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Top3 Processes Recombination Intermediates and Modulates Checkpoint Activity after DNA Damage[D]

Hocine W. Mankouri and Ian D. Hickson

Cancer Research UK Laboratories, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DS, United Kingdom

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Mutation of *TOP3* in *Saccharomyces cerevisiae* causes poor growth, hyperrecombination, and a failure to fully activate DNA damage checkpoints in S phase. Here, we report that overexpression of a dominant-negative allele of *TOP3*, *TOP3^{Y356F}*, which lacks the catalytic (decatenation) activity of Top3, causes impaired S-phase progression and the persistence of abnormal DNA structures (X-shaped DNA molecules) after exposure to methylmethanesulfonate. The impaired S-phase progression is due to a persistent checkpoint-mediated cell cycle delay and can be overridden by addition of caffeine. Hence, the catalytic activity of Top3 is not required for DNA damage checkpoint activation, but it is required for normal S-phase progression after DNA damage. We also present evidence that the checkpoint-mediated cell cycle delay and persistence of X-shaped DNA molecules resulting from overexpression of *TOP3^{Y356F}* are downstream of Rad51 function. We propose that Top3 functions in S phase to both process homologous recombination intermediates and modulate checkpoint activity.

INTRODUCTION

Topoisomerases are highly conserved proteins that catalyze topological rearrangements in the structure of DNA and are required for many aspects of DNA metabolism. Of the three topoisomerases in yeast, the function(s) of the sole type IA topoisomerase, Top3, remains most poorly understood. However, it is likely that Top3 fulfills important roles in vivo, because deletion of *TOP3* in *Saccharomyces cerevisiae* causes hyperrecombination, sensitivity to genotoxic agents, meiotic defects, and poor growth due to accumulation of cells with a late S/G_2 content of DNA (Wallis *et al*., 1989; Gangloff *et al*., 1994, 1999; Chakraverty *et al*., 2001). Similarly, deletion of *top3*$^+$ in *Schizosaccharomyces pombe* causes lethality due to chromosome missegregation and accumulation of DNA double-strand breaks (Goodwin *et al*., 1999; Maftahi *et al*., 1999; Oh *et al*., 2002; Win *et al*., 2004). Whereas lower eukaryotes generally contain only one type IA topoisomerase, human cells, like most vertebrates, possess at least two Top3 homologues, hTOPIIIα and hTOPIIIβ (Hanai *et al*., 1996; Ng *et al*., 1999). In mice, mutation of *TOP3*α causes embryonic lethality (Li and Wang, 1998), whereas mutation of *TOP3*β causes a shortened life span (Kwan *et al*., 2003). Interestingly, in *S. cerevisiae* and *S. pombe*, deletion of *SGS1* or *rqh1*$^+$ can largely suppress the phenotypes caused by deletion of *TOP3* or *top3*$^+$, respectively (Gangloff *et al*., 1994; Goodwin *et al*., 1999; Maftahi *et al*., 1999; Chakraverty *et al*., 2001). Because both *SGS1* and *rqh1*$^+$ belong to the same family of proteins, the RecQ DNA helicases, this intriguing genetic interaction has led to the suggestion that type IA topoisomerases may be functionally associated with RecQ helicases. Indeed, both Sgs1 and Rqh1 physically interact with Top3 in their respective organisms (Gangloff *et al*., 1994; Bennett *et al*., 2000; Fricke *et al*., 2001; Onodera *et al*., 2002; Laursen *et al*., 2003; Ahmad and Stewart, 2005; Ui *et al*., 2005), and a close association between a RecQ helicase and type IA topoisomerase has now been demonstrated in a number of other organisms (Harmon *et al*., 1999; Wu *et al*., 2000; Kim *et al*., 2002).

RecQ helicases are evolutionarily conserved 3→5′ DNA helicase enzymes that are important for the maintenance of genomic stability in all organisms (Hickson, 2003). Of particular interest, defects in three (of the five so far identified) human RecQ helicases cause Bloom's syndrome (*BLM*), Rothmund-Thomson syndrome (*RECQL4*), and Werner's syndrome (*WRN*) (Ellis *et al*., 1995; Yu *et al*., 1996; Kitao *et al*., 1999). Each of these disorders shows genomic instability associated with a predisposition to the development of various cancers. Additionally, Rothmund–Thomson syndrome and Werner's syndrome possess features resembling premature aging. RecQ helicases have, therefore, received much interest due to their putative roles in suppressing cancer and/or aging in humans.

Consistent with an evolutionarily conserved functional interaction between RecQ helicases and a type IA topoisomerases, BLM has been demonstrated to interact with, and stimulate the DNA strand passage activity of, hTOPIIIα in vitro (Johnson *et al*., 2000; Wu *et al*., 2000; Wu and Hickson, 2002). This association is likely to be physiologically important, because BLM and hTOPIIIα act together in vitro to resolve homologous recombination (HR) intermediates containing two Holliday junctions in a process termed "double junction dissolution" (Wu and Hickson, 2003). The physiological consequence of this in vivo is likely to be in the resolution of HR repair intermediates without potentially deleterious crossing over of genetic material. Indeed, elevated levels of sister chromatid exchanges, which arise due to crossing over during or soon after S phase, are currently

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Address correspondence to: Ian D. Hickson (ian.hickson@cancer.org.uk).

Abbreviation used: HR, homologous recombination.

the most reliable diagnostic feature of Bloom's syndrome (German, 1993). Sgs1 and Top3 have also been demonstrated to function in the same HR repair pathway to prevent crossing over during double-strand break repair in *S. cerevisiae* (Ira *et al*., 2003). Therefore, current thinking suggests that RecQ helicases act in concert with type IA topoisomerases in an evolutionarily conserved pathway to process recombination intermediates that arise during DNA replication without the crossing over of genetic material.

Further evidence for a role for Top3 in the late stages of HR repair also comes from the fact that mutation of genes involved in the early steps of HR repair (e.g., *RAD51* in *S. cerevisiae*; *rhp51*$^+$ in *S. pombe*) partially suppresses various *top3* phenotypes in *S. cerevisiae* or *S. pombe* (Fabre *et al*., 2002; Oakley *et al*., 2002; Shor *et al*., 2002). Together with the very low viability of yeast *top3* strains, it has been proposed that DNA intermediates formed by the HR repair pathway are otherwise toxic if Top3 function is impaired (Gangloff *et al*., 1994; Fabre *et al*., 2002). Moreover, it would seem that Top3 catalyzes a step in a late stage of recombination for which there are no redundant activities, because the *top3* mutant phenotype is far more severe than that of cells lacking HR repair altogether.

In addition to a putative role in processing late-stage HR intermediates, Top3 has also been implicated in DNA damage checkpoint signaling in *S. cerevisiae* (Chakraverty *et al*., 2001). Whereas wild-type cells activate the DNA damage checkpoint and slow DNA replication after DNA damage (Paulovich and Hartwell, 1995), *top3* cells fail to fully activate Rad53, and, as a consequence, progress more rapidly through S phase after DNA damage (Chakraverty *et al*., 2001). This defect is apparent only when the damage occurs during S phase, because G_1/S and G_2/M checkpoints are intact in *top3* cells (Chakraverty *et al*., 2001). Interestingly, deletion of *RMI1*, which encodes a Top3-interacting protein, causes a *top3*-like phenotype and also results in a similar failure to fully activate Rad53 in response to DNA damage (Chang *et al*., 2005; Mullen *et al*., 2005). These findings suggest that the Sgs1–Top3–Rmi1 complex (either directly or indirectly) fulfills important DNA damage checkpoint signaling functions after DNA damage. However, it remains to be determined whether this proposed role for Top3 is related to its function in HR repair.

