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# The Swi2–Snf2-like protein Uls1 is involved in replication stress response

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## ABSTRACT

**The *Saccharomyces cerevisiae* Uls1 belongs to the Swi2–Snf2 family of DNA-dependent ATPases and a new protein family of SUMO-targeted ubiquitin ligases. Here, we examine a physiological role of Uls1 and report for the first time its involvement in response to replication stress. We found that deletion of *ULS1* in cells lacking *RAD52* caused a synthetic growth defect accompanied by prolonged S phase and aberrant cell morphology. *uls1*Δ also progressed slower through S phase upon MMS treatment and took longer to resolve replication intermediates during recovery. This suggests an important function for Uls1 during replication stress. Consistently, cells lacking Uls1 and endonuclease Mus81 were more sensitive to HU, MMS and CPT than single *mus81*Δ. Interestingly, deletion of *ULS1* attenuated replication stress-related defects in *sgs1*Δ, such as sensitivity to HU and MMS while increasing the level of PCNA ubiquitination and Rad53 phosphorylation. Importantly, Uls1 interactions with Mus81 and Sgs1 were dependent on its helicase domain. We propose that Uls1 directs a subset of DNA structures arising during replication into the Sgs1-dependent pathway facilitating S phase progression. Thus, in the absence of Uls1 other modes of replication fork processing and repair are employed.**

## INTRODUCTION

Replication forks often stall at specific sites in the genome, e.g. rDNA repeats or DNA lesions resulting from chemical or radiation damage. Homologous recombination repair (HRR) is a conserved process responsible for maintenance of genome stability. Products of genes belonging to the *RAD52* epistasis group act in the repair of single-strand DNA (ssDNA) gaps or double-strand DNA breaks (DSBs) aiding in the restart of damaged or collapsed replication forks (1). However, the repair of such lesions must be tightly regulated because inappropriate, excessive or untimely recombination can lead to deleterious effects such as loss of heterozygosity or chromosome deletions and rearrangements (2). In *Saccharomyces cerevisiae* several proteins have been described as being implicated in the processing of stalled replication forks and control of recombination.

Three helicases were shown to control HRR: Srs2 and Sgs1, two well established helicases with anti-recombinogenic properties (3,4), and recently described Mph1 involved in the dissociation of D-loops formed by Rad51 recombinase (5). *srs2*Δ mutants display hyper-recombination phenotype (6) corroborated by later biochemical data showing that Srs2 protein disrupts Rad51 filament (7). It has been shown that Srs2 acts through interaction with the PCNA complex, a sliding clamp and processivity factor for replicative DNA polymerases, and directs the repair of stalled replication forks away from HRR and into post-replication repair (PRR)-dependent translesion synthesis (TLS) and template switching pathways (4,8). The *sgs1*Δ mutant is also characterized by mitotic hyper-recombination phenotype (9), sensitivity to genotoxins (10) and reduced replicative lifespan (11). These phenotypes can be rescued by overexpression of human BLM gene (12,13) underscoring conservation of function among members of RecQ helicase family. Srs2 and Sgs1, however, are not redundant, even though suppression of *srs2*Δ *sgs1*Δ lethality by deletion of *RAD51* suggests partial functional overlap (14). Sgs1 overexpression can complement hyper-recombination and repair defects of *srs2*Δ mutant (15) but not *vice versa*, and it is suggested (16) that both helicases act preferentially at different stages or on different intermediates

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in HRR. Many functions performed by Sgs1 in DNA metabolism have recently been reviewed (17) and the complexity of phenotypes observed for *sgs1*Δ mutant is further elevated by the fact that Sgs1 can function alone as well as in complex with its interacting partners: DNA topoisomerase III, Top3 (18) and a stimulator of Top3 decatenation activity, Rmi1 (19,20). In DSB repair Sgs1 can be involved both upstream in the resection step of DNA ends (21,22) as well as downstream, with Top3–Rmi1, in the dissolution of double Holliday junctions (HJ) (23). Sgs1 colocalizes with DNA replication sites even in the absence of damage and is involved in the activation of intra-S checkpoint in response to hydroxyurea (HU) induced replication fork stalling. It stimulates checkpoint kinase, Rad53, activation independently of Top3, acting together with the S phase checkpoint mediator, Mrc1, in a pathway synergistic to a clamp loader subunit, Rad24 (10,24). Another important function of Sgs1 at replication forks is the regulation of recombination. Sgs1 slows the progression of replication forks and prevents deleterious HRR, especially in regions rich in natural pause sites, such as an rDNA array (25). In *sgs1*Δ mutant Rad51-dependent X molecules, containing HJ, accumulate at MMS damaged replication forks (26,27) since Sgs1–Top3–Rmi1 complex required for their resolution is compromised.

*MUS81* and *MMS4* have been isolated in a screen for genes required for viability in the absence of Sgs1 (28) and mutants in both were found to be defective in sporulation and sensitive to agents causing replication fork stalling and collapse. Together they encode a heterodimeric structure-specific endonuclease that cleaves branched DNA (29), preferably Y-shaped structures, D-loops and nicked HJ (30). This nuclease activity is enhanced by DNA-dependent ATPase, Rad54, which targets Mus81–Mms4 to substrates at perturbed replication forks (31). In summary, these biochemical data suggest that Mus81–Mms4 could cleave stalled or regressed forks leading to their collapse, but also process structures arising as a result of HRR action at arrested forks (29,31,32), consistent with a role both upstream and downstream in the restart of damaged replication forks. The synthetic lethality of *sgs1*Δ *mus81*Δ double mutant can be suppressed by deletion of HR genes suggesting that both proteins may act in non-redundant but overlapping pathways for the removal of toxic recombination intermediates. However, the generation times of such triple mutants are significantly higher than those of respective *rad* mutants, implying that Mus81 and Sgs1 also have roles that are independent of recombination (33).

