased by a temperature shift during S-phase when compared with a similar treatment in other cell cycle stages. Furthermore, the *top3*-ts mutant was not sensitive to M-phase specific drugs. These results indicate that topoisomerase III may play an important role in DNA metabolism during DNA replication to ensure proper chromosome segregation. Our data are consistent with Top3 acting downstream of Rqh1 to process the toxic DNA structure produced by Rqh1.

INTRODUCTION

Topoisomerase III is a type I topoisomerase engaged in breakage and reunion of single-stranded DNA. Eukaryotic topoisomerase III was first identified as a gene required for suppression of recombination between repeated genes in *Saccharomyces cerevisiae* (1). Partially purified topoisomerase III was found to be much less efficient than yeast DNA topoisomerases I or II in causing relaxation of supercoiled DNA substrates. *In vivo* studies demonstrated that yeast topoisomerase III has, at most, a weak activity in relaxing negatively supercoiled intracellular DNA (2). Null mutation of the *top3⁺* gene in budding yeast results in various phenotypes including slow growth, hyper-recombination between repetitive DNA sequences, and a defect in sporulation (3).

The *S.cerevisiae* *sgs1* mutant was isolated as a suppressor of the slow growth phenotype of the *top3* deletion mutant (4). *SGS1* is a member of the RecQ helicase family which includes fission yeast *rqh1⁺* and human *BLM*, *WRN* and *REQL4* helicases which are implicated in Bloom's, Werner's and Rothmund–Thomson syndromes, respectively (5–8). RecQ-family helicases possess 3' to 5' helicase activity and are required for the maintenance of genomic stability. In *S.cerevisiae*, *sgs1* deletion mutants also show genomic instability including hyper-recombination of the rDNA locus and a reduced life span that correlates with the accumulation of extrachromosomal rDNA circles (9–12). Biochemically, Sgs1 physically interacts with Top3 and with Top2.

Several models have been postulated to explain the interactions between topoisomerase III and RecQ helicases: first, the Sgs1–Top3 complex is proposed to act as a reverse gyrase with a helicase-like domain and a type I topoisomerase (4). Secondly, topoisomerase III and RecQ helicase are thought play a role in recombination (4,13) and thirdly, it has been suggested that the Sgs1–Top3 complex acts at late stages of replication to facilitate the progression of the replication fork by combined unwinding activity of the helicase and decatenation activity of topoisomerase III (14,15).

In *Schizosaccharomyces pombe*, *top3⁺* is essential for viability and plays a role in chromosome segregation (14). The *top3*-deleted spores germinate and divide several times, but fail to maintain long-term growth. The *S.pombe* RecQ family helicase, *rqh1⁺*, was initially isolated as a gene that complemented the *rad12* and *hus2* mutations (the latter found to be allelic), which showed sensitivity to both DNA-damaging agents and to the DNA synthesis inhibitor hydroxyurea (HU). The *rqh1* null mutant is defective in the reversible recovery from the S-phase arrest, and in tolerating DNA damage during S-phase (16,17). Deletion of the *rqh1⁺* gene suppresses the lethality of the *top3* mutant in *S.pombe*, a result reminiscent of the situation in *S.cerevisiae* (14,18).

To further understand the functional roles of *S.pombe* topoisomerase III and its relationship to Rqh1 helicase, we generated *top3* temperature-sensitive (*top3*-ts) mutants. The *top3*-ts mutants are sensitive to DNA-damaging agents, show increased levels of 'cut' (cell untimely torn) phenotypes and elevated rates of minichromosome loss following HU treatment. The viability of wild-type and *rqh1-d* mutant cells is

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Table 1. Strains used in this study

Strain	Genotype	Reference
CF199	<i>h⁻ leu1-32 his3-D1 ura4-D18 ade6-M210</i>	From T. Cech
CF197	<i>h⁺ leu1-32 his3-D1 ura4-D18 ade6-M216</i>	From T. Cech
1589	<i>h⁻ leu1-32 his3-D1 ura4-D18 rqh1::ura4⁺</i>	From T. Enoch
MO134	<i>h⁻ leu1-32 his3-D1 ura4-D18 ade6-M210 top3-134-ts</i>	This study
MO252	<i>h⁻ leu1-32 his3-D1 ura4-D18 ade6-M210 top3-252-ts</i>	This study
MO316	<i>h⁻ leu1-32 his3-D1 ura4-D18 top3::LEU2⁺ rqh1::ura4⁺</i>	This study
MORT132	<i>h⁻ leu1-32 his3-D1 ura4-D18 ade6-M210 top3-134-ts rqh1::ura4⁺</i>	This study
MOL911	<i>h⁻ leu1-32 his3-D1 ura4-D18 ade6-M210</i> containing plasmid pMO19T3	This study
MO797	<i>h⁺/h⁻ leu1-32/leu1-32 his3-D1/his3-D1 ura4-D18/wra4-D18 ade6-M210/ade6-M216</i>	This study
MOL791	<i>h⁺/h⁻ leu1-32/leu1-32 his3-D1/his3-D1 ura4-D18/wra4-D18 ade6-M210/ade6-M216 top3⁺/top3::LEU2⁺</i>	This study
MOU792	<i>h⁺/h⁻ leu1-32/leu1-32 his3-D1/his3-D1 ura4-D18/wra4-D18 ade6-M210/ade6-M216 top3⁺/top3::ura4⁺</i>	This study
TE788	<i>ade6-M210</i> containing Ch ¹⁶	From T. Enoch
TE786	<i>rqh1::ura4⁺ ura4-D18 ade6-M210</i> containing Ch ¹⁶	From T. Enoch
FY1497	<i>h⁺ leu1-32 ura4-DS/E ade6-M210</i> containing Ch ¹⁶	From R. Allshire
MO1841	<i>h⁻ leu1-32 his3-D1 ura4-D18 ade6-M210 top3-134-ts</i> containing Ch ¹⁶	MO134 × FY1497
MO1825	Same with MO1841	MO134 × FY1497

Table 2. Plasmids used in the study

Plasmid	Constructions	Reference
pMO19T3	pUR19N containing a 6 kb <i>EcoRV</i> – <i>NsiI</i> fragment of the <i>top3⁺</i> gene	This work
pMO234	pBluescripts II ks(+) containing a 3.1 kb <i>XbaI</i> – <i>SalI</i> fragment of the <i>top3⁺</i> gene	This work
pMO344	pBG2 containing a 3.1 kb <i>XbaI</i> – <i>SalI</i> fragment of the <i>top3⁺</i> gene	This work
pMO323	<i>top3::LEU2⁺</i> construct	This work
pMO343	<i>top3::ura4⁺</i> construct	This work
pTE436	<i>rqh1::ura4⁺</i> construct	From T. Enoch
pMO322	pBG2 containing 5′-420 bp and 3′-230 bp flanking fragments only	This work

sharply decreased by Rqh1 overproduction. The phenotypes of *rqh1*-overexpression strain are similar to those of *top3*-ts mutant. These results indicate that Top3 has an essential function in processing an intermediate DNA structure produced by Rqh1.

