Requirement for *Schizosaccharomyces pombe* Top3 in the maintenance of chromosome integrity

Thein Z. Win[1], Adele Goodwin[2], Ian D. Hickson[2], Chris J. Norbury[3] and Shao-Win Wang[1,*]

[1]Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3PS, UK
[2]Cancer Research UK Laboratories, University of Oxford, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, OX3 9DS, UK
[3]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, UK
*Author for correspondence (e-mail: shaowin.wang@zoo.ox.ac.uk)

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Summary

In *Schizosaccharomyces pombe*, topoisomerase III is encoded by a single gene, *top3*$^+$, which is essential for cell viability and proper chromosome segregation. Deletion of *rqh1*$^+$, which encodes the sole RecQ family helicase in *S. pombe*, suppresses the lethality caused by loss of *top3*. Here, we provide evidence suggesting that the lethality in *top3* mutants is due to accumulation of aberrant DNA structures that arise during S phase, as judged by pulsed-field gel electrophoresis. Using a *top3* shut-off strain, we show here that depletion of Top3 activates the DNA damage checkpoint associated with phosphorylation of the checkpoint kinase Chk1. Despite activation of this checkpoint, *top3* cells exit the arrest but fail to undergo faithful chromosome segregation. However, these mitotic defects are secondary to chromosomal abnormalities that lead to the lethality, because advance into mitosis did not adversely affect cell survival. Furthermore, *top3* function is required for maintenance of nucleolar structure, possibly due to its ability to prevent recombination at the rDNA loci. Our data are consistent with the notion that Top3 has a key function in homologous recombinational repair during S phase that is essential for ensuring subsequent fidelity of chromosome segregation.

Key words: DNA Topoisomerase III, DNA damage checkpoint, Nucleolus

Introduction

DNA topoisomerases play important roles in DNA metabolism through their ability to catalyse the inter-conversion of topological isomers of DNA (Champoux, 2001; Wang, 1996; Watt and Hickson, 1994; Wigley, 1995). The fission yeast *Schizosaccharomyces pombe* expresses three topoisomerases, designated topoisomerases I, II and III. While the functions of topoisomerases I and II are quite well established, the role of topoisomerase III is not fully understood, in part because this class of enzyme possesses only weak DNA relaxation activity and is thought unlikely to participate in the maintenance of DNA supercoiling homeostasis (Goulaouic et al., 1999; Kim and Wang, 1992). The best-characterised *top3* gene is from the budding yeast *Saccharomyces cerevisiae*. In this organism, deletion of *Top3* results in hyper-recombination between repetitive DNA elements, slow growth due to a propensity to arrest at the G2-M DNA damage checkpoint, and defects in sporulation and S-phase responses to DNA damage (Chakraverty et al., 2001; Gangloff et al., 1999; Wallis et al., 1989). In contrast, deletion of *top3* in *S. pombe* results in defective nuclear division and lethality (Goodwin et al., 1999; Maftahi et al., 1999). Two human topoisomerase III homologues, TOPOIIIα and TOPOIIIβ, have been identified (Hanai et al., 1996; Ng et al., 1999) and murine *top3*α has been shown to be essential for embryonic development (Li and Wang, 1998). Mice lacking *top3*β develop to maturity but show a reduced mean lifespan (Kwan and Wang, 2001).

Accumulating evidence suggests that RecQ helicases act in concert with topoisomerase III. Interestingly, mutation of *SGS1* or *rqh1*$^+$, which encode the sole RecQ homologues found in budding and fission yeast, respectively, can suppress the deleterious effects of loss of *top3* function (Gangloff et al., 1994; Goodwin et al., 1999; Maftahi et al., 1999). There are five human RecQ-like helicase proteins: BLM, WRN, RECQL, RECQ4 and RECQ5. WRN is mutated in the premature ageing disorder Werner's syndrome and RECQ4 is defective in Rothmund-Thomson syndrome (Kitao et al., 1999; Yu et al., 1996). Mutations in BLM cause Bloom syndrome (Ellis et al., 1995), the hallmark of which at the cellular level is an unusually high frequency of sister chromatid exchanges (Chaganti et al., 1974). RECQ5 physically interacts with TOP3α and TOP3β (Shimamoto et al., 2000) and the BLM protein binds to TOP3α (Wu et al., 2000), while overexpressed *Caenorhabditis elegans* TOP3α interacts physically with the RecQ homologue Him6 in vitro (Kim et al., 2002). Rqh1, the single *S. pombe* homologue, exists with Top3 in a high-molecular-weight complex (Laursen et al., 2003). The *S. cerevisiae* Sgs1 and Top3 proteins also interact physically, raising the possibility that Sgs1 may recruit Top3 to its site of action (Bennett and Wang, 2001).

Several observations suggest that the function of the Top3-RecQ complex is required during S phase. *S. cerevisiae sgs1* and *top3* mutants are sensitive to hydroxyurea (HU), which blocks DNA replication by depletion of dNTP pools (Frei and Gasser, 2000; Mullen et al., 2000). In *S. pombe*, treatment of a temperature-sensitive *top3* mutant with HU leads to increased

chromosome segregation defects (Oh et al., 2002) and inactivation of *rqh1* also causes HU sensitivity and a defect in recovery from S phase arrest (Enoch et al., 1992; Stewart et al., 1997). It has been suggested that *rqh1*$^+$ is required to prevent recombination and that suppression of inappropriate recombination is essential for reversible S phase arrest. Consistent with this proposal, expression of a bacterial Holliday junction resolvase can partially suppress the HU and UV sensitivities of *rqh1* mutants (Doe et al., 2000). Moreover in vitro, Sgs1, BLM and WRN proteins can efficiently migrate synthetic four-way DNA structures that represent Holliday junctions (Bennett et al., 1998; Gray et al., 1997; Karow et al., 1997). Such structures can be detected during S phase and perturbation of replication leads to an elevation in their frequency (Zou and Rothstein, 1997). The idea that Top3-RecQ complexes play a role in homologous recombination is further supported by the demonstration that in budding and fission yeast these proteins act in a common pathway, and that inactivation of homologous recombination suppresses defects in *top3* mutants (Laursen et al., 2003; Oakley et al., 2002; Shor et al., 2002). Furthermore, it has been shown that BLM and TOP3α act together in vitro in the resolution of a recombination intermediate containing a double Holliday junction, suggesting that in vivo they may suppress crossing over during homologous recombination (Wu and Hickson, 2003).

To understand the biological function of Top3, we have further characterised the phenotype of a *top3* temperature-sensitive mutant. We show that in *top3* mutants, chromosomes become intertwined during S phase, leading to DNA double-strand break accumulation, checkpoint activation, and chromosome mis-segregation. These data implicate Top3 function in processing aberrant chromosome structures during DNA replication.