Both Sgs1 and Mus81–Mms4 are required for the suppression of gross chromosomal rearrangements (GCR) (3,34). Recently, it has been shown that deletion of genes for *SLX5* and *SLX8* originally isolated by Mullen *et al.* (28), encoding a SUMO-targeted ubiquitin ligase (STUbL) complex (35,36), also resulted in even more substantial increase in GCR rate (37), implicating both proteins in the preservation of genomic stability. In agreement with this notion, it has been reported that Slx5 co-localizes with DNA damage-induced Rad52 foci and is recruited to DSB induced by HO endonuclease (38). The Slx5–Slx8 complex is also involved in the control of DSB repair at nuclear pores (39).

Uls1 (Dis1–Ris1–Tid4), the second putative STUbL in *S. cerevisiae* (35), which belongs to the Swi2–Snf2 family of DNA-dependent ATPases, has been shown to antagonize silencing during mating-type switching (40). Although mutation of *ULS1* causes accumulation of high molecular weight SUMO conjugates and double *uls1*Δ *slx5*Δ mutant shows synthetic growth defect (35), there is no biochemical evidence for a role of Uls1 in SUMO-dependent ubiquitination and/or preservation of genomic stability. Recently it has been shown that Uls1 acts together with Rad54 and Rdh54 to remove Rad51 recombinase from chromatin and that its translocase activity is required for this process (41). Interestingly, two protein paralogs from *Schizosaccharomyces pombe*, Rrp1 and Rrp2, showing 34 and 36% similarity, respectively, to the C-terminal portion of the *S. cerevisiae* Uls1, have been found to function in the Sfr1–Swi5 mediator complex-dependent branch of HR, described in *S. pombe* but conserved in mice and humans (42–44), and play a particularly important role in the rescue of stalled and/or collapsed replication forks in the absence of Rhp57 (Rad57 homolog of *S. cerevisiae*) (45). Based on these results, we sought to determine if Uls1 also shares a role in these processes.

In this study, we found that although the *uls1*Δ mutant does not exhibit sensitivity to genotoxic agents, its progression through S phase and resolution of chromosome replication intermediates is compromised when challenged with MMS. Uls1 is thus crucial for coping with replication stress which is especially evident in cells devoid of HR mediator Rad52 or endonuclease Mus81. In addition, we showed that deletion of *ULS1* results in suppression of *sgs1*Δ phenotypes suggesting that Uls1 acts upstream in the Sgs1-dependent pathway to maintain genomic stability.

## MATERIALS AND METHODS

### Yeast strains, growth conditions and plasmids

Yeast strains used in this study are in the W303 background with a wild-type copy of *RAD5* (Supplementary Table S1). Gene deletions were generated by PCR-based gene replacement method (46). Yeast transformations were done by the lithium acetate procedure (47). Yeast strains were grown in standard rich (YPD) medium or in selective synthetic minimal (SD) medium at 28°C (48). Doubling time calculations were carried out as previously described (49). For DNA damage sensitivity tests, cells were grown to mid-log phase and 10-fold serial dilutions were spotted onto YPD plates containing various concentrations of HU (Calbiochem), methyl methane sulfonate (MMS, Sigma-Aldrich) or camptothecin (CPT, Sigma-Aldrich). Plates were incubated at 28°C for 2–3 days and photographed. DNA damage sensitivity assays were repeated a minimum of three times. Cloning of the *ULS1* gene on a centromeric plasmid (pGURA3_ULS1) was performed by the gap-repair procedure using W303-1A as a host strain and the split-marker vectors, pGRU and pGRA, as described elsewhere (50).

Site-directed mutagenesis of *ULS1* was conducted with QuickChange® kit (Stratagene) and confirmed by DNA sequencing.

### Cell-cycle analysis, pulsed-field gel electrophoresis and microscopy

Cell-cycle synchronization and flow cytometry analysis of DNA content were performed as previously described (51). The fraction of cells remaining arrested in G1 was determined by an α-factor–nocodazole trap assay (51). The pulsed-field gel electrophoresis (PFGE) analysis of yeast chromosomes was performed as previously described (52). To analyze morphology of yeast strains, cells were fixed in 70% ethanol, treated with RNAse, followed by staining with Sytox green (Invitrogen) to visualize nuclei and observed with an Axio Imager M1 upright wide-field fluorescence microscope (Carl Zeiss, Germany) equipped with a 100× oil immersion objective (Zeiss Plan-Neofluar 100×/1.30), a GFP filter set and Nomarski interference contrast. Images were collected using a Zeiss AxioCam MRc digital color camera and processed with Zeiss AxioVision 4.5 software. Microscopy experiments were repeated three times with a minimum of 600 cells counted for each strain.

### Protein analysis

Total protein was extracted by the trichloroacetic acid method as described previously (53). Protein extracts were resolved on 10% SDS–PAGE, blotted onto nitrocellulose membranes (Bio-Rad) and probed with the goat polyclonal anti-Rad53 (Santa Cruz Biotechnology, sc-6749) or the rabbit polyclonal anti-PCNA (kindly provided by Bruce W. Stillman, Cold Spring Harbor Laboratory) antibodies. Blotted membranes were stained for total protein with Ponceau S (Sigma-Aldrich) before immunodetection.

### Recombination assays

The loss of the *ADE2–CAN1* marker genes as a result of recombination between rDNA repeats was measured by scoring for canavanine (Sigma-Aldrich) resistance according to Burgess *et al.* (54). The recombination frequency between the δ repeats at the *SUP4*-**o** locus was determined by detecting the loss of *URA3* marker gene on plates containing 5-flouroorotic acid (5-FOA, Zymo Research), as described elsewhere (55). Recombination experiments were repeated six times and results were subjected to *t*-test analysis.