MATERIALS AND METHODS

Media and reagents

Complete and minimal growth media for fission yeast and chemical reagents were purchased from Difco and Sigma Aldrich. Plasmids were constructed by standard techniques (19) and general procedures for *S.pombe* genetics were carried out according to the methods of Alfa (20).

Yeast strains and plasmids

Yeast strains and plasmids used in this study are listed in Tables 1 and 2. The 6 kb *EcoRV*–*NsiI* DNA fragment of the wild-type *top3⁺* gene derived from cosmid C16G5 (a gift from Rhian Gwilliam at Sanger Center) was cloned into the *SmaI*–*PstI* site of pUR19N to generate plasmid pMO19T3. The 3.1 kb *XbaI*–*SalI* DNA fragment of the *top3⁺* gene was similarly cloned into pBluescripts II KS(+) and pBG2 to create pMO234 and pMO344, respectively. To disrupt the *top3⁺* gene, the 1.7 kb *PstI*–*EcoRI* DNA fragment of pMO234 was replaced by the 2.2 kb *LEU2⁺* gene of *S.cerevisiae* or the 1.8 kb *ura4⁺* gene of *S.pombe* to generate pMO323 and pMO343, respectively. The 3.5 kb *SalI*–*XbaI* DNA fragment of pMO323 or the 2.6 kb *MscI*–*ClaI* DNA fragment of pMO343 was used

to transform the diploid strain MO797. The resulting *top3⁺/top3::LEU2⁺* and *top3⁺/top3::ura4⁺* transformants were designated as MOL791 and MOU792, respectively.

To make a *top3rqh1* double mutant, *top3⁺/top3::LEU2⁺* diploid MOL791 was transformed with *SacI*–*SphI*-linearized pTE436 plasmids (a gift from Tamar Enoch at Harvard Medical Center). The correct transformants were confirmed by Southern blot, sporulated on ME plates and the resulting tetrads were dissected on YES plates. The *top3::LEU2⁺ rqh1::ura4⁺* haploid colonies were selected by replica plating onto minimal medium lacking uracil and leucine. The resulting double mutant was designated as MO316.

Construction of *top3*-ts alleles

To create *top3*-ts mutants by plasmid shuffling, hydroxylamine (HA) mutagenesis (21) and error-prone PCR-based mutagenesis (22) methods were used as described but with some minor modifications. Briefly, *top3⁺/top3::LEU2⁺* diploid MOL791 was transformed with pMO19T3 containing the wild-type *top3⁺* gene. The resulting positive colonies were sporulated on ME plates at 25°C and then *top3::LEU2⁺* haploid cells containing the pMO19T3 were selected (named as MOL911). To specifically mutagenize *top3⁺* gene, 10 µg of pMO344 containing wild-type *top3⁺* was treated with 1 M HA for 5 or 10 min and then was used to transform MOL911. The generated transformants were replica plated onto two 5-fluoroorotic acid (5-FOA) plates to remove pMO19T3. Replica plates were incubated at 25 and 37°C.

For error-prone PCR-based mutagenesis, the 5′- and 3′-flanking DNA fragments of 420 and 230 bp were amplified by

PCR using primers (TOP3-*Sph1*-6175, 5'-GGAACCTACAC-TAATTTAGCATGCTCTA; TOP3-*PstI*-5780, 5'-AAAAAT-GACTGCAGTGTA) and (TOP3-*PstI*-3540, 5'-TAACGC-AGACGTGCAGAGTCTACC; TOP3-*SacI*-3330, 5'-GTG-TATTGTAACCTGAGCTCGTCTTTTCTGA), respectively. The resulting DNA fragments were digested with either *SphI*-*PstI* or *PstI*-*SacI* restriction enzymes and inserted together into the *SphI*-*SacI* site of pBG2 to generate plasmid pMO322. Random mutations in the *top3*⁺ gene were generated by Mn²⁺-buffered error-prone PCR. The PCR mixtures contained 100 pmol of each oligonucleotide (TOP3-CT-R, 5'-GACGATATTAAGTCGACTTG; TOP3-*Sph1*-6175), 50 ng of pMO19T3 DNA as a template, 0.1 mM MnCl₂, 2.5 mM MgCl₂, 0.25 mM dNTP mixtures, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, and 5 U *Taq* polymerase (Promega). The PCR products and *PstI*-linearized pMO322 were cotransformed into MOL911. Total transformants produced by homologous recombination between the gapped plasmids and mutagenized PCR products were replica-plated onto two 5-FOA plates to exclude the *ura4*⁺ marked *top3*⁺ plasmid and replica plates were incubated at 25 or 37°C.

Colonies showing temperature-sensitive growth were identified and the plasmids containing the corresponding mutagenized *top3* genes were extracted from and transferred into the integration vector Yiplac128. The resulting plasmids were linearized with *MscI* restriction enzyme and then transformed into the *top3*⁺/*top3*::*ura4*⁺ diploid strain MOU792. The cells which the mutated *top3* gene was inserted into original *top3*⁺ locus were selected by Southern blot, sporulated, and *ura4*⁺/*LEU2*⁺ colonies were selected. The resulting colonies were subjected to 5-FOA plates to remove the *ura4*⁺ marker by homologous recombination (23).