Materials and Methods

Fission yeast strains and methods

Conditions for growth, maintenance and genetic manipulation of fission yeast were as described previously (Moreno et al., 1991). A complete list of the strains used in this study is given in Table 1. Except where stated otherwise, strains were grown at 30°C in YE5S or EMM2 medium with appropriate supplements. Where necessary, gene expression from the *nmt1* promoter was repressed by the addition of 60 μM thiamine to the growth medium. Cell concentration was determined with a Sysmex F-800 cell counter (TOA Medical Electronic, Japan).

Immunochemistry

Cell extracts were prepared by trichloroacetic acid precipitation following glass bead disruption (Caspari et al., 2000). Immunoblotting was performed essentially as described elsewhere (Ausubel et al., 1995). The mouse anti-influenza haemagglutinin (HA) monoclonal HA-11 (Babco, Berkeley, CA) was used for detection of HA-tagged Top3 and Chk1 proteins. Cdc2 was detected using the mouse monoclonal antibody Y100 (generated by J. Gannon and kindly provided by H. Yamano). Horseradish peroxidase-conjugated anti-mouse antibodies (Sigma, Poole, UK) and enhanced chemiluminescence (ECL, Amersham) were used to detect bound antibody.

Microscopy and flow cytometry

Cells fixed in 70% ethanol were re-hydrated and stained with 4′,6-diamidino-2-phenylindole (DAPI) before examination by fluorescence microscopy. Visualisation of GFP protein in living cells, embedded in 0.6% LMP agarose after staining with Hoechst 33342 (5 μg/ml), was performed at room temperature as previously described (Wang et al., 2002). Images were acquired using a Zeiss Axioplan 2 microscope equipped with a Planapochromat 100× objective, an Axiocam cooled CCD camera and Axiovision software (Carl Zeiss, Welwyn Garden City, UK), and were assembled using Adobe PhotoShop. For flow cytometry, cells fixed with 70% ethanol were re-hydrated in 10 mM EDTA, pH 8.0, 0.1 mg/ml RNase A, 1 μM sytox green, and incubated at 37°C for 2 hours. Cells were analysed using a Coulter Epics XL-MCL (Fullerton, CA).

Pulsed-field gel electrophoresis

DNA plugs were prepared according to the manufacturer's instructions (Bio-Rad, Hercules, CA) with the following modification. 2×10^7 cells for each plug were embedded in 1% low melting point agarose containing 1 mg/ml Zymolyase in suspension buffer (10 mM Tris, pH 7.2, 20 mM NaCl, 50 mM EDTA) and were digested for 1 hour at 37°C in Lyticase buffer (10 mM Tris, pH 7.2, 50 mM EDTA, 1 mg/ml Zymolyase). Plugs were incubated for 90 minutes at 55°C in 1% SDS, 50 mM Tris, pH 7.5, 0.25 mM EDTA before being treated

Table 1. Yeast strains used in this study

Strains	Genotype	Reference
972	*h*$^-$	Lab stock
top3-134	*h*$^-$ *top3-134*	S. D. Park*
Δ*rad1*	*h*$^-$ *rad1::ura4*$^+$ *ade6 leu1 ura4*	A. M. Carr
top3-134 Δ*rad1*	*h*$^-$ *top3-134 rad1::ura4*$^+$ *ura4*	This study
Δ*rad17*	*h*$^-$ *rad17::ura4*$^+$ *ade6 leu1 ura4*	A. M. Carr‡
top3-134 Δ*rad17*	*h*$^-$ *top3-134 rad17::ura4*$^+$ *ade6 leu1 ura4*	This study
Δ*cds1*	*h*$^-$ *cds1::ura4*$^+$ *ade6 leu1 ura4*	A. M. Carr‡
top3-134 Δ*cds1*	*h*$^-$ *top3-134 cds1::ura4*$^+$ *ura4*	This study
Δ*chk1*	*h*$^-$ *chk1::ura4*$^+$ *ade6 leu1 ura4*	A. M. Carr‡
top3-134 Δ*chk1*	*h*$^-$ *top3-134 chk1::ura4*$^+$ *ura4*	This study
Δ*rad3*	*h*$^-$ *rad3::ura4*$^+$ *ade6 leu1 ura4*	A. M. Carr‡
Δ*rad26*	*h*$^-$ *rad26::ura4*$^+$ *ade6 leu1 ura4*	A. M. Carr‡
top3-P41 chk1-HA	*h*$^-$ *top3<< kan*^{r}*-nmt41-HA-top3*$^+$ *chk1-HA ade6 leu1 ura4*	This study
gar2-GFP	*h*$^-$ *gar2*-GFP::*kan*^{r} *leu1 ura4*	M. Yamamoto§
top3-134 gar2-GFP	*h*$^-$ *top3-134 gar2*-GFP::*kan*^{r} *leu1*	This study

*School of Biological Science, Seoul, Republic of Korea; ‡Genome Damage and Stability Centre, Brighton, UK; §Department of Biophysics and Biochemistry, Tokyo, Japan.

with proteinase K in 1% lauroyl sarcosine, 0.5 M EDTA pH 8.0 for 48 hours at 55°C. Before electrophoresis, plugs were equilibrated in TE for at least 1 hour. Pulsed-field gel electrophoresis was carried out with a 0.8% chromosomal grade agarose gel in 1× TAE buffer (40 mM Tris-acetate, 2 mM EDTA) by using a CHEF III apparatus (Bio-Rad, Hercules, CA). The settings were as follows: 2 V/cm; switch time, 30 minutes; angle, 106°; 14°C, 48 hours.