## RESULTS

### Uls1 shows synthetic growth interaction with Rad52

We have recently reported that two *S. pombe* protein paralogs, Rrp1 and Rrp2, have a role in the rescue of stalled and/or collapsed replication forks in the Sfr1–Swi5-dependent branch of HR, which acts in parallel to the second HR mediator complex Rhp55–Rhp57 (Rad55–Rad57 in *S. cerevisiae*) (45). Rrp1 and Rrp2 show significant similarity to the C-terminal portion of the *S. cerevisiae* Uls1 (45), a member of Swi2–Snf2 DNA-dependent ATPase family (40) and putative STUbL (35) (Supplementary Figure S1). In order to determine if Uls1 also has a role in replication-associated DNA damage response, we deleted the *ULS1* gene in the W303 background and a growth of the resulting mutant was studied in the presence of DNA damaging agents. Similarly to single *Δrrp1* and *Δrrp2* mutants, the *uls1*Δ mutant had wild-type sensitivities to HU, MMS and CPT, agents that stall or collapse replication forks (Supplementary Figure S2). However, our *S. pombe* study demonstrated that both $rrp1^+$ and $rrp2^+$ genes exhibit genetic interactions with the HR mediator gene $rhp57^+$; the double *Δrrp1 Δrhp57* and *Δrrp2 Δrhp57* mutants were more sensitive to HU, MMS and CPT than the single *Δrhp57* mutant (45). On the other hand, both genes were epistatic to the recombinase gene, *rhp51*+ (*RAD51* in *S. cerevisiae*). Thus, next we analyzed the sensitivity of double *uls1*Δ *rad51*Δ and *uls1*Δ *rad57*Δ mutants to DNA damaging agents. Double mutants were not more sensitive to HU, MMS and CPT than single *rad51*Δ and *rad57*Δ mutants, suggesting either a role of Uls1 in the Rad51-dependent HRR pathway or a function unrelated to DNA repair by HR (Supplementary Figure S2). We also examined the phenotype of *uls1*Δ in a *rad52*Δ background lacking the main HR mediator involved in multiple pathways of DSB repair, including Rad51-independent modes of DNA repair, as well as in a *rad59*Δ background devoid of single-strand annealing repair pathway (1). Deletion of *ULS1* in the *rad59*Δ mutant did not change its level of sensitivity to DNA damaging agents (Supplementary Figure S2). Interestingly, we found that the double *uls1*Δ *rad52*Δ mutant showed a severe slow-growth phenotype with generation time of 270 min, significantly >170 min for *rad52*Δ and 110 min for *uls1*Δ and wild-type (Figure 1A). In contrast, deletion of *ULS1* in *rad52*Δ did not affect the strain's sensitivity to DNA damaging agents.

To further examine the synthetic growth interaction between Rad52 and Uls1, we compared cellular morphology in asynchronously growing wild-type, *rad52*Δ, *uls1*Δ, and *uls1*Δ *rad52*Δ strains. Wild-type and *uls1*Δ cells showed distribution of G1 (unbudded), S (small-budded) and G2–M (large-budded) phase cells typical for log-phase cultures. *rad52*Δ and *uls1*Δ *rad52*Δ mutants, however, accumulated in G2–M phase of cell cycle as enlarged cells with buds similar in size to the mother cell and a single DNA mass, indicative of prolonged G2–M cell-cycle arrest and impaired chromosome segregation (Figure 1B). In addition, abnormalities in cell morphology typical for *rad52*Δ were greatly exacerbated in *uls1*Δ *rad52*Δ as 25% of these cells formed elongated, tubular buds, virtually absent in wild-type and *uls1*Δ mutant (Figure 1B and C). Moreover, deletion of *ULS1* in *rad52*Δ further increased phosphorylation of checkpoint effector kinase Rad53 (Figure 1D), suggesting accumulation of spontaneous DNA damage in these strains.

It has been shown that slowing down replication in *S. cerevisiae* by sublethal levels of HU or MMS induces pseudofilamentous growth dependent on checkpoint proteins Mec1, Rad53 and Swe1 (56). Thus, severe slow