UV, methyl methane-sulfonate (MMS) and HU sensitivity tests

Two *top3*-ts mutants and the parental (control) strain CF199 were grown in YES medium to mid-log phase and the cell number counted using a hemocytometer. To analyze the sensitivity to HU and MMS, appropriate numbers of cells in the exponential growth phase were spread onto YES plates with or without DNA-damaging agents of various concentrations in duplicate. To test UV sensitivity, cells were spread on YES agar plates and then irradiated with different doses of UV light in duplicate. Plates were incubated either at 25°C or at 25°C following 12 h incubation at 37°C. The number of viable colonies in each plate treated with the DNA-damaging agents was normalized with respect to that of an untreated control for each temperature regime.

Minichromosome loss assay

The minichromosome loss rate of the *rqh1-d* mutant was determined with TE788 and TE786 and for the *top3*-ts mutants with FY1497 and MO1841. The loss rate of minichromosome Ch¹⁶ from wild-type and mutant cells was determined by two independent methods (16,17) with slight modifications. In both methods, the number of cells changed from *ade*⁺ to *ade*⁻ by the loss of Ch¹⁶ during one generation was ascertained. Each cell culture was either untreated or treated with 10 mM HU at 30°C and appropriate numbers of cells were spread onto YES plates not supplemented with adenine. The plates of MO1841 and FY1497 were prepared in duplicate and one of

the plates incubated at 37°C for 12 h before being transferred to 25°C. The other plate was incubated directly at 25°C. The plates of TE786 and TE788 were incubated at 30°C. After 5–7 days incubation, the proportion of red colonies was calculated. The loss rates estimated by the two methods were averaged.

Cell viability test after G1 cell cycle arrest

Exponentially growing cells were washed three times with EMM media lacking a nitrogen source, resuspended in the same media at 2×10^6 cells/ml and then incubated at 25°C for 16 h. After changing to fresh YES media, cell aliquots were then sampled at 30 min intervals, transferred to 37°C for 2 h and the appropriate number of cells were then spread onto YES plates. An aliquot of the same cells was fixed for FACS analysis or septation index counts. The relative colony-forming units were calculated by comparison with the number of colonies evident when plates were incubated at 25°C for 2 h after cell cycle restart.

Sensitivity test to thiabendazole (TBZ) and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)

Cells were grown to mid-log phase, washed with fresh YES medium, and then resuspended at a concentration of 1×10^7 cells/ml. In duplicate, serial 10-fold dilutions were spotted onto YES plates containing the relevant agent and onto a control plate. TBZ plates contained 10 µg/ml TBZ. TPCK plates were composed of 1% DMSO, 5 µg/ml phloxine B and 120 µM TPCK. As before, each plate was incubated at 25°C or at 25°C following 12 h incubation at 37°C.

The preparation of *Rqh1* overexpression strains

The pREP41-HAN vector was used for *Rqh1* overexpression. Cells bearing *rqh1*-overproducing plasmid were first grown in EMM medium containing 4 µM thiamine (transcription repressed), washed three times with EMM medium, and further grown in EMM lacking thiamine for 20 h (transcription induced at ~14–16 h). The overexpression of HA-*Rqh1* at 20 h was confirmed by western blot using anti-HA antibody (Boehringer Mannheim).

RESULTS

Isolation of *top3*-ts mutants

Using the methodology described (see Materials and Methods) we generated two independent *top3*-ts alleles on multi-copy plasmids designated as *top3-ts134* and *top3-ts252* (Fig. 1A). The wild-type *top3*⁺ gene compensated for the temperature-sensitive phenotype, whereas the empty vector did not (Fig. 1B), indicating that the temperature-sensitive phenotype of the mutants resulted from mutation of the *top3*⁺ gene. To confirm this, the DNA sequences of the mutated *top3* genes were analyzed (Fig. 1C). Only one amino acid change, Ile155 to Thr, was evident in *top3-ts134* (silent mutations occurred at several sites). Ile155 is conserved between *S.pombe* and *S.cerevisiae* Top3, human Top3α, and *Escherichia coli* Top1. In *top3-ts252*, three amino acid changes were identified Ser18 to Asn, Phe42 to Ser and Cys274 to Tyr. Both mutated *top3* genes were cloned into an integration vector (see Materials and Methods) and stable temperature-sensitive mutants were constructed. The strains whose original *top3*⁺ gene was