Results

Top3 mutants accumulate defects during S phase

In contrast to its homologue in *S. cerevisiae*, the *S. pombe top3*$^+$ gene is essential for cell viability. Fission yeast cells deficient in *top3* survive only a limited number of cell divisions before arresting as highly elongated cells with aberrant chromosome morphologies indicative of defects in chromosome segregation (Goodwin et al., 1999; Maftahi et al., 1999). To explore the role of *top3* in chromosome segregation, *top3-134* cells were synchronized in G1 by nitrogen starvation and then released from the arrest by transfer to nitrogen-rich medium at the non-permissive temperature. As shown in Fig. 1A, *top3-134* cells entered and proceeded through S phase with kinetics similar to those of wild-type cells. Both strains initiated DNA replication by 2 hours after nitrogen re-feeding and completed S phase by 5 hours. These results indicate that *top3* is not required for bulk DNA replication. However, we observed a significant delay in entry into mitosis in *top3-134* cells (57% reduction in mitotic index as compared with wild-type cells at 6 hours; Fig. 1B). Moreover, *top3-134* cells that underwent mitosis frequently mis-segregated their chromosomes (Fig. 1C) with chromosomes lagging between the separating daughter nuclei (Fig. 1D). After 8 hours, *top3-134* cells became highly elongated and displayed aberrant chromosome morphologies. These cells often contained numerous DAPI-staining bodies indicative of fragmented chromosomes (data not shown). It has been proposed that Top3 functions together with RecQ helicase in processing DNA lesions that arise during S phase (Wu and Hickson, 2003). To determine whether passage through S phase elicited the defects in *top3-134* cells, we performed analogous experiments in which the cells were allowed to complete S phase (5 hours in nitrogen-rich medium at 26°C) before inactivation of Top3 (Fig. 2A). In contrast to the results shown in Fig. 1B, *top3-134* traversed mitosis with kinetics similar to those of wild-type cells (Fig. 2B). Furthermore, mis-segregation defects were dramatically reduced in *top3-134* cells, which only occurred as cells went through the next cell cycle (Fig. 2C). Taken together, these results indicate that some aspect of S phase is defective in *top3-134* cells and that these defects manifest themselves only later in the cell cycle, during mitosis, as aberrant chromosome segregation.

Activation of the DNA damage checkpoint in *top3* mutants

The observed mitotic delay and cell elongation suggest that inactivation of *top3* leads to a checkpoint-mediated cell cycle arrest, as shown previously for *S. cerevisiae top3* deletion strains (Chakraverty et al., 2001). To explore the relationship between the loss of *top3* function and checkpoint pathways, we analysed the effects on the phenotype of *top3-134* cells with deletion of the genes encoding effector kinases Chk1, which

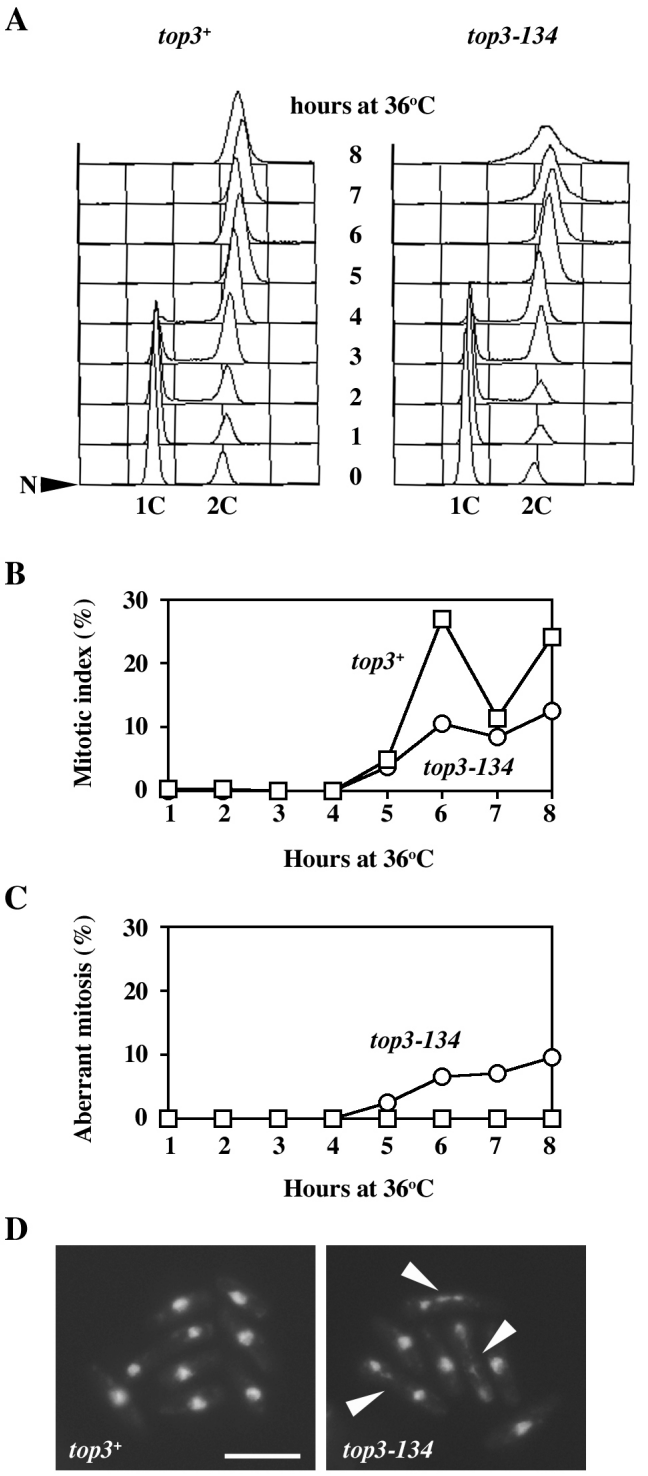

Fig. 1. *top3-134* mutants accumulate defects during S phase. Wild type (*top3*$^+$) and *top3-134* cells were arrested in G1 by nitrogen starvation and released into nitrogen-rich medium to restart the cell cycle at the non-permissive temperature of 36°C. Cells harvested at hourly intervals were processed for flow cytometry (A), and mitotic index was assessed by scoring bi-nucleate cells (B). The percentage of cells displaying aberrant mitosis with chromosomes lagging between the separating daughter nuclei (indicated by arrowheads in D) was also determined (C). (D) Fluorescence micrographs of DAPI-stained wild type and *top3-134* cells, 6 hours after release from nitrogen starvation at 36°C. Bar, 10 μm.

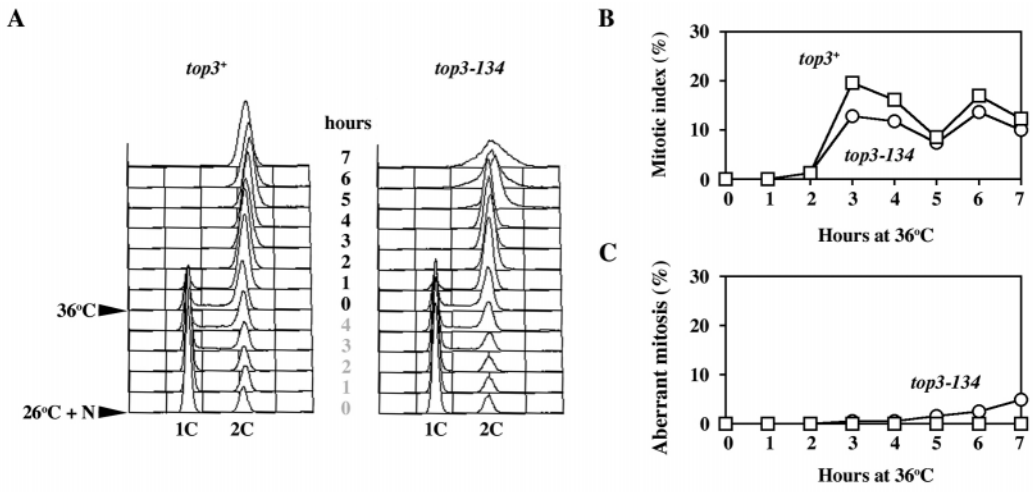

Fig. 2. Chromosome segregation defects in *top3-134* cells require passage through S-phase. G1-arrested wild type (*top3*$^+$) and *top3-134* cells were released into nitrogen-rich medium to restart the cell cycle at the permissive temperature of 26°C for 5 hours before shifting to the restrictive temperature of 36°C. Cells harvested at hourly intervals were processed for flow cytometry (A), and assessed for mitotic index (B) and aberrant mitosis (C) as in Fig. 1.