To further investigate the putative role(s) of Top3 in yeast, we have used an allele of *TOP3*, *TOP3*^{Y356F}, that fails to complement a *top3* strain (Bennett and Wang, 2001) and causes an inducible, dominant-negative, *top3*-like phenotype when overexpressed in wild-type cells (Oakley *et al*., 2002). Because a *top3* mutant strain is very poor growing and readily acquires suppressor mutations (most notably in *SGS1*), use of this dominant-negative system allowed us to investigate the acute effects of Top3 impairment and permitted more technically challenging experiments than would be possible with a *top3* strain. Additionally, because the *TOP3*^{Y356F} allele used in this study possesses a mutation in the active site tyrosine residue that abolishes the catalytic activity, but not the DNA-binding capability, of hTOPIIIα (Goulaouic *et al*., 1999), we reasoned that overexpression of catalytically dead Top3^{Y356F} protein might reveal selective, separable functions of Top3 that could otherwise be overlooked in a *top3* strain.

We report that, unlike *top3* mutants (Chakraverty *et al*., 2001), cells overexpressing *TOP3*^{Y356F} fully activate Rad53 after DNA damage. Therefore, the checkpoint defect previously reported in *top3* cells (Chakraverty *et al*., 2001) is likely due to loss of Top3 function(s) other than loss of catalytic activity. Moreover, cells overexpressing *TOP3*^{Y356F} demonstrate a persistent DNA damage checkpoint-mediated cell cycle delay in the presence of MMS. Therefore, although the catalytic activity of Top3 is not required for checkpoint activation, it is required for some aspect of S-phase progression after DNA damage. We also demonstrate that overexpression of *TOP3*^{Y356F} in wild-type cells causes the accumulation of HR repair intermediates after DNA damage. Because phenotypes caused by overexpression of *TOP3*^{Y356F} are downstream of Rad51 activity, our results further verify a late role for Top3 in HR repair. We propose a model in which Top3 acts late in HR repair to both process repair intermediates and (either directly or indirectly) modulate checkpoint activation/maintenance.

MATERIALS AND METHODS

S. cerevisiae Strains and Plasmids

All the strains used in this study are isogenic derivatives of T344 (Hovland *et al*., 1989). The *rad51* and *sgs1* deletion strains were constructed using a polymerase chain reaction (PCR)-based gene disruption method (Wach *et al*., 1994). Plasmids pYES2-*TOP3* and pYES2-*TOP3*^{Y356F} have been described previously (Oakley *et al*., 2002).

Growth Conditions, Cell Synchronization, and Flow Cytometry Analysis

Strains were grown at 30°C in CSM-Ura medium (Formedium) containing glucose [2% (wt/vol)]. For overexpression of *TOP3* or *TOP3*^{Y356F} from the pYES2 plasmid, 2% galactose was added. Cell cycle synchronization in the G_1 phase was performed using α-factor mating pheromone (Cancer Research UK peptide synthesis laboratory, Clare Hall, United Kingdom) at 30°C for 4–5 h. Release from α-factor arrest was achieved by centrifugation, washing, and resuspension of cells in fresh medium. Cell cycle progression was monitored at 25°C using flow cytometry (fluorescence-activated cell sorting; FACS), as described previously (Chakraverty *et al*., 2001).

Protein Extraction and Western Blot Analysis

Protein extracts from yeast cells were prepared using a modified trichloroacetic acid (TCA) protein extraction technique (Foiani *et al*., 1994). Approximately 10^8 cells were harvested, washed once, and resuspended in 20% TCA. An equal volume of glass beads was added, and cells were disrupted using a BIO101/Savant FastPrep FP120 cell disrupter (4 × 20 s cycles at full speed; Thermo Electron Corporation, Waltham, MA). The supernatant was then removed, and the glass beads were washed twice with 200 μl of 5% TCA. Extracts were then clarified by centrifugation at 5700 rpm for 5 min, and the pellet was resuspended in 2× Laemmli buffer (Bio-Rad, Hercules, CA) diluted with an equal volume of 0.5 M Tris-HCl, pH 8.0. Samples were boiled for 3 min and then further clarified by centrifugation. Proteins were separated by SDS-PAGE using 3–8 or 7% NuPAGE Tris-acetate gels (Invitrogen, Paisley, United Kingdom). Rad53 phosphorylation status was analyzed using a mouse monoclonal antibody (EL7; kindly provided by Dr. Marco Foiani, FIRC Institute of Molecular Oncology, Milan, Italy) at a final dilution of 1:10. Top3 and Top3^{Y356F} proteins were detected using a mouse anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO) at a final concentration of 10 μg/ml. Horseradish peroxidase-linked secondary antibody (Sigma-Aldrich) was used at 1:4000, and chemiluminescent detection was performed with an ECL kit (GE Healthcare, Little Chalfont Buckinghamshire, United Kingdom).

Two-Dimensional (2D) Gel Electrophoresis

The hexadecyltrimethylammonium bromide (CTAB) method of DNA extraction and two-dimensional gel procedures were described previously (Brewer and Fangman, 1987; Allers and Lichten, 2000; Lopes *et al*., 2003). DNA was digested with NciI and NcoI before running the first-dimension gels.

RESULTS

Overexpression of TOP3^{Y356F} *Causes Poor Growth*

Mutation of the conserved tyrosine residue in the active site of hTOPIIIα abolishes catalytic activity, but not an ability to bind DNA (Goulaouic *et al*., 1999). The corresponding mutation in the *S. cerevisiae TOP3* gene results in a *TOP3* allele (*TOP3*^{Y356F}) that fails to complement a *top3* strain (Bennett and Wang, 2001). We confirmed that a construct, pYES2-*TOP3*, could complement the poor growth and DNA damage sensitivity of a *top3* strain, whereas pYES2-*TOP3*^{Y356F} could not (our unpublished data; Oakley, 2001). Therefore,

complementation of the phenotype of a *top3* mutant requires the catalytic (decatenase) activity of Top3.

When overexpressed from the *GAL1* promoter of the high copy vector pYES2, $TOP3^{Y356F}$ (but not *TOP3*) causes poor growth in the wild-type *S. cerevisiae* YP1 strain background (Oakley, 2001; Oakley *et al*., 2002). In the present study, we used the T344 strain, which contains mutations in the *reg101* and *gal1* genes, permitting the use of galactose as a gratuitous inducer and allowing 1,500-fold induction of genes regulated by the *GAL1* promoter, even in the presence of 2% glucose (Hovland *et al*., 1989). We confirmed that *TOP3* and $TOP3^{Y356F}$ were overexpressed to an equivalent level in the wild-type T344 strain after exposure to 2% galactose (Supplemental Figure 1). Consistent with previous analysis (Oakley *et al*., 2002), overexpression of $TOP3^{Y356F}$ also caused poor growth in the wild-type T344 strain, whereas overexpression of *TOP3* had no effect (Supplemental Figure 2). Furthermore, the poor growth caused by overexpression of $TOP3^{Y356F}$ was prevented in a T344 strain lacking *SGS1* (Supplemental Figure 2). Western blotting again revealed that *TOP3* and $TOP3^{Y356F}$ were similarly overexpressed in the presence of 2% glucose in our *sgs1* strain (our unpublished data).