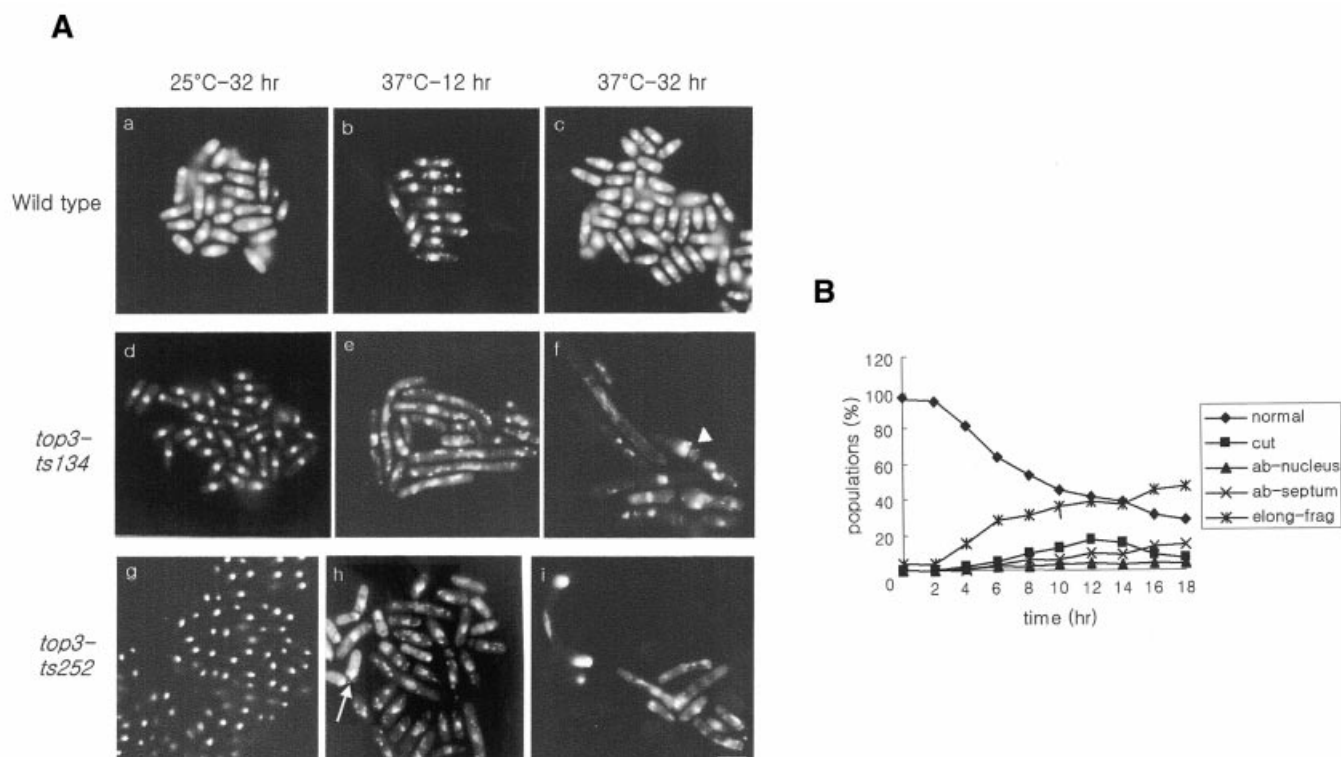


Figure 2. (A) Cellular and nuclear morphologies of *top3-ts* mutants. Parental strain CF199 (panels a–c) and *top3-ts* mutant strains MO134 (panels d–f) and MO252 (panels g–i) were grown at the permissive temperature (25°C) and then shifted to the restrictive temperature (37°C). The arrowhead in panel f indicates an abnormal septum. The arrow in panel h indicates a ‘cut’ cell. Bar, 10 μ m. (B) Cells with each abnormality were counted at 2 h intervals. cut, ‘cut’ phenotype; ab-nucleus, anucleated or off-centered nuclei; ab-septum, multiple septa or a septum formed at one side of cell; elong-frag, elongated cells with fragmented nuclei.

possibly slightly elevated, in an *rqh1-d* mutant background (Fig. 3D). The DNA damage sensitivity of *top3-ts* mutants, combined with the DNA damage inducibility of the *top3⁺* transcript implies an involvement of *top3⁺* in the DNA damage response, possibly during repair. It is likely that Top3 and Rqh1 function in the same DNA damage response pathway(s) because the damage sensitivities of the *top3rqh1* double mutant is equivalent to that of the *rqh1-d* single mutant (14).

The *top3-ts* mutant is defective in recovery from S-phase arrest by HU

A previous report demonstrated that the *rqh1* null mutant was HU sensitive and defective in recovery from S-phase arrest (17). Therefore, we examined if the HU sensitivity of *top3-ts* mutants was similarly related to a recovery defect. Flow cytometry analysis (Fig. 4A) showed that the wild-type, *rqh1-d* mutant, and *top3-ts* mutant could resume and complete bulk DNA replication with similar kinetics following release from HU arrest at 37°C. However, subsequent chromosome segregation in both *rqh1* null and *top3-ts* mutants was defective, indicating that, while bulk DNA replication appeared complete, the chromosomes were either incompletely replicated or remained entangled (Fig. 4B). At 140 min after HU release, both *rqh1-d* and *top3-ts* mutants showed an elongated cell morphology and an increased frequency of ‘cut’ phenotypes

when compared with either controls without HU treatment (Fig. 4B) or to *top3⁺rqh1⁺*-treated controls (data not shown) (17).

We also examined the *top3rqh1* double mutant in the same assay (Fig. 4B). When released from HU arrest, the number of cells with the ‘cut’ phenotype also increased with kinetics similar to the *rqh1-d*. These observations indicate that Rqh1 and Top3 are required for the same late step of DNA replication.

Both Top3 and Rqh1 are required for the fidelity of chromosome segregation

Using a minichromosome loss assay (see Materials and Methods) we measured the loss rate of a non-essential minichromosome in wild-type, *top3-ts*, and *rqh1-d* mutant cells (Fig. 4C). The *rqh1-d* mutant exhibited a 75-fold increased frequency of Ch¹⁶ loss compared with wild-type controls. This was further increased 3.3-fold following 4 h of treatment with 10 mM HU. These observations imply that *rqh1-d* mutant cells are defective in chromosome segregation and that this is exacerbated after a temporary block in S-phase.

The loss rates of *top3-ts* mutant cells were elevated at the permissive temperature by HU treatment (44-fold, compared with wild-type, 1.5-fold) and increased with either a 12 h incubation at the restrictive temperature (62-fold, compared with wild-type cells, 2.2-fold) or transient HU treatment