acts in the DNA damage checkpoint (Walworth et al., 1993) and Cds1, which acts in the S-M checkpoint (Murakami and Okayama, 1995). Interestingly, deletion of *chk1*$^+$ but not *cds1*$^+$ suppressed the cell cycle arrest in *top3-134* cells. Whereas Δ*cds1 top3-134* cells became highly elongated to the same extent as *top3-134* cells at the restrictive temperature, Δ*chk1 top3-134* cells were smaller and of a more uniform size (Fig. 3A). These data suggest that the elongation and cell cycle arrest are DNA damage checkpoint responses that signal through Chk1.

Recently, it was shown that Top3 exists in a high-molecular-weight complex even in the absence of Rqh1 (Laursen et al., 2003). It is therefore possible that the phenotype of *top3* temperature-sensitive mutants reflects the dissociation of such a complex rather than the loss of Top3 activity per se. We therefore analysed further the effect of depletion of Top3 by generating a 'shut-off' strain *top3-P41* containing *top3* under the control of the thiamine-repressible attenuated *nmt41* promoter, at the same time introducing an HA epitope tag sequence fused in-frame to the 5′ end of the *top3* open reading frame. An HA epitope-tagged *chk1* allele was also introduced into this strain. The growth defects of *top3* shut-off cells were investigated by measuring their growth rate in liquid medium containing thiamine (Fig. 4). These cultures were incubated at 36°C to improve the synchrony of appearance of the *top3* phenotype. Anti-HA immunoblotting showed that most of the Top3 was depleted by 3 hours after addition of thiamine (Fig. 4C). The generation time of cultures without thiamine was 4.24 hours. Depletion of Top3 inhibited cell proliferation such that the generation time measured 9 hours after addition of thiamine was 20 hours (Fig. 4A). By 18 hours after thiamine addition, these cells had completely ceased dividing. DAPI staining and microscopy revealed that cells depleted for Top3 became highly elongated 12 hours after addition of thiamine (Fig. 4B). After prolonged incubation a variety of nuclear defects were observed in these cells, including the 'cut' phenotype as well as extensive nuclear DNA fragmentation, identical to the phenotype of *top3* temperature-sensitive mutants. In addition, deletion of *chk1* but not *cds1* suppressed the cell cycle arrest in these cells (data not shown). In line with these data, immunoblot analysis revealed the appearance of a slower-migrating band of phosphorylated Chk1, coincidently with the disappearance of Top3 signal following addition of thiamine (Fig. 4C). Phosphorylation of Chk1 is associated with activation of its protein kinase activity and is used as a surrogate marker of checkpoint activation (Walworth et al., 1993). These data suggest that the elongation and cell cycle arrest following depletion of Top3 (Fig. 4A,B) are DNA damage checkpoint responses.

Given the effects that deletion of checkpoint genes had on the cell cycle distribution in *top3-134* cells, we investigated the consequences for cell viability. We reasoned that the G2 checkpoint arrest following inactivation of *top3* would be important for cell survival and loss of this checkpoint control would lead to a more rapid loss of viability. However, as shown in Fig. 3B, shortening the period of G2 arrest by deletion of *rad1* did not appear to affect survival adversely, because Δ*rad1 top3-134* double mutants and *top3-134* single mutants showed comparable levels of cell survival after shift to the restrictive temperature (Fig. 3C). These data suggest that the lethality in *top3-134* cells results from the failure to resolve DNA structures rather than the defect in chromosome segregation or the failure to re-enter the cell cycle.

During the course of construction of double mutants, we identified a specific interaction between *top3* and *rad3* or *rad26*, but not with other checkpoint genes. As shown in Fig. 5, whereas deletion of *rad3* or *rad26* resulted in synthetic lethality in *top3-134* cells, deletion of genes encoding the checkpoint sliding clamp protein Rad1 or clamp loader Rad17 had no effect on the growth of *top3-134* cells at the permissive temperature. Consistent with the genetic interaction between *top3* and *rqh1*, similar results have been described for strains combining Δ*rqh1* and checkpoint mutations. Deletion of *rqh1* is synthetic lethal in combination with either Δ*rad3* or Δ*rad26* but not with other checkpoint mutations (Murray et al., 1997). These data indicate that *rad3* might have a function in addition

to its role in checkpoint control that is required for the survival of *rqh1* and *top3* mutants.

Top3 is required for maintenance of chromosome structure

The results presented above suggest that the lethality in *top3-134* cells results from a failure to resolve DNA structures arising during S phase. To determine the nature of these abnormal structures, we used pulsed-field gel electrophoresis (PFGE) to assess the integrity of the three *S. pombe* chromosomes. Incomplete DNA replication and the accumulation of unresolved replication forks yield *S. pombe* DNA samples that cannot enter the gel (Waseem et al., 1992). If *top3-134* cells accumulate abnormal structures during S-phase, we would expect to see a reduction in the amount of DNA entering the gel. Indeed, we consistently observed that the total amount of DNA entering the gel was lower for *top3-134* cells (from four independent isolates), even when grown at permissive temperature, than for wild-type cells (Fig. 6A). In addition, a diffuse zone of faster migrating fragmented DNA running below chromosome III, indicating DNA double-strand breaks, was also observed. These results are consistent with the chromosome segregation defects (see below) as attempts to segregate these entangled chromosomes would lead to chromosome fragmentation. Significantly, no obvious difference was seen between the intensity of chromosomes isolated from *top3-134* and wild-type cells arrested in G1 (Fig. 6B). This suggests that the pattern of chromosomal abnormality seen in exponentially growing *top3-134* cultures is specifically associated with the S and G2-M phases of the cell cycle. Furthermore, we consistently observed anomalous migration of chromosome III from *top3-134* cells, which had a significantly faster mobility than the wild-type chromosome, and often showed a greater reduction in the intensity of signal compared with that of chromosome I or II (Fig. 6A, *top3-134* number 2 and 4). A similar result was recently described in Δ*rqh1* cells, suggesting that Rqh1 and Top3 might function together in the maintenance of the rDNA repeats located at the ends of chromosome III (Coulon et al., 2004).