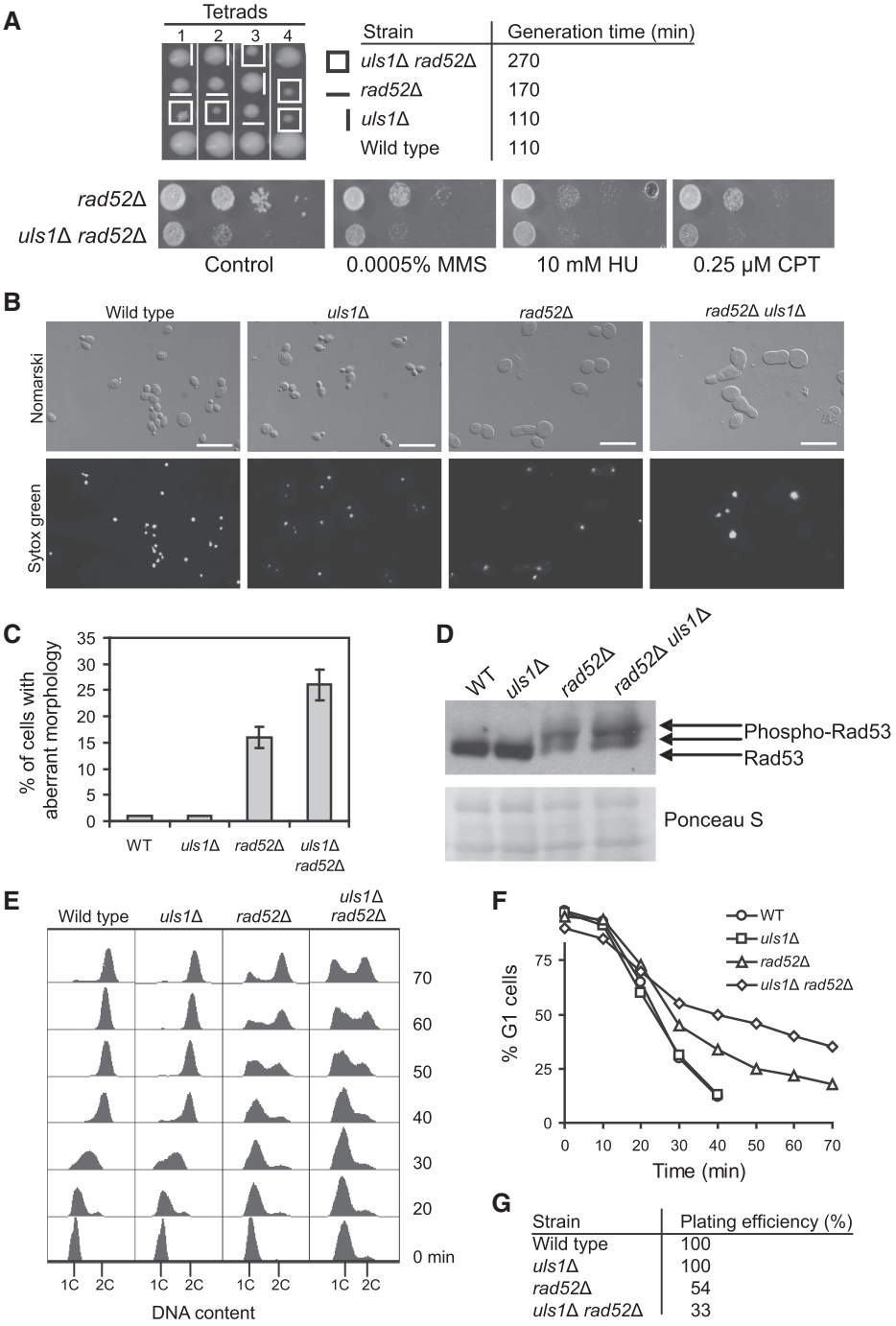


**Figure 1.** Synthetic growth defect of *uls1*Δ *rad52*Δ cells. (**A**) The *uls1*Δ *rad52*Δ double mutant exhibits severe slow growth phenotype as determined by tetrad analysis, measurements of doubling time and serial dilutions of indicated strains plated on YPD plates in the absence or presence of DNA-damaging agents. (**B** and **C**) The *rad52*Δ and *uls1*Δ *rad52*Δ strains accumulate in G2–M phase with aberrant morphology (enlarged cells with elongated, tubular buds). Asynchronously growing log-phase cells of indicated strains were fixed and stained with Sytox Green to visualize nuclei followed by microscopic observation to score the number of cells in each cell-cycle phase and characterize cell morphology. (**D**) Level of Rad53 phosphorylation as a marker of DNA damage checkpoint activation in the indicated strains. Rad53 was analyzed by western blotting with anti-Rad53 antibodies. Slow-migrating forms of Rad53 in *rad52*Δ and *uls1*Δ *rad52*Δ represent hyperphosphorylated Rad53. (**E**) Flow cytometry analysis reveals severely prolonged S phase in *uls1*Δ *rad52*Δ cells. Cells were synchronized in G1 by α-factor and released into fresh YPD media at 28°C to monitor S phase progression by flow cytometry. The positions of G1 (1C) and G2–M (2C) peaks are indicated below. (**F**) Percentage of cells remaining in G1 after release from α-factor synchronization was determined by an α-factor–nocodazole trap assay. (**G**) To determine number of cells able to form colonies, G1-synchronized cells used for analysis by an α-factor–nocodazole trap assay (Figure 1F) were diluted and approximately 100 cells were plated on YPD medium.