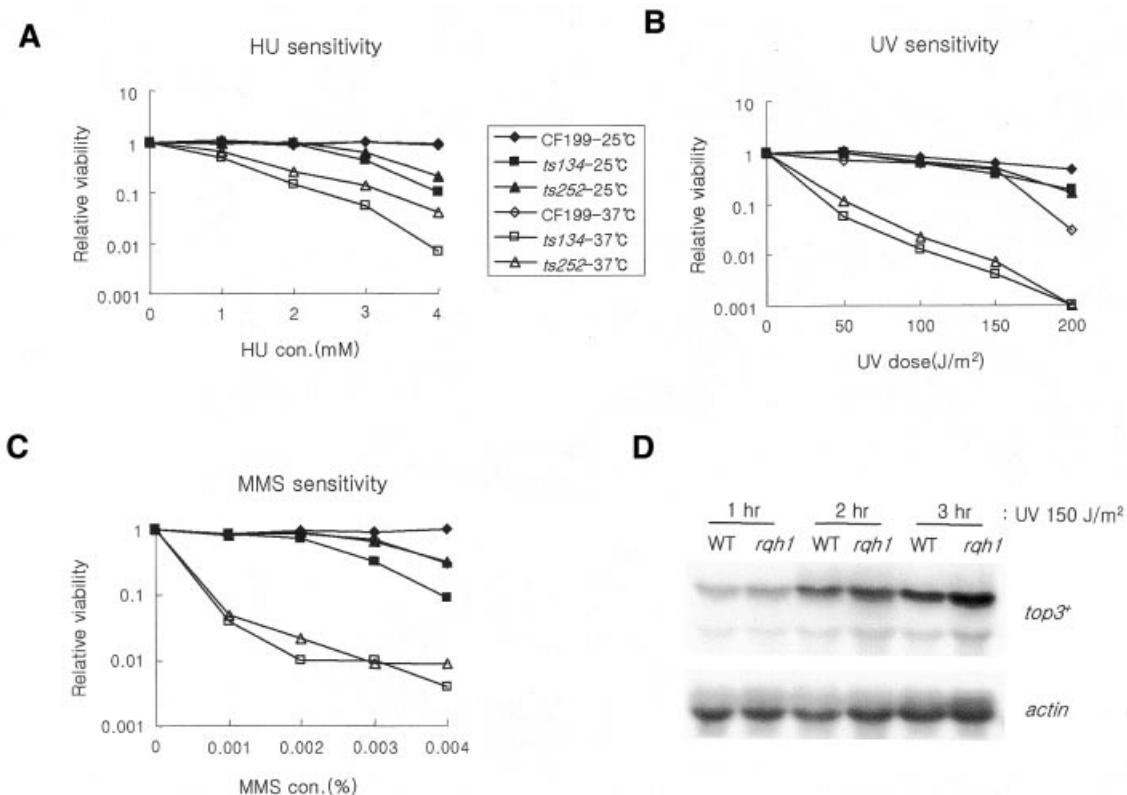


Figure 3. The sensitivity of *top3*-ts mutants to HU (A) and DNA-damaging agents, UV (B) and MMS (C). The sensitivity of wild-type and *top3*-ts mutant strains MO134 and MO252 was measured by colony formation on YES plates containing the indicated concentrations of HU and MMS either at the permissive temperature or at the restrictive temperature for 12 h followed by incubation at 25°C. The relative viability of each strain (25 and 37°C) was calculated by dividing the number of viable cells on plates containing the damaging agents by the number of cells on untreated control plates. (D) The relative levels of *top3*⁺ transcripts in wild-type (WT) and *rqh1*-d mutant 1589 (R1) after UV irradiation were examined by northern blot. After irradiation, total RNA was extracted at 1 h intervals and probed with *top3* cDNA and an actin gene as a loading control.

(106-fold, compared with wild-type, 2-fold). Since *rqh1-d* and *top3*-ts mutants showed very low viability after the HU treatment and *top3*-ts cells lose viability after a temperature shift, the minichromosome loss rates probably underestimate the mis-segregation problem.

The viability of cells in S-phase slightly decreased with a rise in temperature

Because the *top3-ts134* mutant is sensitive to HU, defective in recovery after S-phase arrest and loses Ch¹⁶ dramatically after HU treatment, we examined at which point in the cell cycle the *top3*-ts mutant cells lost viability. Cells were synchronized in G1-phase by nitrogen starvation and aliquots were transferred to 37°C for 2 h at 30 min intervals. The viability was plotted with the septation index of the unshifted culture (Fig. 5A). In *S.pombe* there is a temporal correlation between septation and S-phase and so the septation index is taken as a measure of the number of replicating cells. The viability of the *top3*-ts mutant was modestly reduced by a transient temperature shift during G1-S-phase, but not by a shift during G2. Although these differences are modest [which is not unexpected given the fact that *top3*-ts cells lose viability slowly following a shift of an asynchronous cell culture to 37°C (data not shown)], these results are consistent with the lethality of the *top3*-ts mutant being due to defects during DNA replication.

Chromosome mis-segregation is not due to defects in the anaphase promoting complex (APC) or microtubules

Explanations for chromosome mis-segregation defects in *top3*-ts mutant cells could be that Top3 functions to maintain the architecture of the centromere, or that it affects microtubule interactions with chromosomes, and has an uncharacterized function in APC-catalyzed anaphase initiation. To examine these possibilities, we tested *top3-ts134* mutants for sensitivity to the microtubule-destabilizing agent TBZ and the protease inhibitor TPCK.

At 25°C, TBZ and TPCK had little or no effect on growth of *top3*-ts, *rqh1-d* and *rqh1top3* double mutant cells (Fig. 5B). A 12 h transient shift to the restrictive temperature similarly had little or no effect on *top3*-ts or *rqh1top3* double mutants in the presence of TBZ or TPCK. A modest decrease in viability was observed for *rqh1* null cells after this treatment in the presence of TPCK. These results provide strong evidence that the chromosome mis-segregation defect of *top3*-ts mutant is not due to defects in M-phase.

Rqh1 overexpression causes cell inviability

Rqh1 and Top3 are thought to function together, and to biochemically interact. Our data demonstrate similarities in some phenotypes when Rqh1 is lost or Top3 function is impaired. However, loss of the *rqh1* gene is not lethal, whereas