Overexpression of $TOP3^{Y356F}$ Causes Impaired S-Phase Progression after DNA Damage

To analyze the effects of $TOP3^{Y356F}$ overexpression on DNA replication, wild-type strains transformed with pYES2, pYES2-*TOP3*, or pYES2-$TOP3^{Y356F}$ plasmids were synchronized in G_1 with α-factor, and overexpression of *TOP3* or $TOP3^{Y356F}$ was induced simultaneously by the addition of 2% galactose (Supplemental Figure 1). Cultures were then released from G_1 arrest, and samples were taken at fixed intervals to analyze DNA content by flow cytometry (FACS). Wild-type strains transformed with empty vector or those overexpressing *TOP3* traversed S phase and apparently completed DNA replication (as measured by a doubling of DNA content) within ~60 min (Figure 1A). Wild-type cells overexpressing $TOP3^{Y356F}$ consistently demonstrated a slight delay in the rate of S-phase progression (Figure 1A, note DNA content at 40 min), but they nevertheless successfully completed DNA replication with only marginally delayed kinetics. Therefore, we conclude that overexpression of $TOP3^{Y356F}$ mildly affects some aspect of S-phase progression in unperturbed cells, but it does not noticeably affect bulk DNA synthesis per se.

Previous studies demonstrated that *top3* cells are sensitive to the DNA-damaging agent methylmethanesulfonate (MMS) (Chakraverty *et al*., 2001; Shor *et al*., 2002). We also observed that cells overexpressing $TOP3^{Y356F}$ demonstrated sensitivity to MMS, whereas overexpression of *TOP3* had no effect (our unpublished data). Therefore, we analyzed cell cycle progression in wild-type cells overexpressing *TOP3* or $TOP3^{Y356F}$ in the presence of 0.0167% MMS. Under these conditions, wild-type cells overexpressing *TOP3* completed DNA replication by ~2 h (Figure 1B). Similar kinetics of DNA replication were also observed for wild-type strains transformed with empty pYES2 vector (our unpublished data). This increased S-phase duration (relative to untreated cells; Figure 1A) is a consequence of MMS-induced replication fork stalling and subsequent activation of the DNA damage checkpoint (Paulovich and Hartwell, 1995; Santocanale and Diffley, 1998; Shirahige *et al*., 1998; Tercero and Diffley, 2001). Wild-type cells overexpressing $TOP3^{Y356F}$ demonstrated much more severely delayed S-phase progression, completing DNA replication only by ~4 h (Figure 1B). Therefore, we conclude that overexpression of $TOP3^{Y356F}$ increases the duration of S phase in the presence of MMS, suggesting that Top3 is required for some aspect of S-phase progression after DNA damage.

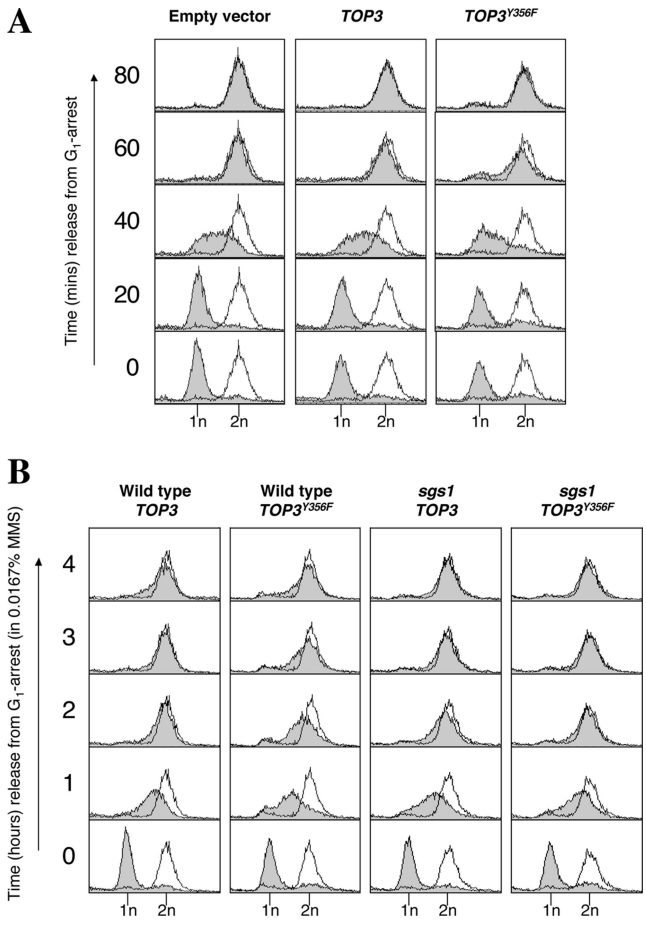

Figure 1. Overexpression of $TOP3^{Y356F}$ causes impaired S-phase progression after DNA damage. (A) Wild-type strains transformed with pYES2, pYES2-*TOP3*, or pYES2-$TOP3^{Y356F}$ were arrested in G_1 with α-factor and simultaneously treated with 2% galactose (to induce overexpression from the pYES2 *GAL1* promoter). Cultures were released into fresh medium under normal growth conditions, and DNA content was analyzed by flow cytometry at the indicated times. The shaded peaks represent experimental data, whereas the unshaded peak is a reference to indicate a normal G_2/M peak (at 2-h release from G_1 arrest). The positions of the 1n (G_1) and 2n (G_2/M) peaks are indicated below. (B) Wild-type and *sgs1* strains overexpressing *TOP3* or $TOP3^{Y356F}$ were released from G_1 arrest into fresh medium containing 0.0167% MMS, and DNA content was analyzed by flow cytometry at the indicated times.

Mutation of SGS1 Suppresses the Impaired S-Phase Progression Phenotype Caused by Overexpression of $TOP3^{Y356F}$

It has been reported previously that mutation of *SGS1* suppresses all known *top3* mitotic phenotypes (Gangloff *et al*., 1994; Chakraverty *et al*., 2001). Therefore, we examined whether this also holds true for the novel phenotype of impaired S-phase progression caused by overexpression of $TOP3^{Y356F}$. In agreement with a previous report (Liberi *et al*., 2005), we found that mutation of *SGS1* alone did not noticeably affect unperturbed S-phase progression, but it did cause a slight delay in S-phase progression in the presence of MMS (our unpublished data). Similar to what was observed in wild-type cells, *sgs1* cells overexpressing *TOP3* traversed S phase and apparently com-

pleted DNA replication ~2 h after G_1 release in the presence of 0.0167% MMS (Figure 1B). Interestingly, in contrast to wild-type cells overexpressing *TOP3*^{Y356F}, *sgs1* cells overexpressing *TOP3*^{Y356F} completed DNA replication with kinetics indistinguishable from that of *sgs1* cells overexpressing *TOP3*. We conclude, therefore, that overexpression of *TOP3*^{Y356F} does not cause impaired S-phase progression after DNA damage when *SGS1* is mutated. Furthermore, this suggests that impaired S-phase progression after DNA damage caused by *TOP3*^{Y356F} overexpression, like previously reported *top3* mitotic phenotypes (Gangloff *et al*., 1994; Chakraverty *et al*., 2001), is likely to be the consequence of Sgs1 activity uncoupled from that of Top3.

The Impaired S-Phase Progression Caused by Overexpression of TOP3^{Y356F} *after DNA Damage Is Due to a Persistent Checkpoint-mediated Cell Cycle Delay*

The finding that *TOP3*^{Y356F} cells show an impaired ability to progress through S phase is in contrast with previous data demonstrating that *top3* cells traverse S phase more rapidly than wild-type cells in the presence of MMS due to a failure to fully activate Rad53 (Chakraverty *et al*., 2001). This intriguing finding suggested that the previously reported *top3* phenotype could be a consequence of lack of Top3 protein rather than abolition of Top3 catalytic activity per se. We investigated, therefore, if the impaired S-phase progression observed in *TOP3*^{Y356F} cells is a consequence of proficient checkpoint activation in the presence of catalytically-dead Top3^{Y356F} protein.