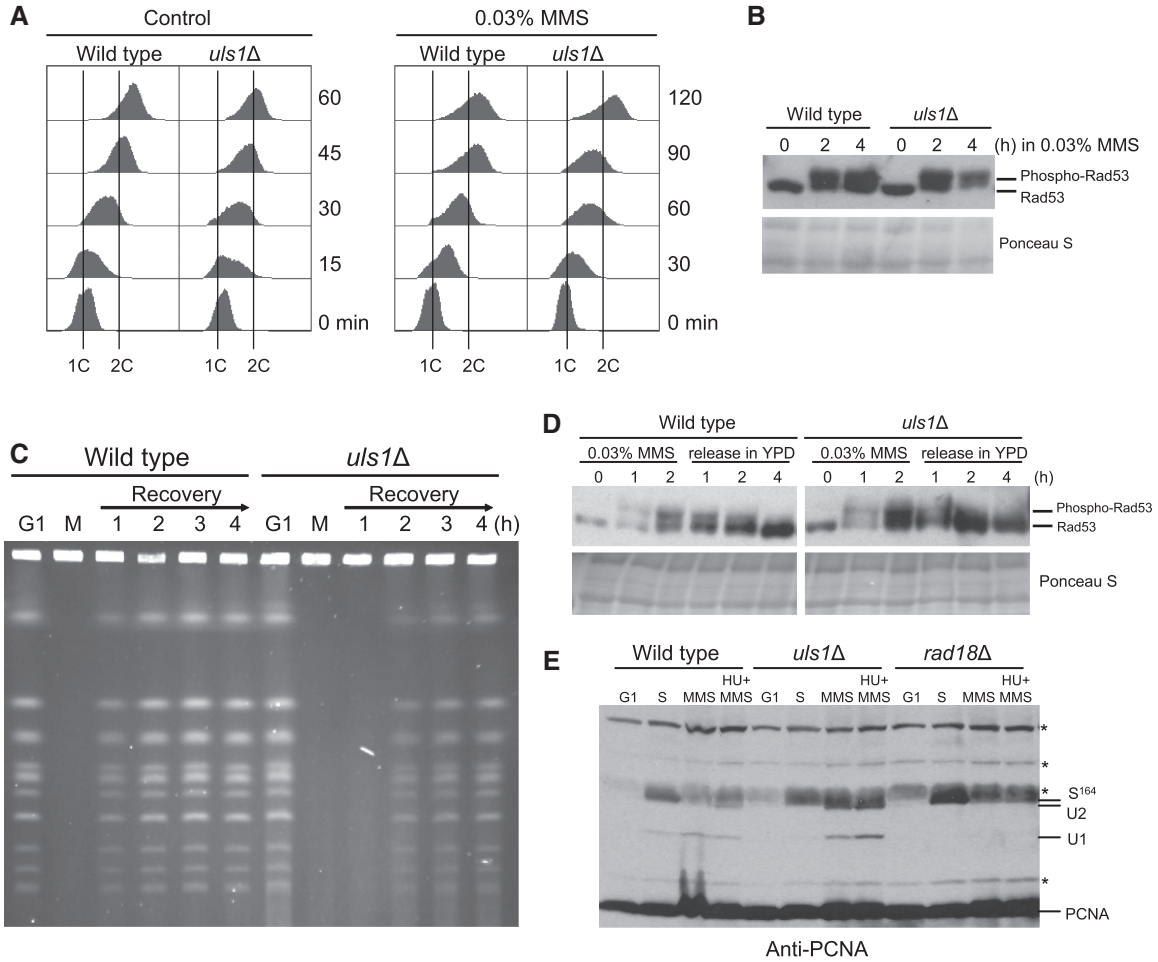


**Figure 2.** DNA synthesis defect in the *uls1*Δ mutant. (**A**) *uls1*Δ cells exhibit S phase delay in the presence of MMS. G1-synchronized cells of wild-type and *uls1*Δ were released in the presence or absence of MMS and DNA content was measured by flow cytometry at the indicated time-points. (**B**) Rad53 phosphorylation is increased in *uls1*Δ cells in response to MMS. Protein samples were prepared from G1-synchronized cells before and after release in the presence of MMS at indicated time-points and analyzed by western blotting with anti-Rad53 antibodies. (**C**) Completion of DNA replication is delayed in *uls1*Δ after MMS treatment. DNA samples from cells arrested in G1 by α factor (G1), released and treated with 0.03% MMS (M) for 60 min and then recovering in fresh YPD media were subjected to PFGE analysis. (**D**) DNA damage checkpoint activation does not persist in *uls1*Δ following MMS treatment. Protein extracts were prepared from the wild-type and *uls1*Δ cultures treated with 0.03% MMS for 2 h, then washed and allowed to recover in fresh media. (**E**) PCNA mono- and polyubiquitination is increased in the absence of Uls1. Cells were synchronized in G1, allowed to enter S phase and after 30-min incubation in YPD media exposed to 0.03% MMS or 0.03% MMS plus 0.1 M HU for 2 h. The *rad18*Δ mutant lacking PCNA ubiquitination was analyzed as a control. Non-specific bands are depicted by asterisks.

growth phenotype, increase in number of G2–M cells with elongated buds and hyperactivation of Rad53 in *uls1*Δ *rad52*Δ suggest that cells of this double mutant experience severe perturbations in replication progression. To test this hypothesis, we synchronized wild-type, *uls1*Δ, *rad52*Δ and *uls1*Δ *rad52*Δ cells in G1 with α-factor and released them into fresh YPD media to monitor cell-cycle progression by flow cytometry (Figure 1E). Both wild-type and *uls1*Δ completed S phase and reached 2C DNA content by 40 min, albeit *uls1*Δ with a slight delay. In contrast, *rad52*Δ cells replicated more slowly and accumulated in G2–M at 60–70 min after release, while the double *uls1*Δ *rad52*Δ mutant had not completed DNA synthesis even by that time. Moreover, deletion of *ULS1* in the *rad52*Δ mutant increased the number of cells failing to enter S phase (Figure 1F), which is in a good agreement with reduced plating efficiency (Figure 1G) and growth (Figure 1A) observed for the double *uls1*Δ *rad52*Δ mutant when compared to the single *rad52*Δ mutant. This suggests that cells lacking Rad52 and Uls1 accumulate DNA damage leading to perturbation of cell-cycle progression and loss of viability. In sum, our results indicate that Uls1 function becomes especially important when cells suffer from replication stress due to the absence of Rad52.

### Uls1 is required for S phase progression in the presence of DNA damage

To test the involvement of Uls1 in replication progression through damaged DNA template, we examined replication kinetics in wild-type and *uls1*Δ cells in the presence of MMS. Both strains were synchronized in G1, released into YPD or YPD with 0.03% MMS and analyzed by flow

cytometry at 30-min intervals for DNA content (Figure 2A). Under control conditions the *uls1*Δ mutant progressed slightly slower through S phase than wild-type. When cells were treated with MMS, both wild-type and *uls1*Δ replicated slowly but the *uls1*Δ mutant reproducibly completed DNA synthesis with at least 30-min delay. A defect in DNA synthesis observed in the *uls1*Δ mutant was accompanied by slightly increased level of phosphorylated Rad53 in MMS-exposed cells indicating impaired processing of stalled replication forks (Figure 2B). These results suggest that Uls1 facilitates DNA replication, especially when DNA template is damaged.