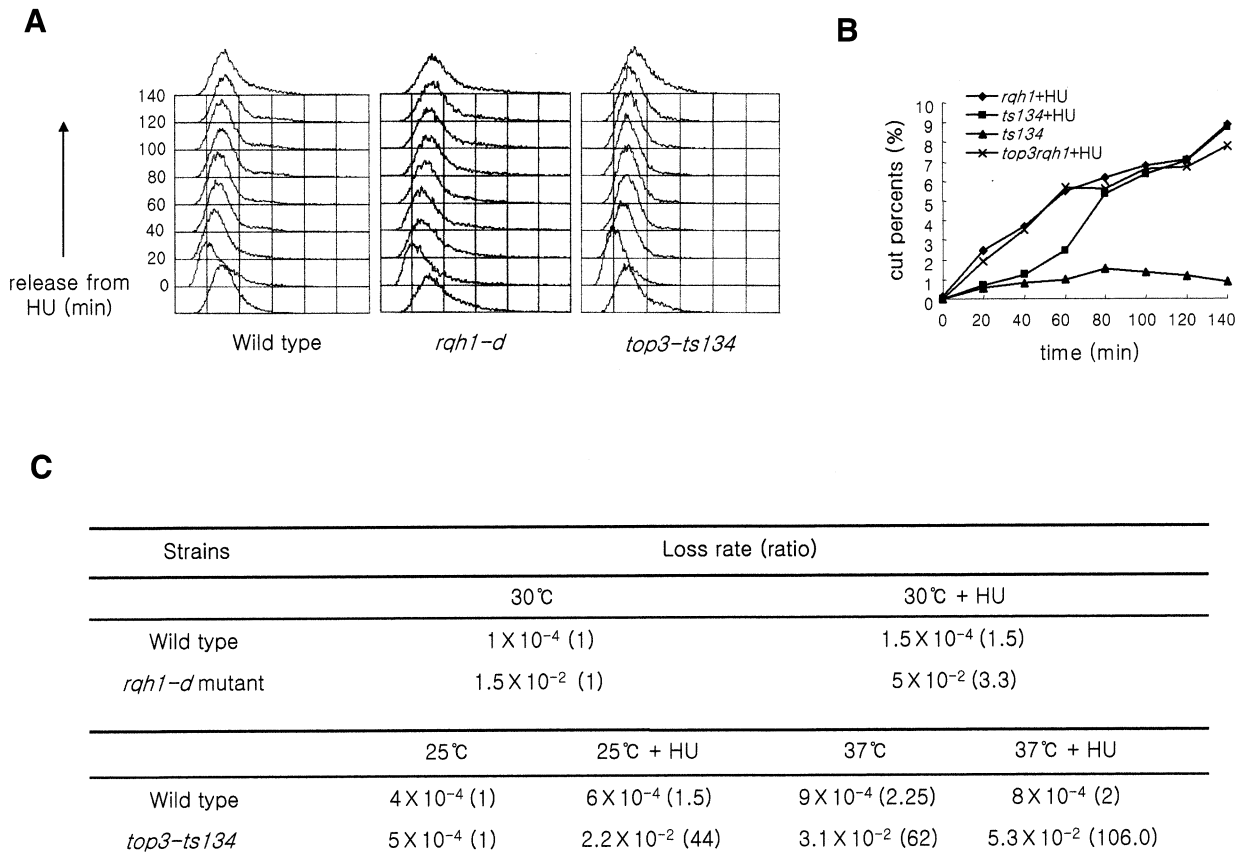


Figure 4. Analysis of DNA contents and the percentage of cells showing the 'cut' phenotype following release from HU arrest. (A) After S-phase arrest (10 mM HU at 37°C for 2 h) cells were fixed for FACS analysis at 20 min intervals. (B) *rqh1-d*, *top3rqh1* double and *top3-ts* mutant cells were treated with 10 mM HU as above and then incubated at 30°C for 4 h in fresh YES media. Cells were sampled every 20 min for DAPI staining. The proportions of cells showing the 'cut' phenotype were plotted against the incubation times after HU removal. (C) The minichromosome loss rates per generation for *rqh1* and *top3-ts* mutants.

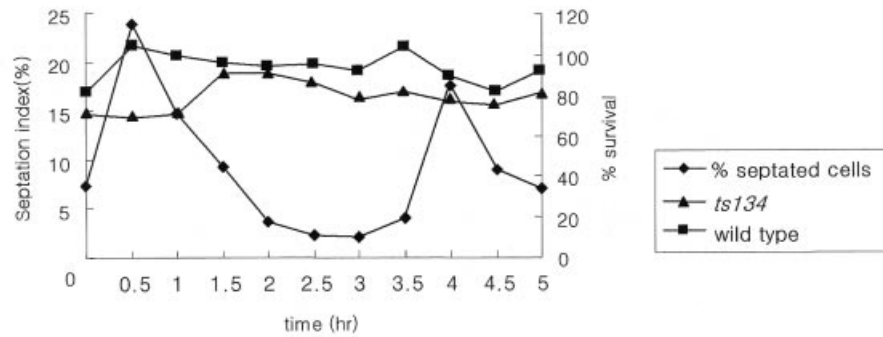
loss of the *top3* gene is. Because the *top3rqh1* double mutant is viable (14,18) and *sgs1* mutants suppress the slow growth of *top3-d* mutant in *S.cerevisiae*, it has thus been suggested (4) that Top3 resolves potentially lethal DNA structures that are produced by Rqh1. If this is the case, we hypothesized that Rqh1 overexpression may itself be a lethal event, as it may perturb the balance of Rqh1–Top3 functions, causing a phenotype similar to that seen with the loss of *top3* function. Indeed, when we expressed *rqh1* from an inducible exogenous promoter (Fig. 6A), both wild-type and *rqh1-d* mutants were unable to grow (Fig. 6B). The morphology of *rqh1*-overexpressing cells was reminiscent of *top3-ts* mutant cells at the restrictive temperature (Fig. 6C). They were elongated, had double or triple septa, abnormal nuclei and showed 'cut' phenotypes. The percentage of 'cut' cells was, however, slightly lower than that seen for *top3-ts* mutant cells at 37°C. The *rqh1*-overexpressing cells also showed an increase in 'cut' cells when they were transiently treated with HU, reminiscent of *top3-ts* cells (data not shown). From these results, we suggest that Top3 has a role in the maintenance of an optimum level of Rqh1 activity or in resolving structures created by Rqh1 helicase, and may function downstream of Rqh1.

We also attempted to examine the sensitivity of *rqh1*-overproducing cells to DNA-damaging agents (Fig. 6D). However, when *rqh1* was overexpressed from the pREP41 promoter in wild-type or *rqh1-d* mutant cells, viability was lost. When the *nmt* promoter was repressed by the addition of thiamine (Fig. 6D, left panels), cells were viable and the DNA damage sensitivity of the *rqh1* null mutant disappeared. These results indicate that the low level expression of Rqh1 from the repressed *nmt* promoter could compensate for the damage sensitivity of the *rqh1-d* mutant.