DNA damage checkpoint activation was assessed in cells exposed to a slightly higher concentration of MMS (0.033%) than used above in order to permit robust activation of the DNA damage checkpoint. Under these conditions, wild-type strains transformed with empty vector or those overexpressing *TOP3* complete DNA replication by ~5 h, whereas cells overexpressing *TOP3*^{Y356F} still only demonstrate a mid-S-phase DNA content by this time (Figure 2A). DNA damage checkpoint activation was assessed 1 h after MMS treatment by an analysis of the phosphorylation status of the Rad53 checkpoint effector kinase. We observed that DNA damage-induced Rad53 phosphorylation (as indicated by a slower migrating species on protein gels) occurred to a similar extent in cells overexpressing *TOP3* or *TOP3*^{Y356F} (Figure 2B). This activation of Rad53 was dependent on DNA damage, because overexpression of *TOP3* or *TOP3*^{Y356F} did not promote activation of Rad53 in the absence of MMS (Supple-

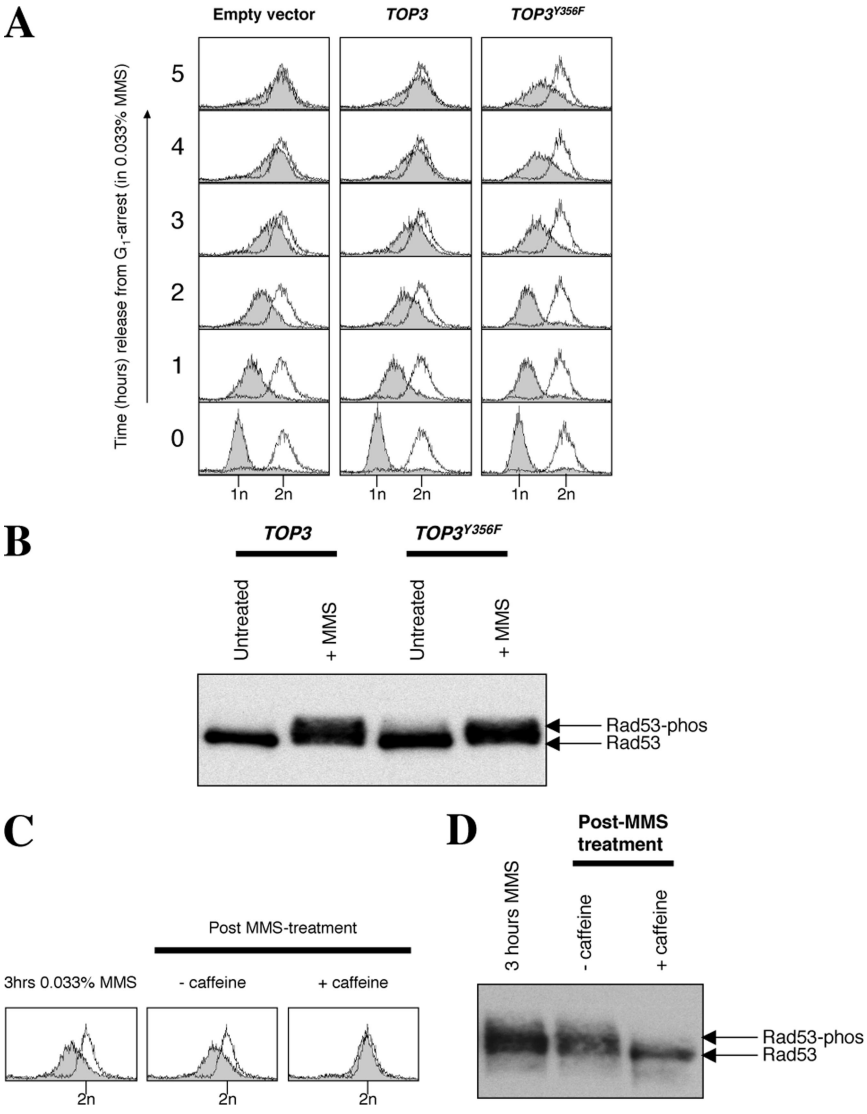

Figure 2. The *TOP3*^{Y356F}-induced impaired S-phase progression phenotype is due to a persistent checkpoint-mediated cell cycle arrest. (A) Wild-type strains transformed with pYES2 or overexpressing *TOP3* or *TOP3*^{Y356F} were released from G_1 arrest into fresh medium containing 0.033% MMS, and S-phase progression was monitored by FACS. (B) Cells overexpressing *TOP3* or *TOP3*^{Y356F} were collected before treatment with α-factor and 2% galactose (untreated), and 1 h after cells were released from α-factor arrest into medium containing 0.033% MMS. Protein extracts were prepared, and Rad53 phosphorylation status was monitored by Western blotting. The positions of the unphosphorylated Rad53 and slower migrating phosphorylated forms are shown on the right. (C and D) After 3-h treatment with 0.033% MMS, cells overexpressing *TOP3*^{Y356F} were harvested and resuspended in fresh medium ± 5 mg/ml caffeine. Samples were taken 1 h later for analysis of DNA content by flow cytometry (C) and Rad53 phosphorylation status (D).

mental Figure 1 and Figure 2B). Analysis of a time course of Rad53 activation also verified that Rad53 phosphorylation occurred with similar kinetics and to a similar level in cells transformed with empty pYES2 vector or cells overexpressing *TOP3*^{Y356F} after exposure to MMS (Supplemental Figure 3). We conclude that, unlike in *top3* cells (Chakraverty *et al*., 2001), Rad53 becomes fully activated in response to DNA damage in cells overexpressing *TOP3*^{Y356F}.

Next, we analyzed whether a persistent checkpoint-mediated cell cycle delay might be responsible for the impaired S-phase progression observed in cells overexpressing *TOP3*^{Y356F}. Caffeine has been demonstrated to override DNA damage checkpoints in a number of organisms by inhibiting the DNA damage checkpoint transducer ATR (*Homo sapiens*)/Mec1 (*S. cerevisiae*)/Rad3 (*S. pombe*) (Schlegel and Pardee, 1986; Osman and McCready, 1998; Hall-Jackson *et al*., 1999; Moser *et al*., 2000; Vaze *et al*., 2002; Liberi *et al*., 2005). Therefore, we compared the effects of caffeine addition to a culture overexpressing *TOP3*^{Y356F} after 3 h of MMS treatment. We found that cells resuspended in fresh medium lacking caffeine for 1 h failed to show any significant recovery and remained with a mid-S DNA content (Figure 2C). In contrast, cells treated with 5 mg/ml caffeine seemed to successfully traverse S phase, because a high proportion of cells possessed a 2n DNA content 1 h after the addition of caffeine (Figure 2C). To verify that this effect was due to override of the DNA damage checkpoint by caffeine, we analyzed Rad53 phosphorylation in cells before and after caffeine treatment. Consistent with the above-mentioned proposal, Rad53 was phosphorylated after 3 h of MMS treatment and remained phosphorylated 1 h later in cells released into fresh medium lacking caffeine (Figure 2D). In contrast, Rad53 became dephosphorylated in cells released into fresh medium for 1 h in the presence of 5 mg/ml caffeine (Figure 2D). Therefore, 5 mg/ml caffeine promotes DNA damage checkpoint override. Interestingly, the "resetting" of Rad53 by caffeine addition implies that Rad53 is dynamically phosphorylated and dephosphorylated with rapid kinetics. We conclude that the impaired S-phase progression observed in cells overexpressing *TOP3*^{Y356F} is a consequence of a persistent DNA damage checkpoint-mediated delay caused (either directly or indirectly) by the catalytically dead Top3^{Y356F} protein.