To determine whether S phase delay observed in *uls1*Δ is caused by prolonged persistence of unresolved replication intermediates, wild-type and *uls1*Δ cells were arrested in G1 by α-factor, released in 0.03% MMS for 1 h and then allowed to recover in MMS-free media to monitor the fate of chromosomes by PFGE (Figure 2C). As expected, the intact chromosomal DNA isolated from G1 cells was separated as individual, well-defined bands, while exposure to MMS resulted in retention of the chromosomal DNA in the loading wells because of incomplete replication and presence of branched structures (57,58). During recovery period wild-type cells quickly resumed replication indicated by re-entry of intact chromosomes into the gel after 1 h from MMS release. Consistently with the flow cytometry data, chromosomes from the *uls1*Δ mutant were retained in the wells much longer suggesting a delay in resolving stalled replication forks and/or recombination intermediates generated in response to MMS (Figure 2C). After release from MMS no persistent activation of Rad53 was observed in *uls1*Δ mutant (Figure 2D) implying that in the absence of Uls1 MMS-induced replication intermediates do not lead to the generation of replication-associated DNA damage, which is in a good agreement with the lack of *uls1*Δ sensitivity to genotoxic agents (Supplementary Figure S2).

In *S. cerevisiae*, replication of damaged DNA requires the *RAD6* pathway, which involves monoubiquitination of proliferating cell nuclear antigen (PCNA) on Lys164 by the Rad6–Rad18 E2–E3 ubiquitin-conjugating complex, which activates error-prone TLS, and/or polyubiquitination by the heterodimeric E2 Ubc13–Mms2 enzyme in concert with the RING finger E3 ubiquitin ligase Rad5, which triggers error-free lesion bypass pathway (59). Because of the putative role of Uls1 as a STUbL (35) belonging to the Swi2–Snf2 family of DNA-dependent ATPases with high similarity to Rad5 (40), we decided to examine post-translation modification status of PCNA in the *uls1*Δ mutant exposed to MMS in S phase (Figure 2E). We found that deletion of *ULS1* causes elevated levels of mono- and polyubiquitinated PCNA when compared to wild-type cells. First, this suggests that Uls1 does not contribute to PCNA ubiquitination and does not channel DNA damage bypass to PRR pathway. Second, increased PCNA ubiquitination may reflect activation of a rescue pathway to cope with elevated replication stress caused by *ULS1* deletion and/or may constitute one of alternative DNA damage bypass pathways triggered specifically in the absence of Uls1. As *ULS1* is epistatic to *RAD18*, *RAD5* and *REV3* (Supplementary Figure S3), we favor the hypothesis that Uls1 has an upstream role in channeling replication fork processing and repair into the *RAD6*-independent pathway.

### Uls1 shows genetic interactions with Mus81 and Sgs1

Many proteins are involved in replication fork progression, stabilization and restart, sometimes additionally, positively or negatively regulating HR events during replication. It has been shown that this function is fulfilled, among others, by anti-recombinogenic helicases such as Sgs1 (3) and Srs2 (4). Together with Mus81–Mms4 and Yen1 nucleases (58,60) as well as a third helicase, Mph1 (61), they play a crucial role in stalled replication fork processing. Having established that cells lacking Uls1 display replication defect in the presence of MMS, we decided to identify Uls1-mediated DNA repair pathway by examining the genetic interactions among Mph1, Mus81, Sgs1, Srs2, Yen1 and Uls1 in the presence of HU, MMS and CPT (Figure 3 and Supplementary Figure S4). We found that lack of *ULS1* had no effect on the phenotype of *mph1*Δ, *srs2*Δ, *yen1*Δ mutants (Supplementary Figure S4). In contrast, deletion of *ULS1* in the *mus81*Δ background conferred pronounced additional sensitivity to all DNA-damaging agents tested. This synergistic effect was comparable to that described recently for the *mus81*Δ *yen1*Δ mutant (58,60), which together with the epistatic relationship between *uls1*Δ and *yen1*Δ (Supplementary Figure S3), could point to the role of Uls1 in a Yen1-related pathway. However, we also observed partial rescue of HU and MMS sensitivity of *sgs1*Δ by concomitant deletion of *ULS1*, which has not been observed in *sgs1*Δ *yen1*Δ (60). Interestingly, this suppression was specific to Sgs1 function not shared with Rmi1–Top3 complex as it was not observed in the double *uls1*Δ *top3*Δ and *uls1*Δ *rmi1*Δ mutants (Figure 3).

Next we checked whether the observed increase of sensitivity in the *uls1*Δ *mus81*Δ double mutant and the suppression of DNA damage sensitivity in the *uls1*Δ *sgs1*Δ double mutant were indeed the result of deleting *ULS1*. We cloned the *ULS1* gene from genomic DNA by a plasmid rescue as described in Materials and Methods, and used it to transform single *mus81*Δ and double *uls1*Δ *mus81*Δ mutant strains. We showed that acquisition of wild-type copy of *ULS1* restored the sensitivity to HU, MMS and CPT of the double mutant to the level of the single *mus81*Δ (Figure 3). Similarly, we found that introduction of wild-type copy of *ULS1* into the *uls1*Δ *sgs1*Δ double mutant increased its sensitivity to HU and MMS to the level of single *sgs1*Δ, confirming the role of *ULS1* deletion in the suppression of *sgs1*Δ sensitivity to replication inhibitors (Figure 3). Interestingly, the presence of extra copies of *ULS1* in the single *sgs1*Δ mutant further increased its sensitivity to HU and MMS (Figure 3). Both observations suggest that Uls1 may act upstream in the Sgs1-dependent DNA repair pathway during replication stress.