DISCUSSION

Goodwin *et al.* (14) examined the phenotypic consequences of germinating *top3* deleted spores and observed abnormal nuclei, multiple septa and elongated cells. However, they were not able to directly examine the role of Top3 in DNA damage repair or chromosome stability because the only viable *top3* defective cells examined were, by necessity, also deleted for the *rqh1* gene. In our study, we have examined the phenotype of *top3* deficient cells by creating *top3-ts* mutants. Following a temperature shift to the restrictive temperature, we see morphological phenotypes that are very similar to

A



B

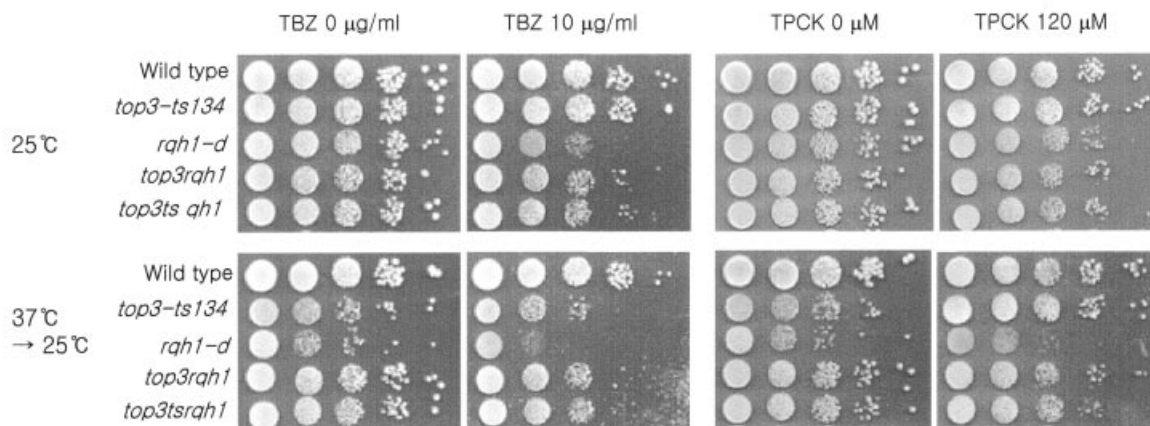


Figure 5. (A) Wild-type and *top3-ts134* mutant cells were arrested in G1 by nitrogen starvation and released into nitrogen-containing media to restart the cell cycle. Upon release, at 30 min intervals, aliquots of cells were transferred to 37°C for 2 h. Cell viability was then checked by plating cells at 25°C. An aliquot was used for counting the septation index at the permissive temperature. (B) The sensitivity of the indicated mutant cells to TBZ and TPCK. Wild-type, the *top3-ts* mutant strain MO134, *rqh1*, and *top3ts rqh1*, and *top3rqh1* double mutant cells were serially diluted and spotted onto duplicate plates contained either 10 µg/ml TBZ or 120 µM TPCK, and onto control plates. One copy of each plate was incubated at 25°C and another was transferred to 25°C after incubation at 37°C for 12 h.

those reported by Goodwin *et al.* (14). The percentage of normal looking cells decreases within a few generations and an increase is seen in cells with an abnormal septum, mispositioned nuclei and 'cut' nuclei. Cells also elongate and ultimately many cells appear to contain fragmented DAPI-staining material.

Top3 is required for chromosome stability

By studying minichromosome stability in *top3-ts* mutants we have been able to demonstrate that *S.pombe top3⁺* is required for the fidelity of chromosome segregation. After a brief shift to the restrictive temperature, we found that minichromosome loss rates were significantly increased. Interestingly, both at the permissive temperature and a brief temperature shift to 37°C, the loss rate of the minichromosome was also increased following a transient incubation with HU, which arrests cells in S-phase. This result is reminiscent of the data from Stewart *et al.* (17), who demonstrated increased chromosome loss in *rqh1* null cells after HU treatment. Because Stewart *et al.* (17) concluded that *rqh1-d* mutant cells were unable to effectively recover viability following a transient arrest in S-phase, we examined this recovery response in more detail. Indeed, we

observed that, like *rqh1* null cells, *top3-ts* cells at the restrictive temperature were ultimately able to complete bulk DNA synthesis, but that this did not represent intact, accurately or completely replicated DNA because the subsequent mitosis and cytokinesis were delayed and ultimately resulted in numerous mitotic defects.

Evidence for an essential function of Top3 during S-phase

On the basis of the sensitivity of *top3-ts* mutants to the S-phase inhibitor HU, we also examined the point in the cell cycle at which *top3-ts* cells lost viability at the restrictive temperature. Our data indicate that, if cells proceed through S-phase with impaired Top3 function, viability is modestly decreased. However, if Top3 function is impaired in G2 for several hours (by a transient temperature shift), viability is not lost. While these effects are quite subtle—which is consistent with the fact that *top3-ts* cells continue cycling for several generations and lose viability quite slowly at the restrictive temperature—we feel that, in concert with the HU sensitivity of *top3-ts* mutants and the fact that transient S-phase delay greatly increases minichromosome loss rates, these data point to an essential