Overexpression of TOP3^{Y356F} Causes Abnormal DNA Replication Intermediates (X-Molecules) to Persist after Exposure to MMS

Next, we sought to determine the cause of the persistent checkpoint-mediated cell cycle delay observed in cells overexpressing *TOP3*^{Y356F}. For this, we used the neutral-neutral 2D gel electrophoresis method to monitor DNA replication fork progression (Brewer and Fangman, 1987; Lopes *et al*., 2003). To monitor DNA replication under normal (unperturbed) growth conditions, wild-type strains overexpressing *TOP3* or *TOP3*^{Y356F} were released from G_1 arrest into fresh medium, and samples were taken at fixed intervals to observe DNA replication intermediates on 2D gels originating from an early firing replication origin, *ARS305*. Genomic DNA was prepared using the CTAB method of DNA extraction to restrain branch migration of joint (X-shaped) molecules (Lopes *et al*., 2003). We observed that origin firing at *ARS305* was detectable after 30 min in wild-type cells overexpressing either *TOP3* or *TOP3*^{Y356F} by the appearance of bubbles, Y-molecules, and origin-associated X-spikes (Figure 3A). Previous studies have indicated that the origin-associated X-spikes are normal DNA replication intermediates that are not dependent on Rad51 or Rad52 for their formation and are not, therefore, HR intermediates (Lopes *et al*., 2003). After 60 min, all of the *ARS305* replication intermediates detectable at 30 min had disappeared in both strains, consistent with the completion of bulk DNA replication by this time (Figure 1A). We conclude, therefore, that overexpression of *TOP3*^{Y356F} does not noticeably affect early replication origin firing or replication fork progression in the region adjacent to *ARS305* in unperturbed cells.

Next, we analyzed DNA replication in the presence of 0.033% MMS. We observed that origin firing at *ARS305* was again detectable at 30 min after G_1 release in wild-type strains harboring either the pYES2 plasmid or pYES2-*TOP3*^{Y356F} (Figure 3B). In MMS-treated cells harboring the empty pYES2 vector, all of the replication intermediates detectable at 30 min at *ARS305* had largely disappeared by 90 min, consistent with completion of DNA replication in this particular region of the genome (in the presence of 0.033% MMS) by this time. In contrast, whereas cells overexpressing *TOP3*^{Y356F} also revealed comparable kinetics of *ARS305* origin activation (at 30 min) and bubble and Y-molecule disappearance (at 90 min), a structure corresponding to a joint molecule (X-molecule) persisted for at least 120 min. These MMS-induced X-molecules are not merely transient DNA replication intermediates, because they were still detectable in cells overexpressing *TOP3*^{Y356F} (but not *TOP3*) 4 h after release from G_1 arrest (Figure 3C). Furthermore, once formed, persistence of these X-molecules was not dependent on DNA damage checkpoint activity or cell cycle stage/progression, because driving cells into G_2/M with caffeine did not promote their resolution (Figure 3D). We conclude that overexpression of *TOP3*^{Y356F} causes abnormal DNA replication intermediates (X-molecules) to persist and/or accumulate during S phase in MMS-treated cells.

Mutation of RAD51 Causes Impaired S-Phase Progression after Exposure to MMS

Because Top3 has been implicated in HR repair (Gangloff *et al*., 1994; Fabre *et al*., 2002; Oakley *et al*., 2002; Shor *et al*., 2002), we considered the possibility that the X-molecules detected in MMS-treated cells overexpressing *TOP3*^{Y356F} might represent unprocessed/aberrant HR repair intermediates. We analyzed, therefore, whether the phenotypes caused by *TOP3*^{Y356F} overexpression were suppressed by deletion of *RAD51*. Rad51 catalyzes the early strand invasion step of HR and has been demonstrated to partially suppress the growth defect of a *top3* mutant (Fabre *et al*., 2002; Oakley *et al*., 2002; Shor *et al*., 2002). In agreement with previous reports, we also observed that mutation of *RAD51* in the T344 strain background partially suppressed the growth defect caused by *TOP3*^{Y356F} overexpression (Supplemental Figure 4).

Next, we investigated whether the impaired S-phase progression caused by overexpression of *TOP3*^{Y356F} is suppressed by mutation of *RAD51*. We observed that unperturbed *rad51* cells completed bulk DNA replication by ~60 min (our unpublished data), which are kinetics similar to that observed for wild-type cells (Figure 1A). Unlike wild-type cells, however, *rad51* cells were unable to successfully traverse S phase in the presence of 0.033% MMS (Figure 4A). This finding was also verified in an independent (BY4741) strain background (our unpublished data) and suggests that the Rad51-dependent HR repair pathway is important for the repair/tolerance of MMS-lesions during S phase.

We note that the impaired S-phase progression observed in MMS-treated *rad51* cells is actually more severe than that