To determine whether the lack of ATP-dependent DNA helicase activity of Uls1 is responsible for the phenotypes observed in the *uls1*Δ *mus81*Δ and *uls1*Δ *sgs1*Δ double

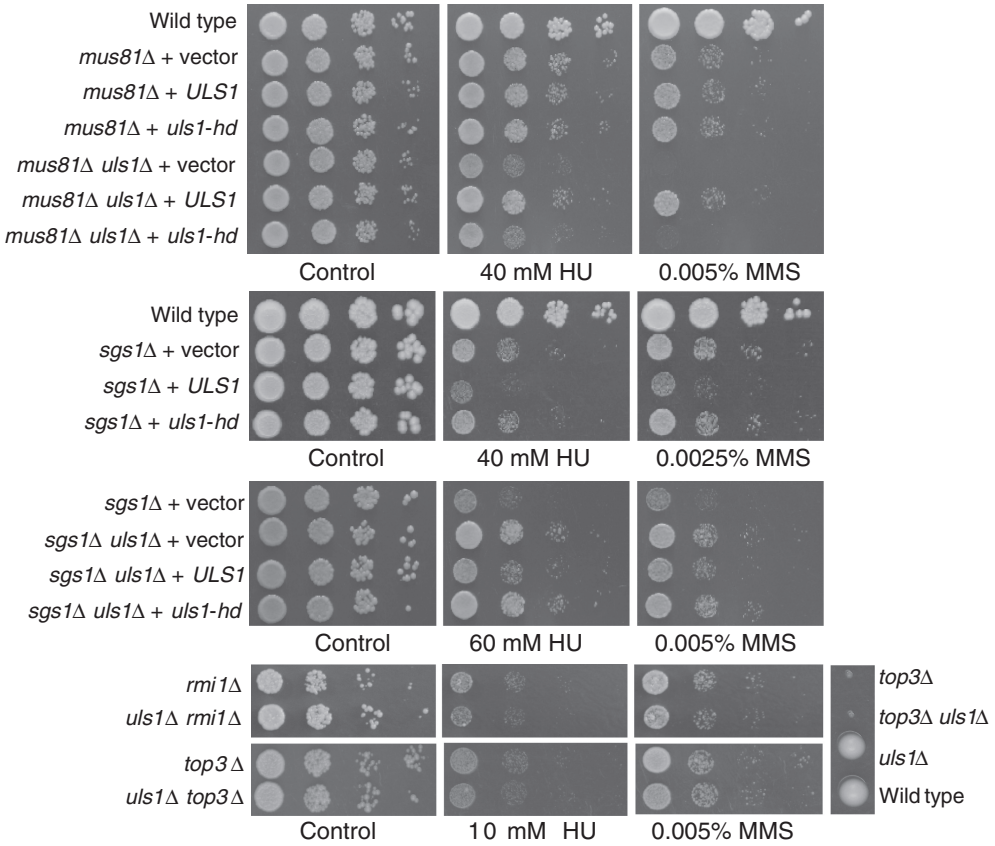


**Figure 3.** Deletion of *ULS1* suppresses DNA damage sensitivity of *sgs1*Δ but not *rmi1*Δ–*top3*Δ and shows synergistic effect with *mus81*Δ for decreased resistance to DNA damaging agents. Mutants were transformed with the centromeric plasmid bearing the wild-type *ULS1* gene (pGURA3_ULS1) or the mutated version *uls1-D1108A,E1109A* (pGURA3_uls1-hd) encoding the catalytic inactive Uls1 (*uls1-hd*). Growth test in the presence of HU, MMS and CPT was performed as in Figure 1.

mutants, we generated two point mutations in the conserved ATPase motif located in the N-terminal part of Uls1 helicase domain (*uls1-D1108A,E1109A* or *uls1-hd*) to obtain helicase–ATPase deficient protein (62) (Supplementary Figure S1). Expression of *uls1-hd* mutant neither complemented the increased sensitivity of *uls1*Δ *mus81*Δ to DNA damaging agents nor reversed suppression of DNA damage sensitivity in *uls1*Δ *sgs1*Δ (Figure 3). Moreover, expression of *uls1-hd* was not toxic in *sgs1*Δ in the presence of HU and MMS. This strongly suggests that DNA translocase activity of Uls1 is required for regulation of replication-associated DNA repair.

### Cells lacking both Uls1 and Mus81 exhibit prolonged G2–M delay

To investigate in greater detail the interactions of Uls1 with Mus81, we monitored the effect of MMS on the cell-cycle progression of wild-type and *uls1*Δ, *mus81*Δ and *uls1*Δ *mus81*Δ mutants (Figure 4A). Asynchronous cultures were used, since it has been shown that G1-arrested *mus81*Δ cells released into various concentrations of MMS could not be distinguished from wild-type (60). In the presence of low concentration of MMS (0.0015%), wild-type and single mutants showed no perturbation of cell-cycle progression, while the *uls1*Δ *mus81*Δ double mutant exhibited G2–M arrest (Figure 4A). When 0.015% MMS was added, wild-type cells first accumulated in G2–M phase but were able to adapt and resume cycling. The *uls1*Δ mutant progressed much slower than wild-type through DNA replication in the presence of high concentration of MMS, confirming our data obtained for cultures synchronized in G1 (Figure 2). In contrast, both *mus81*Δ and *uls1*Δ *mus81*Δ strains did not exhibit this slowing phenotype but accumulated with a 2 C DNA content during 4-h incubation in MMS. However, after 8 h in MMS *mus81*Δ restarted cell cycle, while *uls1*Δ *mus81*Δ showed a broadened peak of 2C DNA content, suggesting cell segregation defects and aberrant mitosis due to the persistence of unresolved recombination intermediates formed in S phase (Figure 4A) (63). Consistently, in the G2–M arrested population of *uls1*Δ *mus81*Δ we observed a 3-fold increase in the number of cells with morphology defects as compared to the *mus81*Δ single mutant (Figure 4B). Apart from enlarged cells with big buds and unsegregated DNA characteristic for *mus81*Δ and *mus81*Δ *yen1*Δ (60), many cells with multiple, elongated buds or filament-like projections were present (Figure 4B). The level of Rad53 phosphorylation induced by the incubation in 0.015% MMS was comparable in all four studied strains, indicating that the observed increase of