To substantiate the link between DNA replication and aberrant chromosome structures in *top3* mutants, *top3-134* cells were treated with HU for 3 hours at 36°C. Consistent with previous findings (Oh et al., 2002), both *top3-134* and wild-type cells responded to HU and arrested in S phase (Fig. 6D). As exponentially growing cells are mostly in G2, these data

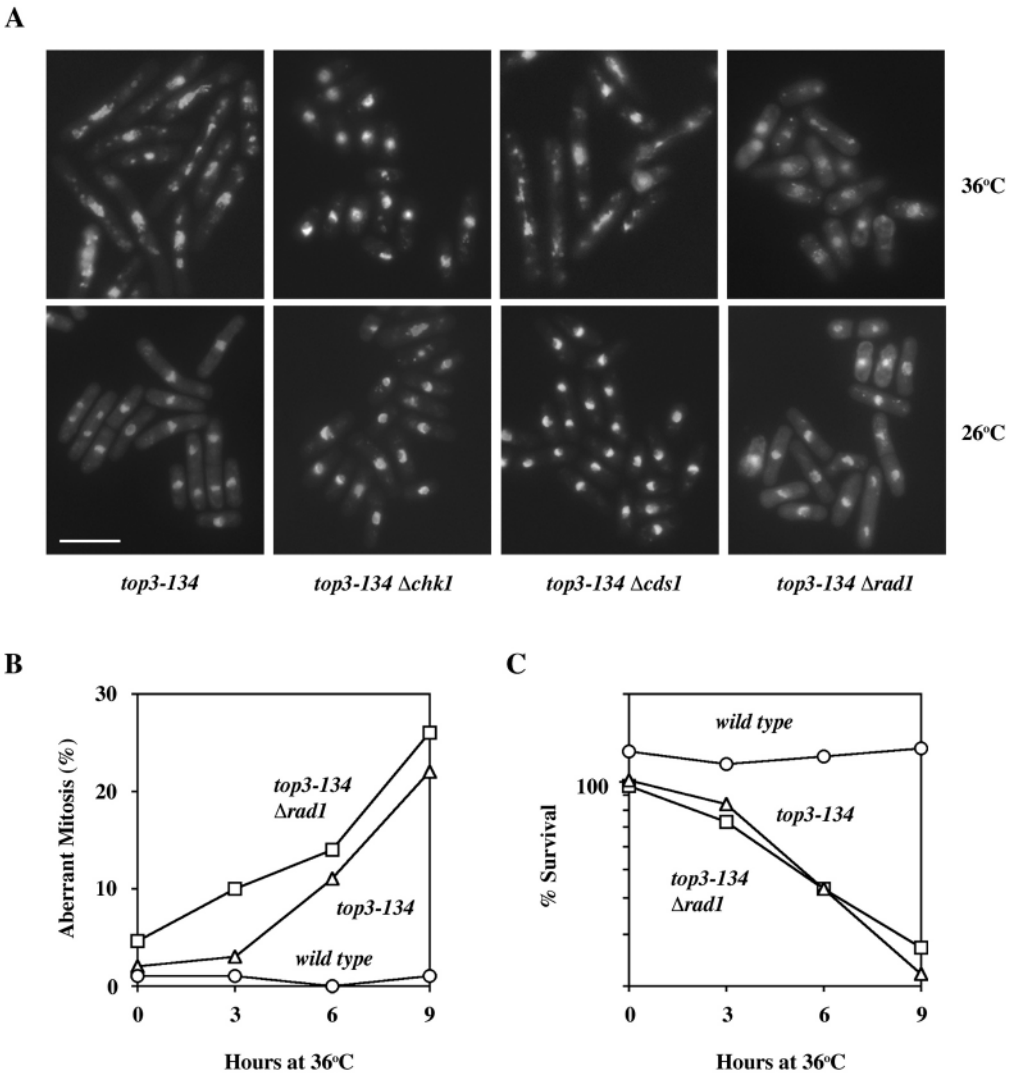

Fig. 3. The DNA damage checkpoint is activated in *top3* mutants. (A) Fluorescence micrographs of DAPI-stained *top3-134*, *top3-134* Δ*chk1*, *top3-134* Δ*cds1 and top3-134* Δ*rad1* cells grown at the permissive temperature of 26°C or after shift to 36°C for 9 hours. Bar, 10 μm. (B) At 3-hour intervals, samples from cultures of wild type (972), *top3-134* and Δ*rad1 top3-134* cells incubated at the restrictive temperature of 36°C were taken to score the percentage of aberrant mitosis in each culture. (C). One thousand cells from B at the times indicated were plated onto YES plates to assess cell survival. Colonies were counted after 5 days growth at 26°C.

further support the idea that *top3-134* cells must undergo S phase to accumulate chromosome segregation defects in the subsequent mitosis. This was further confirmed by PFGE, which showed that chromosomes from both strains remained in the well due to unresolved replication intermediates (Fig. 6C, time 0). After release from the HU block, both wild type and *top3-134* cells resumed the cell cycle and completed DNA replication with similar kinetics at 1 hour (Fig. 6D). However, a significant difference in chromosome integrity was observed on PFGE analysis. In contrast to the DNA from wild-type cells that re-entered the gel and separated into three chromosomes, DNA from *top3-134* cells failed to enter the gel even after 3 hours when the cells had entered mitosis (Fig. 6C,E). These data suggest that *top3-134* cells undergo mitosis with unresolved DNA structures and are not able to separate their chromosomes effectively (Fig. 6E). Similar observations have been made in *rqh1* mutants upon treatment of HU (Stewart et al., 1997), in line with the idea that Top3 functions together with Rqh1 in processing DNA lesions that arise during S-phase.