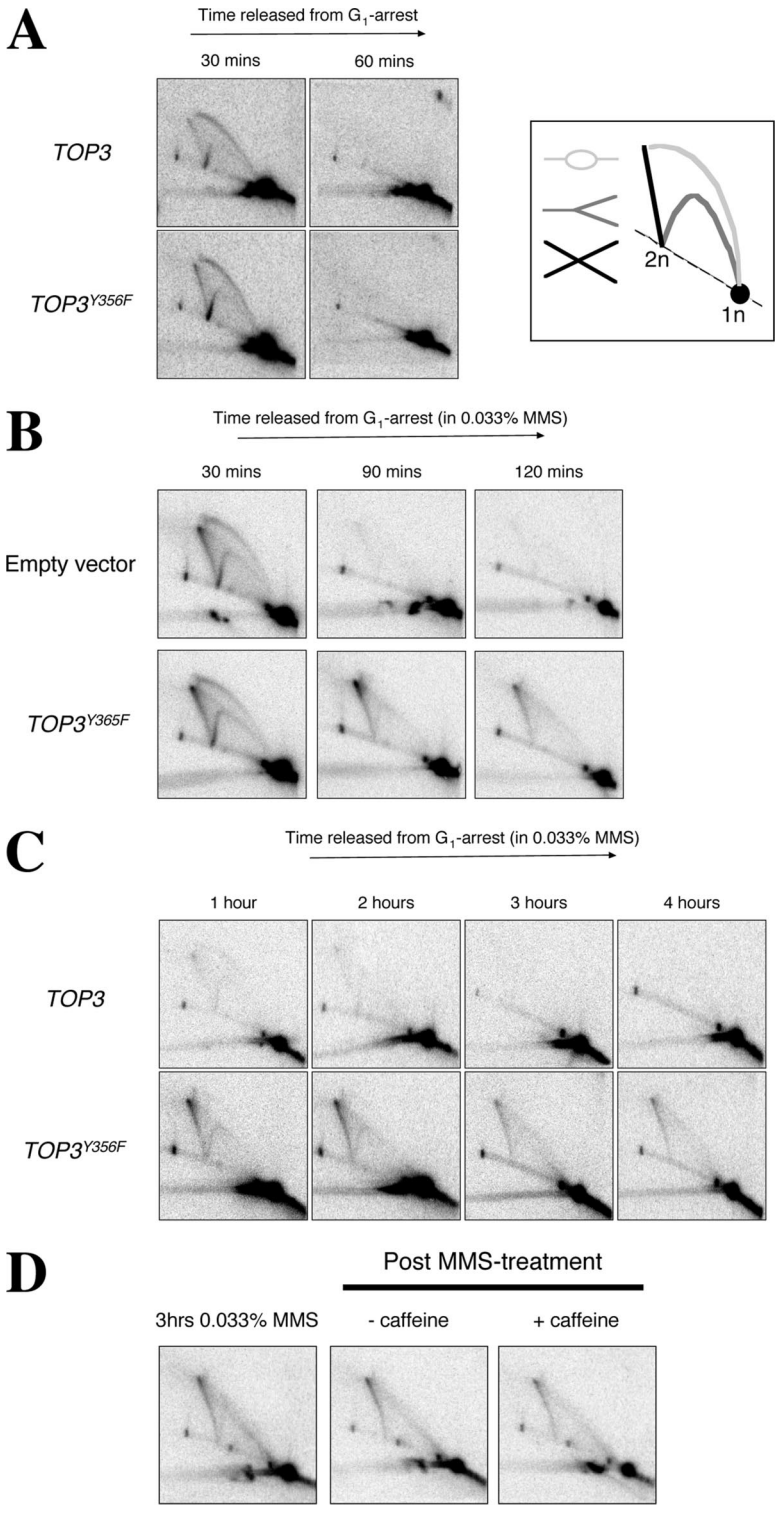

Figure 3. Overexpression of *TOP3*^{Y356F} causes abnormal DNA replication intermediates (X-molecules) to persist after exposure to MMS. (A–C) Wild-type strains transformed with pYES2, or overexpressing *TOP3* or *TOP3*^{Y356F} were released from G_1 arrest into fresh medium under normal growth conditions (A) or containing 0.033% MMS (B and C). DNA replication intermediates were analyzed by 2D gel electrophoresis at the times indicated. DNA samples were analyzed with a probe for the early firing *ARS305* replication origin. The key in A (right) denotes DNA structures that can be identified by the 2D gel technique. (D) After 3-h treatment with 0.033% MMS, *TOP3*^{Y356F}-overexpressing cells were harvested and resuspended in fresh medium ± 5 mg/ml caffeine. Samples were taken 1 h later for analysis of DNA replication intermediates by 2D gel electrophoresis.

observed in cells overexpressing *TOP3*^{Y356F} (Figure 5). Nevertheless, like *TOP3*^{Y356F} cells, it seems that the impaired S-phase progression observed in MMS-treated *rad51* cells is also, at least partially, caused by the DNA damage checkpoint, because addition of caffeine (after 3 h of MMS treatment) could largely override this phenotype (Figure 4B). Analysis of Rad53 phosphorylation status also confirmed that the DNA damage checkpoint is proficient and that addition of caffeine overrides the DNA damage checkpoint in *rad51* cells (our unpublished data). These findings are consistent with a previous report demonstrating that Rad53 becomes fully activated earlier in *rad51* cells than in wild-type cells after treatment with MMS (Liberi *et al.*, 2005). Taken together, these data suggest that, in the absence of Rad51, the DNA damage checkpoint is both rapidly and robustly activated after exposure to MMS.

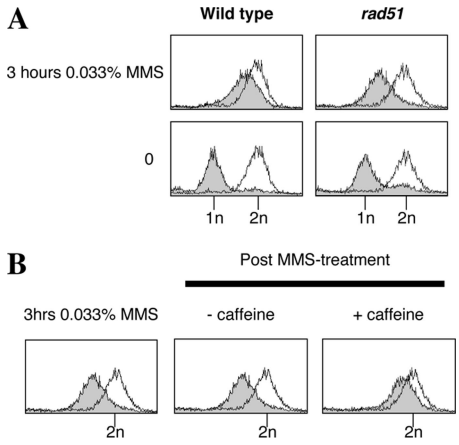

Figure 4. Mutation of *RAD51* causes impaired S-phase progression after DNA damage. (A) Wild-type and *rad51* strains transformed with the pYES2 plasmid were released from G_1 arrest into fresh medium containing 0.033% MMS, and DNA content was analyzed by flow cytometry at the indicated times. (B) After 3-h treatment with 0.033% MMS, the *rad51*-pYES2 culture was harvested and resuspended in fresh medium ± 5 mg/ml caffeine. Samples were taken 1 h later for analysis of DNA content by flow cytometry.

Impaired S-Phase Progression in rad51 Cells after Exposure to MMS Predominates Over That Caused by Overexpression TOP3^{Y356F}

To determine whether the impaired S-phase progression phenotypes observed in *rad51* mutants and cells overexpressing *TOP3*^{Y356F} are epistatic or additive, we compared the effects of overexpressing *TOP3* or *TOP3*^{Y356F} on S-phase progression in *rad51* cells in the presence of 0.0167% MMS. Under these conditions, wild-type cells overexpressing *TOP3* complete DNA replication by ~2 h, whereas wild-type cells overexpressing *TOP3*^{Y356F} complete DNA replication by ~4 h (Figures 1B and 5). In contrast, *rad51* cells overexpressing *TOP3* still failed to complete DNA replication by 5 h in medium containing 0.0167% MMS, confirming that the impaired S-phase progression phenotype of *rad51* cells is more severe than that observed in wild-type cells overexpressing *TOP3*^{Y356F}. Interestingly, we observed that the FACS profile of *rad51* cells overexpressing *TOP3*^{Y356F} was essentially indistinguishable from that of *rad51* cells overexpressing *TOP3*. Therefore, overexpression of *TOP3*^{Y356F} did not further impair S-phase progression in a *rad51* mutant, suggesting that the impaired S-phase progression phenotypes observed in *rad51* mutants or cells overexpressing *TOP3*^{Y356F} are epistatic. Taken together, these data suggest that Rad51 and Top3 function in the same pathway and that the (more severe) impaired S-phase progression phenotype conferred by mutation of *RAD51* predominates over that caused by overexpression of *TOP3*^{Y356F}. This finding is consistent with previous reports that *RAD51* and *TOP3* are epistatic and that Top3 acts downstream of Rad51 in HR repair (Fabre *et al*., 2002; Oakley *et al*., 2002; Shor *et al*., 2002).

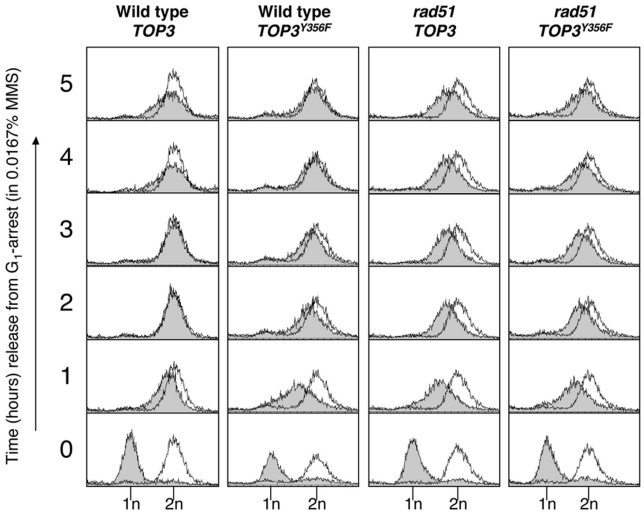

Figure 5. Impaired S-phase progression in *rad51* cells predominates over that caused by overexpression of *TOP3*^{Y356F}. Wild-type and *rad51* cells overexpressing *TOP3* or *TOP3*^{Y356F} were released from G_1 arrest into fresh medium containing 0.0167% MMS, and DNA content was analyzed by flow cytometry at the times indicated.