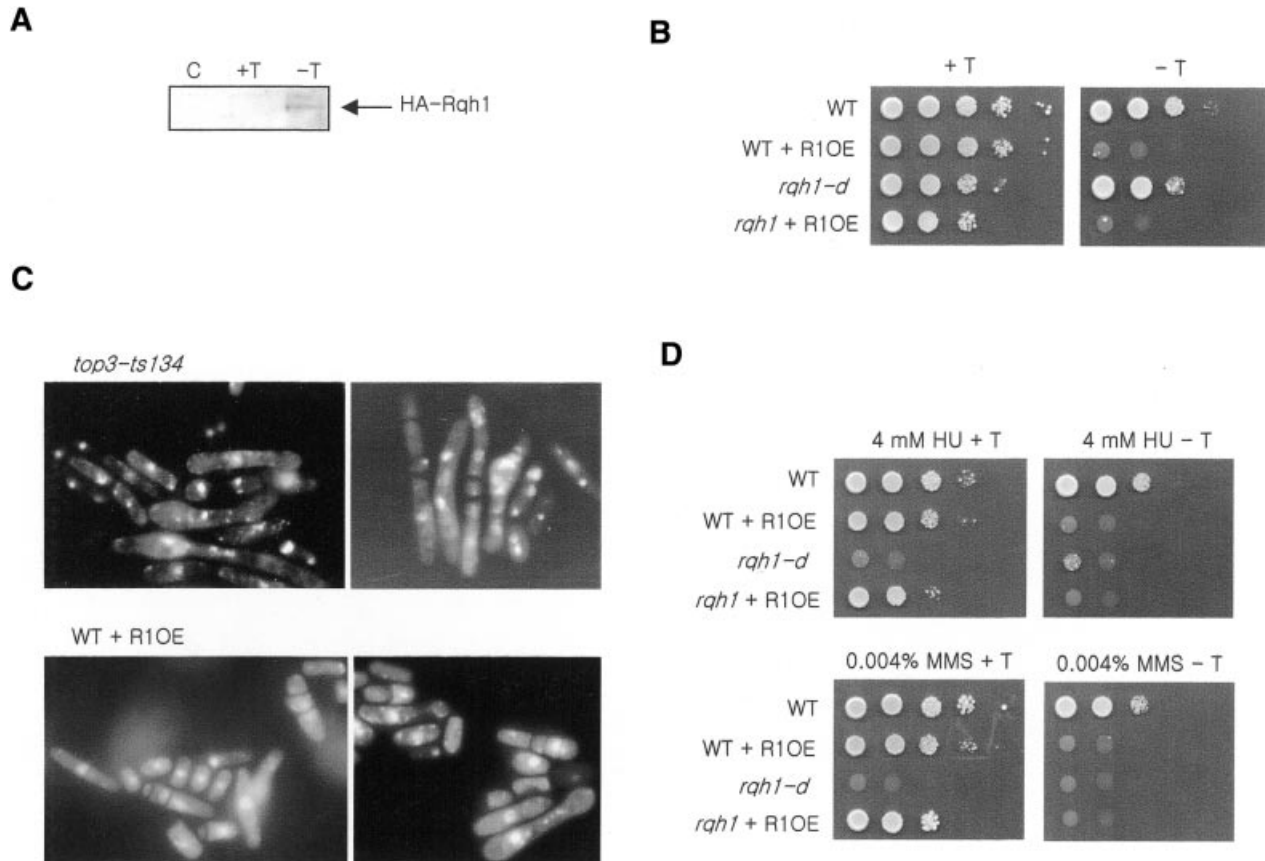


Figure 6. Preparation and examination of *rqh1*-overexpression strains. (A) Rqh1 was fused to three HA epitopes in the pREP41-HAN vector. After induction for 20 h by the removal of thiamine, HA-Rqh1 was detected by western blot analysis using anti-HA antibody. (C) Extract of cells harboring pREP41-HAN control vector induced for 20 h without thiamine; (+T) uninduced pREP41-HR1; (-T) pREP41-HR1 induced for 20 h in the absence of thiamine. The 150 kDa HA-Rqh1 band was detected only in induced pREP41-HR1 extracts. (B) Spotting assay to examine the cell growth and damage sensitivity of Rqh1-overexpressing strains. When Rqh1 was overexpressed (-T) in the wild-type and *rqh1-d* mutant, cell growth was blocked. (C) Cell morphologies of *top3-ts* and *rqh1*-overexpression strains. The *top3-ts* mutant was grown for 32 h at the restrictive temperature. The *rqh1*-overexpression strain was induced for 20 h in the absence of thiamine. Both cultures showed 'cut' phenotypes, abnormal cytokinesis and aberrant nuclear structures. (D) The sensitivity to HU and MMS of the *rqh1* null mutant was complemented by the repressed pREP41-HR1 (+T).

function for *S.pombe* Top3 in S-phase. A recent report revealed that *top3* deletion mutants were highly sensitive to HU and the level of *TOP3* mRNA peaked at late G1-phase in *S.cerevisiae*, suggesting a specific role of Top3 in S-phase (24).

Minichromosome loss mutants fall into a number of classes, including DNA replication and repair genes, genes affecting centromere function and genes affecting the progress through mitosis such as those involved with the APC. In order to provide evidence to support our hypothesis that Top3 function is important in S-phase and not directly within mitosis, we examined the sensitivity of *top3-ts* mutants to microtubule polymerization inhibitor TBZ and to the proteasome inhibitor TPCK. Since *top3-ts* has increased minichromosome loss at the restrictive temperature, we reasoned that, should *top3*⁺ be required for centromere architecture or have an uncharacterized role within mitotic progression, it should (like many mutants affecting mitotic processes) be significantly sensitive to either TBZ or TPCK. In fact, we found no evidence of such sensitivity at either the permissive temperature or combined with a transient temperature shift. We thus conclude that it is

most likely that the essential function for *top3*⁺ is during S-phase.

Top3 is involved in DNA repair

Both the *top3-ts* mutants were sensitive to the DNA-damaging agents UV and MMS at the restrictive temperature. This sensitivity is similar to that seen for the *rqh1-d* single mutant and for *top3rqh1* double mutants. Previously, in *S.pombe* it has not been possible to interpret the observation that *rqh1-d* single and *rqh1top3* double mutants have the same sensitivity to DNA-damaging agents because it was not known if a *top3* mutant alone was DNA damage sensitive. Our data demonstrate that this is the case, and thus allow the conclusion that Top3 is involved in DNA damage survival, that the *top3-ts* mutant is epistatic with the *rqh1-d* mutant and thus Top3 acts in the same DNA repair pathway(s) as Rqh1, as characterized by Murray *et al.* (16). In support of the contention that Top3 has a significant role in the DNA damage responses, we also observed that *top3*⁺ transcript levels were significantly induced by DNA-damaging agents. In addition, Chakraverty *et al.* (24) demonstrated that *top3* deletion mutants were

defective in Rad53 phosphorylation following DNA damage during S-phase in *S.cerevisiae*, suggesting the role of Top3 in DNA damage response during S-phase.

Conclusions

Several models have been proposed to explain the function of the Top3–Sgs1 complex in *S.cerevisiae*. One possibility is that the complex acts at the termination step of replication (15,25). Another possibility is that the Top3–Sgs1 complex is involved in the processing of structures arising at stalled replication forks (26). Our results are consistent with both these possibilities, which are not themselves mutually exclusive. The high frequency of ‘cut’ phenotypes and the elevated minichromosome loss rate we see in the *top3-ts* mutant after a transient temperature shift or following HU treatment support a role in the termination step of replication by implying that Top3 is involved in chromosome decatenation and separation. However, *top3-ts* and *rqh1* mutants are both sensitive to DNA-damaging agents, which is consistent with a role in the repair of stalled replication forks. It is, of course, possible that Top3–Rqh1 play roles in both processes or that incorrect repair at sites of stalled replication forks can cause an irreversible catenation event in the absence of Top3. Significant further work to explore these possibilities will be necessary to understand the role of Top3 and Rqh1.

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