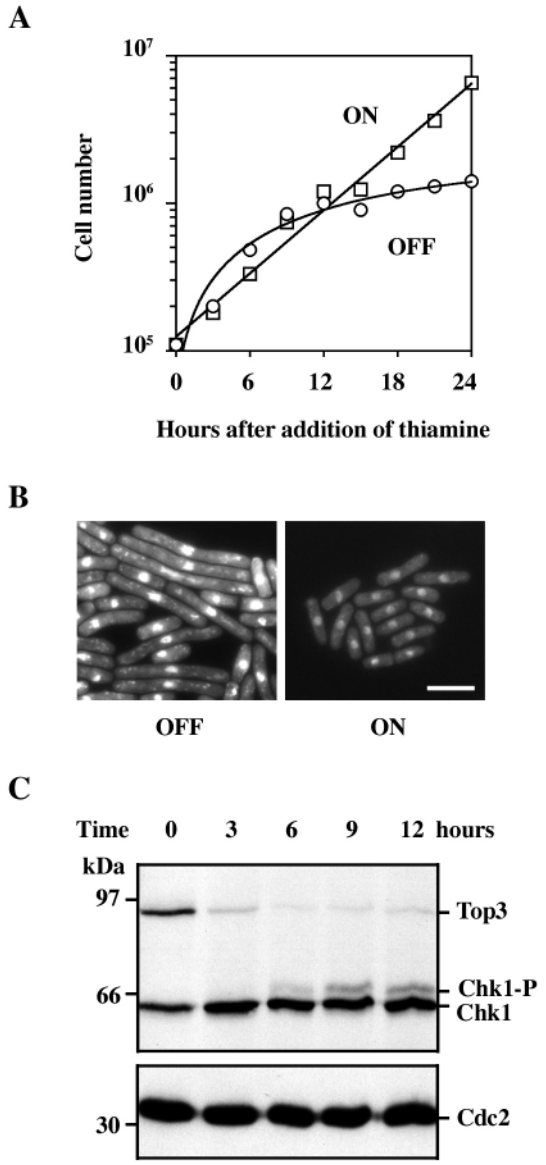

Fig. 4. Depletion of Top3 leads to phosphorylation of Chk1. (A) Growth curves of exponentially growing cultures of the *top3-P41* strain in the presence (○) or absence (□) of thiamine at 36°C. (B) Cell and nuclear morphologies of samples from A were determined by fluorescence microscopy of DAPI-stained cells 12 hours after the addition of thiamine. Bar, 10 μm. (C) Whole-cell protein extracts were prepared by trichloroacetic acid precipitation following glass bead disruption. The extracts were separated by SDS-PAGE and subjected to immunoblotting using anti-HA (upper panel) or anti-Cdc2 (loading control) antibodies as indicated.

Nucleolar segregation is defective in *top3-134* mutants

We explored the nature of the abnormal DNA structures further by using proteins tagged with green fluorescent protein (GFP). It has been shown that the organization of rDNA genes strongly influences the organization and localization of the nucleolus (Oakes et al., 1998). We therefore reasoned that the abnormal chromosome structure in *top3-134* cells might affect nucleolar architecture. To address this point, nucleolar structure was

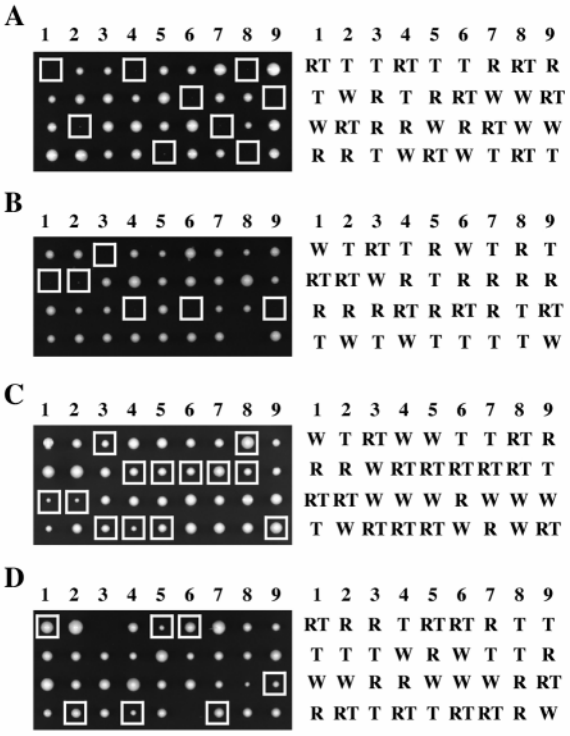

Fig. 5. *top3-134* is synthetically lethal in combination with deletion of *rad3*/*rad26* but not with deletion of other checkpoint genes. (A-D) Tetrads derived from diploid strains of the following genotypes were microdissected onto YES agar: (A) h^+/h^- *rad3*::*ura4*$^+$/*rad3*$^+$ *top3-134*/*top3*$^+$; (B) h^+/h^- *rad26*::*ura4*$^+$/*rad26*$^+$ *top3-134*/*top3*$^+$; (C) h^+/h^- *rad1*::*ura4*$^+$/*rad1*$^+$ *top3-134*/*top3*$^+$; and (D) h^+/h^- *rad17*::*ura4*$^+$/*rad17*$^+$ *top3-134*/*top3*$^+$. Colonies resulting from nine tetrads in each case were photographed after seven days growth at 26°C. The genotypes of the segregants were determined by replica plating and are indicated schematically [right: (A) W, *top3*$^+$ *rad1*$^+$ *rad3*$^+$ *rad17*$^+$ *rad26*$^+$; T, *top3-134*, R, Δ*rad3*; (B) Δ*rad26*; (C) Δ*rad1*; or (D) Δ*rad17*; (A) RT, *top3-134* Δ*rad3*; (B) *top3-134* Δ*rad26*; (C) *top3-134* Δ*rad1*; (D) or *top3-134* Δ*rad17*]. Boxes (left) indicate the position of *top3-134* double mutants with *rad1*, *rad3*, *rad17* and *rad26*, respectively.