Mutation of RAD51 Prevents MMS-induced X-Molecules from Persisting in Cells Overexpressing TOP3^{Y356F}

Next, we analyzed whether *RAD51* mutation also prevents X-molecules from forming in cells overexpressing *TOP3*^{Y356F} after exposure to MMS. We found that, despite an inability of *rad51* cells to complete bulk DNA replication in the presence of 0.033% MMS (Figure 4A), these cells usually completed DNA replication (as measured by disappearance of

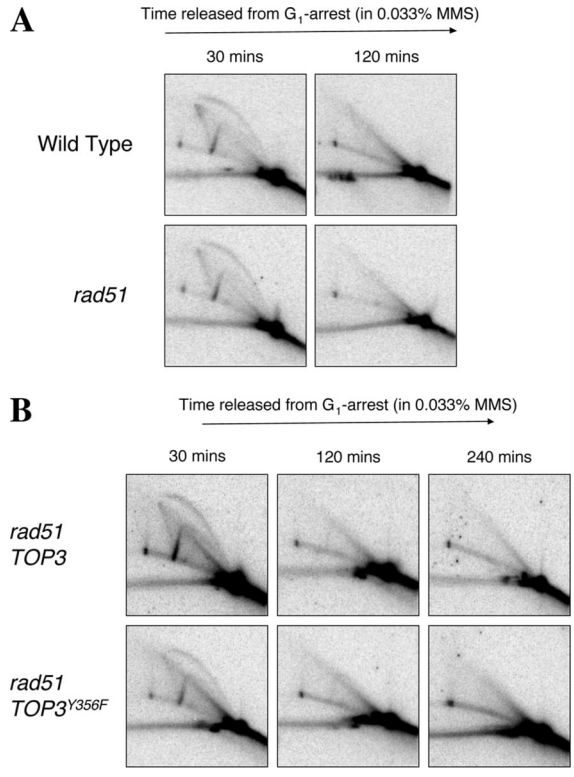

Figure 6. Mutation of *RAD51* suppresses the MMS-induced X-spikes caused by overexpression of *TOP3*^{Y356F}. (A) Wild-type and *rad51* strains transformed with pYES2 were released from G_1 arrest into fresh medium containing 0.033% MMS. DNA replication intermediates were analyzed by 2D gel electrophoresis at the times indicated. (B) *rad51* cells overexpressing *TOP3* or *TOP3*^{Y356F} were released from G_1 arrest into fresh medium containing 0.033% MMS, and DNA replication intermediates were analyzed by 2D gel electrophoresis at the times indicated.

replication intermediates on 2D gels) around the site of the *ARS305* origin under routine analysis within 2 h (Figure 6A). Therefore, mutation of *RAD51* does not noticeably affect early origin firing, the formation or subsequent disappearance of the (HR-independent) origin-associated X-spike, or replication fork progression around *ARS305*. We propose that the impaired S-phase progression after DNA damage observed in *rad51* cells arises only once a (checkpoint-activating) threshold level of unrepaired, MMS-induced discontinuities in DNA synthesis is attained.

Similar kinetics of *ARS305* origin firing (at 30 min) and disappearance of replication intermediates (at 120 min) were observed in MMS-treated *rad51* mutants overexpressing either *TOP3* or $TOP3^{Y356F}$ (Figure 6B). However, we found that the persistent X-molecules normally caused by overexpression of $TOP3^{Y356F}$ were not evident in *rad51* strains. We conclude that mutation of *RAD51* prevents MMS-induced X-molecules from persisting and/or accumulating in cells overexpressing $TOP3^{Y356F}$, suggesting that Rad51 is required for their formation and/or stabilization. Together, these data are consistent with the proposal that unprocessed HR repair intermediates exist in MMS-treated cells overexpressing $TOP3^{Y356F}$.

DISCUSSION

To investigate the role(s) of Top3 in the maintenance of genome stability, we have used a dominant-negative allele of *TOP3*, $TOP3^{Y356F}$, that causes poor growth when overexpressed in wild-type cells (Oakley *et al*., 2002). The $TOP3^{Y356F}$ construct used in this study possesses a mutation in the active site tyrosine that abolishes the catalytic (decatenation) activity of Top3 and fails to complement the poor growth of a *top3* mutant (Bennett and Wang, 2001). Interestingly, $TOP3^{Y356F}$ overexpression did not cause an identical phenocopy of a *top3* mutation, because cells overexpressing $TOP3^{Y356F}$ exhibited impaired S-phase progression after DNA damage. Conversely, *top3* cells fail to fully activate Rad53 in the presence of MMS, and, as a consequence, progress more rapidly through S phase than wild-type cells (Chakraverty *et al*., 2001). We propose that the checkpoint-signaling defect in *top3* cells arises due to absence of Top3 protein, rather than loss of Top3 catalytic activity, because Rad53 activation seemed normal in cells overexpressing $TOP3^{Y356F}$ after exposure to MMS. Moreover, the impaired S-phase progression observed in cells overexpressing $TOP3^{Y356F}$ is due to a persistent checkpoint-mediated cell cycle delay, which can be overridden by caffeine. Therefore, although the catalytic activity of Top3 is apparently not required for normal DNA damage checkpoint activation, it is required for normal S-phase progression after DNA damage. We note, however, that although cells overexpressing $TOP3^{Y356F}$ demonstrate a persistent checkpoint-mediated cell cycle delay, Rad53 activation seemed qualitatively similar (as detected by Western blotting) in cells overexpressing *TOP3* or $TOP3^{Y356F}$ after MMS treatment. However, we cannot exclude the possibility that subtle alterations to Rad53 activity, such as an altered phosphorylation pattern and/or differences in Rad53 turnover rate (dephosphorylation) in cells overexpressing $TOP3^{Y356F}$ might not have been evident during our analyses.

In addition to causing impaired S-phase progression, we have demonstrated that overexpression of $TOP3^{Y356F}$ also causes abnormal DNA structures (X-shaped DNA molecules) to persist after MMS treatment. During the course of our study, Liberi *et al*. (2005) also demonstrated that MMS-induced X-molecules persist in *sgs1*, *sgs1top3*, and *top3* cells (Liberi *et al*., 2005). However, because *top3* mutants readily acquire suppressor mutations in *SGS1* (Gangloff *et al*., 1994), and *sgs1top3* cells exhibit X-molecule accumulation (Liberi *et al*., 2005), we reasoned that these findings required independent verification by using the dominant-negative $TOP3^{Y356F}$ system. Our data demonstrate conclusively that impairment of Top3 function does indeed cause X-molecules to persist in MMS-treated cells. Furthermore, these MMS-induced X-molecules are Rad51-dependent and therefore likely represent unprocessed recombination intermediates. It should be noted, however, that these X-molecules differ from those normally observed at firing origins. Unlike origin-associated X-molecules (Lopes *et al*., 2003), the X-molecules observed in cells overexpressing $TOP3^{Y356F}$ are both MMS and Rad51 dependent. However, it remains to be determined whether the persistent X-molecules we detect in cells overexpressing $TOP3^{Y356F}$ arise independently after origin X-molecule resolution, or whether they arise due to the interconversion of the origin-associated X-molecules into bona fide HR repair intermediates via the action of Rad51/Rad52 etc.