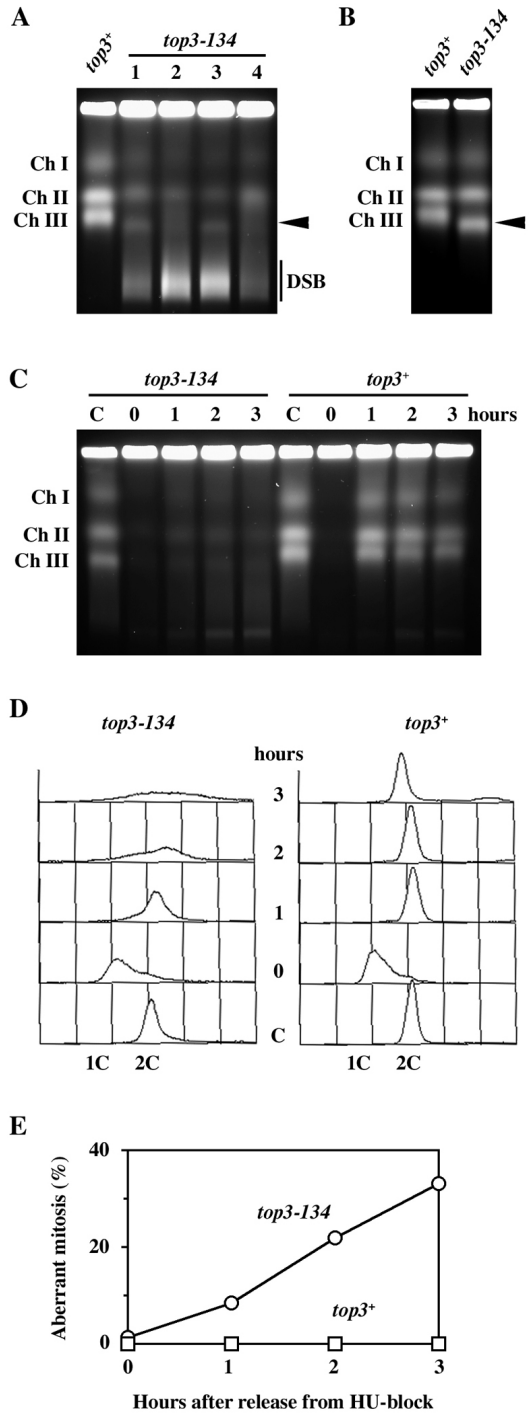

Fig. 6. Pulsed-field gel electrophoresis analysis of chromosomes from the *top3-134* mutant. (A-C) Equal numbers of cells were prepared in agarose gel plugs from exponentially growing cultures of wild type (*top3*$^+$) or *top3-134* cells (four independent isolates) at the permissive temperature of 26°C (A), after nitrogen starvation to arrest cells in G1 (B), or following HU block (3 hours at 36°C) and release (C). Cells were harvested at the time of HU addition (C) and at hourly intervals after removal of HU for up to 3 hours. Arrowheads indicate the aberrant chromosome III band seen in *top3-134* samples. (D) Flow cytometric analysis of the DNA content of ethanol-fixed, Sytox Green-stained samples from the experiment shown in C. (E) Samples from C taken at the times indicated were used to score the percentages of aberrant mitosis in each culture.

visualized in living cells using a fusion protein between the nucleolar protein Gar2 and GFP (Shimada et al., 2003). In wild-type cells, Gar2-GFP occupied roughly half of the nucleus, in a discrete region distinct from the bulk chromosomal DNA, against a background of fainter nuclear signal (Fig. 7A,B). In contrast, a variety of abnormal morphologies were observed in *top3-134* cells, even at the permissive temperature. Some cells showed a reduction or complete loss of the discrete signal (Fig. 7A, example 1), while others appeared to have increased and more diffuse Gar2-GFP fluorescence (examples 2 and 3). Dispersion of Gar2-GFP in the nucleoplasm was also observed (example 4). In addition, Gar2-GFP fluorescence that was largely or wholly separate from the bulk chromosomal DNA was frequently seen in binucleate cells, indicating segregation defects during mitosis (Fig. 7B; 17% in cells grown at the permissive temperature of 26°C, which increased to 49.6% 4 hours after shift to the restrictive temperature, 36°C). This phenomenon was further explored by time-lapse microscopy. As shown in Fig. 7C, in contrast to wild-type cells in which Gar2-GFP separated equally into the daughter cells at mitosis, in *top3-134* cells an extended bridge of Gar2-GFP often persisted for some time between the nascent daughter nuclei. These data, although indirect, suggest that Top3 function is required at the rDNA loci, presumably to process aberrant chromosome structures arising as a result of DNA replication, to allow proper chromosome segregation during mitosis.

Discussion

In a previous study, we identified *S. pombe top3*$^+$ as an essential gene, in contrast to the situation in *S. cerevisiae*, where *top3* mutants are viable despite their slow growth compared with wild-type cells (Goodwin et al., 1999; Maftahi et al., 1999). To understand the growth defects and determine the cell cycle stage at which these problems arise, we have further characterised the phenotype of a *top3* temperature-sensitive mutant (Oh et al., 2002). We have shown that *top3* mutants accumulate defects during S phase. Like *S. cerevisiae top3* mutants (Chakraverty et al., 2001), these cells arrest at G2/M in a manner that is dependent on the DNA damage checkpoint, as deletion of *chk1*$^+$ abolishes the cell cycle arrest (Fig. 3). Despite activation of the DNA damage checkpoint, these cells can exit the arrest but fail to segregate their chromosomes effectively. However, these mitotic defects appear secondary to the fundamental defects in chromosome stability that lead to the lethality in these cells, as advance into mitosis does not adversely affect cell survival.

The phenotype of *top3* mutants shares a striking similarity to that of the *rqh1* mutants treated with HU (Stewart et al., 1997). In each case, the cells are not able to separate their chromosomes after a cell cycle delay: despite the chromosome segregation defect, the aberrant mitosis is not the primary cause of cell death in these cells. Moreover, the lethality of *top3* mutation can be suppressed by inactivation of homologous recombination (Laursen et al., 2003; Oakley et al., 2002; Shor et al., 2002) and the HU sensitivity of *rqh1* mutants is also suppressed by deletion of the homologous recombination gene *rhp51* (our unpublished data). Given the connection between *rqh1* and homologous recombination, we propose that the growth defects in *top3* strains arise from the failure to resolve

and process recombination intermediates rather than a direct role in chromosome segregation. Entering mitosis with sister chromatids entangled by unresolved recombination intermediates would make subsequent chromosome segregation difficult or impossible. Consistent with these data, pulsed-field gel electrophoresis showed that chromosomes isolated from *top3* mutants contain elevated levels of DNA double-strand breaks, probably as a consequence of failed chromosome segregation.