Because overexpression of $TOP3^{Y356F}$ does not cause any (additive) impaired S-phase progression in *rad51* cells, it is likely that the checkpoint-mediated cell cycle delay caused by overexpression of $TOP3^{Y356F}$, like the persistent X-molecules, is also downstream of Rad51 activity. We propose, therefore, that the futile engagement of catalytically-dead $Top3^{Y356F}$ protein in HR repair is inhibitory to repair and causes a more persistent and/or robust activation of the DNA damage checkpoint (Figure 7). Because mutation of the active site tyrosine of hTOPIIIα abolishes the catalytic activity, but not an ability to bind DNA (Goulaouic *et al*., 1999), one possibility is that catalytically dead $Top3^{Y356F}$ remains bound to unprocessed HR intermediates, and that the DNA damage checkpoint then recognizes this as incomplete HR repair. Conversely, it is possible that, in *top3* cells, unprocessed HR intermediates persist (Liberi *et al*., 2005), but they cannot be recognized by the checkpoint machinery (Chakraverty *et al*., 2001). Therefore, we speculate that the extent of checkpoint activation after MMS treatment varies in cells depending on whether Top3 is absent (as in *top3* cells), catalytically active with normal turnover (as in wild-type cells), or catalytically dead and unable to turnover (as in cells overexpressing $TOP3^{Y356F}$). Although this model is consistent with our data, we cannot rule out the possibility that the effects we observed could also be explained either by Top3 influencing events at stalled replication forks (e.g., the processing of stalled forks; Hishida *et al*., 2004), or the stabilization of DNA polymerases at stalled forks (Bjergbaek *et al*., 2005)], or by the fact that persistent X-molecules in *top3* cells represent different DNA structures from those in cells overexpressing $TOP3^{Y356F}$.

It remains to be determined whether the activation/modulation of Rad53 activity by Top3 is direct (i.e., mediated by Top3 itself) or indirect (i.e., mediated by a Top3-binding protein). Interestingly, deletion of *RMI1*, which encodes a Top3-interacting protein, also causes a *top3*-like phenotype and results in a similar failure to fully activate Rad53 in response to DNA damage (Chang *et al*., 2005; Mullen *et al*., 2005). It is worth noting that deletion of either *TOP3* or *RMI1* adversely affects the stability of Sgs1 and causes a reduction in Sgs1 protein levels (Chang *et al*., 2005). It is possible, therefore, that Sgs1 mediates the activation of Rad53 after DNA damage via its ability to physically interact with Rad53 (Frei and Gasser, 2000; Bjergbaek *et al*., 2005).

Consistent with previous data (Gangloff *et al*., 1994; Chakraverty *et al*., 2001), we also found that mutation of

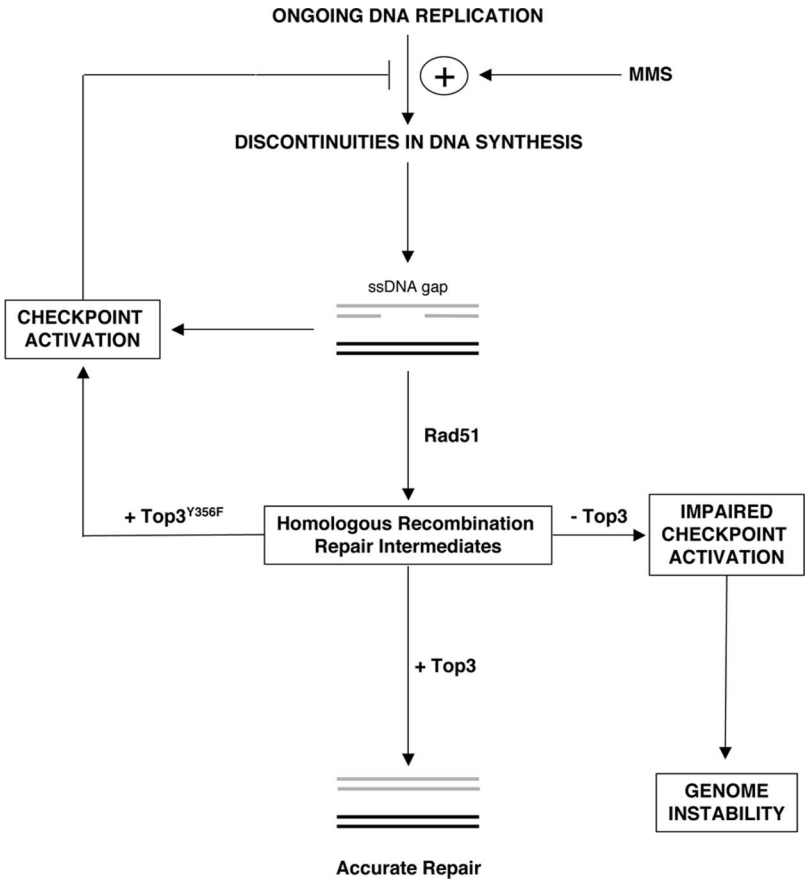

Figure 7. Model for role of Top3 in resolving homologous recombination intermediates and modulating checkpoint activity after DNA damage. Replication forks stall when encountering MMS lesions, leading to discontinuous DNA synthesis and the accumulation of single-stranded DNA (ssDNA) gaps. Replication protein A (RPA) binds to ssDNA and then activates the DNA damage checkpoint, which promotes the stabilization of stalled replication forks at MMS lesions and inhibits further origin firing. By doing this, the DNA damage checkpoint limits further formation of recombinogenic ssDNA gaps caused by ongoing DNA replication in the presence of DNA damage. Rad51 catalyzes the early strand invasion step of homologous recombination repair of ssDNA gaps, and, through its ability to displace RPA, deactivates the checkpoint signal. Top3 acts downstream of Rad51 to resolve homologous recombination repair intermediates via its catalytic activity. Additionally, the checkpoint machinery (either directly or indirectly) senses the engagement of Top3 in homologous recombination repair and modulates checkpoint activity accordingly. Absence of Top3 protein causes unprocessed/unresolved HR intermediates to persist, and a failure to adequately delay S-phase progression after DNA damage. Conversely, catalytically dead Top3^{Y356F} protein inhibits resolution of homologous recombination repair intermediates and promotes a persistent checkpoint-mediated cell cycle delay. DNA replication resumes at stalled replication forks once MMS-induced lesions have been removed/repaired or due to checkpoint activity falling below a threshold level.

SGS1 suppresses the poor growth and impaired S-phase progression after DNA damage caused by overexpression of *TOP3*^{Y356F}. This suggests that phenotypes caused by *TOP3*^{Y356F} overexpression, like other previously reported *top3* phenotypes (Gangloff *et al*., 1994; Chakraverty *et al*., 2001), may arise due to the uncoupling of Sgs1 activity from that of Top3. However, we could not directly test whether mutation of *SGS1* abolishes the X-molecules we observed in cells overexpressing *TOP3*^{Y356F}, because *sgs1* cells also accumulate (MMS-induced) Rad51-dependent X-molecules (Liberi *et al*., 2005; our unpublished data). Given the very different catalytic activities of Sgs1 and Top3, it seems likely that X-molecules detected in *sgs1* mutants or cells overexpressing *TOP3*^{Y356F} could represent different types of HR intermediates. Consistent with this idea, it has been proposed that RecQ helicases act in concert with a type IA topoisomerase to resolve key HR repair intermediates containing double Holliday junctions, in a process termed double junction dissolution (Ira *et al*., 2003; Wu and Hickson, 2003). This process is thought to consist of a two-step process: branch migration of individual Holliday junctions by a RecQ helicase to form a hemicatenane intermediate, which a type IA topoisomerase then decatenates via its strand passage activity. We speculate that the X-molecules detectable in *sgs1* cells may represent double Holliday junctions, whereas those caused by Top3 impairment represent hemicatenanes. Ongoing experiments are aimed at testing this hypothesis by attempting to discriminate between, or selectively resolve, X-molecules present in *sgs1* mutants and cells overexpressing *TOP3*^{Y356F}.

Of wider significance, if the proposed functions of Top3 in resolving HR repair intermediates and modulating checkpoint activity reported here also holds true for hTOPOIIIα, this suggests that a putative hTOPOIIIα inhibitor that selectively inhibits the catalytic activity of hTOPOIIIα alone, could provide a means to greatly sensitize rapidly proliferating cells to DNA damage. Therefore, we propose that hTOPOIIIα might represent an attractive anticancer drug target in human cells.

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