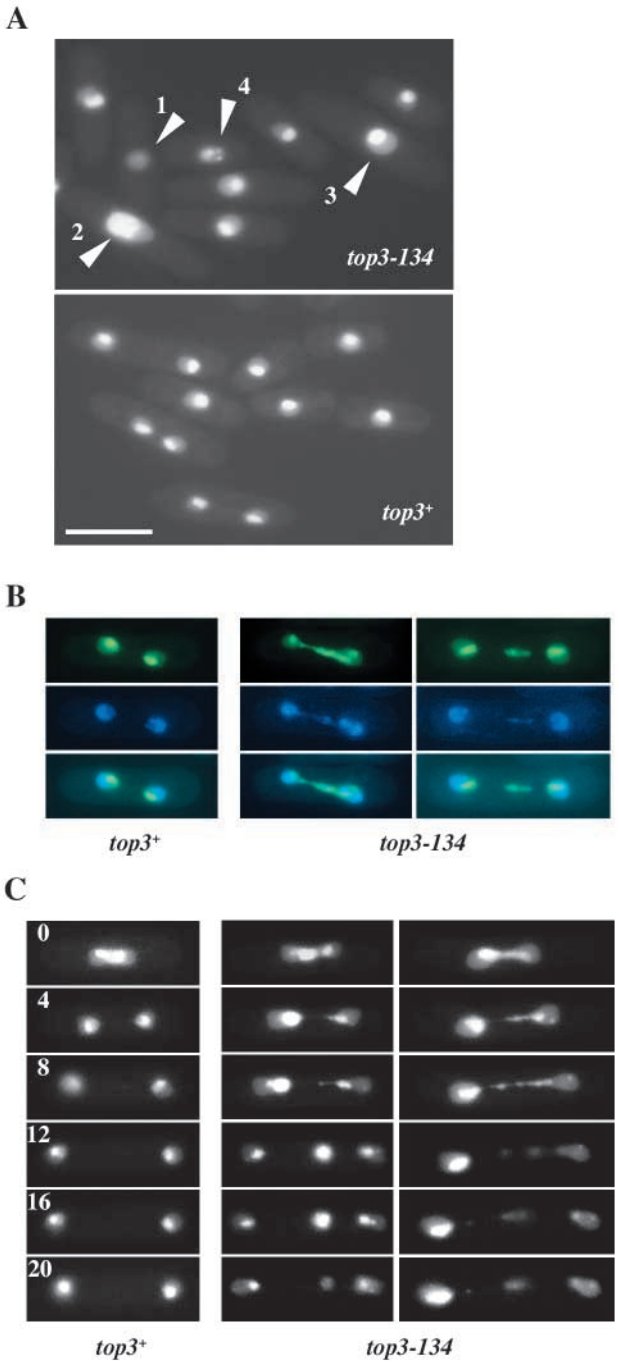

Fig. 7. Aberrant nucleolar structures in the *top3-134* mutant. (A) Living *gar2-GFP* (*top3*$^{+}$) and *gar2-GFP top3-134* (*top3-134*) cells were observed by green fluorescence microscopy. Arrowheads indicate cells with aberrant nucleolar structures. (B) Merged images (lower panels) of fluorescence micrographs showing Gar2-GFP (upper panels) and DNA (Hoechst 33342, middle panels) localisation in living cells. (C) Visualisation of lagging Gar2-GFP signal in *top3-134* cells. Individual *gar2-GFP* and *gar2-GFP top3-134* cells were observed as in A, over a 20 minute period, with images collected every 4 minutes. Bar, 10 μm.

The results presented here, together with our previous data on the interaction between *rqh1* and *top3*, are consistent with an essential function of *top3* during S phase. Top3, together with Rqh1, is presumably required to process or disrupt aberrant recombination structures that arise during S-phase, in a manner similar to that proposed for RecQ, the *Escherichia coli* equivalent of Rqh1 (Harmon et al., 1999). One interpretation of the conserved genetic interaction is that RecQ helicases act upstream of Topoisomerase III in a common biochemical pathway and generate a DNA structure that requires resolution by Top3. In the absence of Top3, this DNA structure would be toxic. However, in the absence of Rqh1 and Top3, the toxic structure would not arise. In line with this idea, we observed an accumulation of aberrant DNA structures in *top3* mutants by PFGE analysis, which only occurred following DNA replication in the absence of *top3* function. Determination of the exact nature of the aberrant DNA structures would be useful in understanding the function of Top3. Based on a recent biochemical analysis of a combined RecQ helicase/topoisomerase III reaction, the candidate for this toxic structure could be an unresolved double Holliday junction (Wu and Hickson, 2003). Intriguingly, the chromosome abnormality in *top3* mutants appears to be more pronounced in chromosome III, where the rDNA loci are located, than in chromosome I or II. This interpretation is further supported by the aberrant nucleolar structures and segregation defects observed in *top3-134* mutants (Fig. 7). The rDNA array in *S. cerevisiae* contains a high density of replication fork barriers (Brewer and Fangman, 1988), which represent a potential source of homologous recombination. Recently, Versini et al. (Versini et al., 2003) showed that DNA replication is specifically retarded at the rDNA locus in *sgs1* cells and suggested that this could be due to their inability to prevent recombination at the abundant stalled forks in that region. Consistent with these data, loss of function of either *SGS1* or *TOP3* results in increased recombination in the multiple tandem rDNA array (Gangloff et al., 1994; Watt et al., 1996). Taken together, these data suggest a function for Top3-RecQ complexes in maintenance of the rDNA structure, presumably processing aberrant chromosome structures arising from DNA replication, to allow proper chromosome segregation during mitosis.

We have identified a specific interaction between *top3* and *rad3* or *rad26*, but not other checkpoint genes. Deletion of *rad3*$^{+}$, but not the downstream kinase genes *cds1*$^{+}$ and/or *chk1*$^{+}$ (Fig. 5 and data not shown), leads to a more severe growth defect in *top3* cells, indicating that *rad3*$^{+}$ might have an function in addition to checkpoint control that is required for the survival of *top3* mutants. Similar results have been described in Δ*rqh1* and Δ*rhp51* mutants. Deletion of *rad3* is synthetically lethal in combination with either Δ*rqh1* or Δ*rhp51* (Murray et al., 1997). In the model discussed above, we place homologous recombination upstream of the *rqh1* and *top3* pathway, as inactivation of homologous recombination, preventing the channelling of damaged DNA into this pathway, suppresses the lethality of *top3* mutants. Clearly in the absence

of Top3-Rqh1 and homologous recombination, repair of DNA damage must proceed via an alternative route. Like its mammalian orthologue *ATM*, *rad3*$^+$ might have an additional function in regulation of DNA damage repair (Foray et al., 1997). Further work will be required to identify the nature of this alternative pathway. In addition, *top3* deletion mutants are defective in Rad53 phosphorylation following DNA damage specifically during S phase in *S. cerevisiae* (Chakraverty et al., 2001), suggesting a role of Top3 in DNA damage responses during but not outside of S phase. Consistent with this suggestion, Chk1 is phosphorylated following DNA damage in Δ*rqh1* Δ*top3* mutants (our unpublished data), suggesting that these cells are not checkpoint defective, at least at G2. Whether or not *S. pombe top3*$^+$ is required for responses to exogenous DNA damage during S phase remains to be determined.

In summary, we have shown that accumulation of aberrant DNA structures in *top3* mutants activates the DNA damage checkpoint, leading to a cell cycle delay at G2 and failure of these cells to segregate their chromosomes as they exit the arrest. Despite the chromosome segregation defects, the phenotype of *S. pombe top3* bears a striking resemblance to its counterpart in *S. cerevisiae*. Together with the conserved relationship between Top3-Rqh1 and homologous recombination, these data strongly suggest that this class of enzyme executes a conserved function in these two highly divergent eukaryotic species and probably in higher eukaryotes.

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