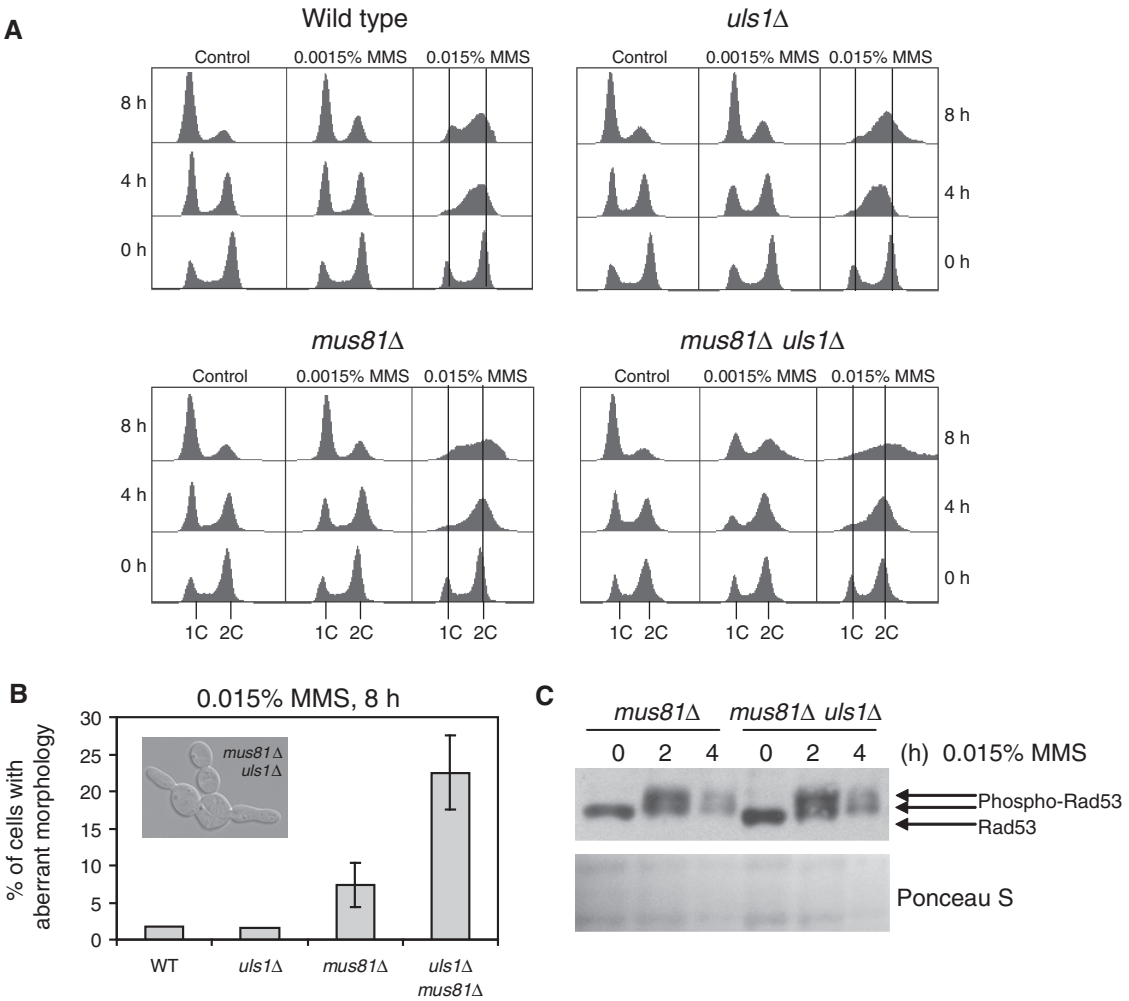


**Figure 4.** The *mus81*Δ *uls1*Δ double mutant arrests at G2–M with aberrant cell morphology during MMS treatment. (**A**) The *mus81*Δ *uls1*Δ double mutant accumulates with the G2–M DNA content after 8-h incubation in MMS. (**B**) The *mus81*Δ *uls1*Δ strain exhibits 3-fold increase of cells with morphology defects in the presence of MMS. (**C**) Western blotting of Rad53 reveals intact checkpoint activation in tested strains after MMS treatment. Asynchronous cells of indicated strains were exposed to MMS and analyzed by flow cytometry to measure DNA content, by microscopy to score the number of cells with altered morphology and used for protein extraction to detect Rad53.

sensitivity of *uls1*Δ *mus81*Δ did not result from a checkpoint defect (Figure 4C). We conclude that Uls1 is involved in an alternative Mus81-independent pathway for repair of replication-associated DNA damage.

### Deletion of *ULS1* suppresses several defects of *sgs1*Δ

Next we investigated the mechanism of DNA damage sensitivity suppression conferred by *ULS1* deletion in the *sgs1*Δ background suggesting the role of Uls1 in the Sgs1-dependent DNA damage bypass. Since *uls1*Δ cells exhibited a significant delay of S phase progression in the presence of MMS (Figure 2A) and in the absence of Rad52 (Figure 1E), we compared the effect of MMS on the cell-cycle progression of asynchronous cultures of *sgs1*Δ and double *uls1*Δ *sgs1*Δ mutants (Figure 5A). In the presence of 0.015% MMS, we did not see any slowing defect in the *sgs1*Δ mutant, only persisting G2–M arrest, resembling that of *uls1*Δ *mus81*Δ strain (compare Figure 4A). The *uls1*Δ *sgs1*Δ double mutant also accumulated with a 2C DNA content at 8 h in 0.015% MMS but in a much sharper peak, typical for G2–M checkpoint arrest suggesting decrease of unsolved recombination intermediates. Interestingly, in the presence of higher concentration of MMS (0.03%), S phase progression was significantly slower in the *uls1*Δ *sgs1*Δ double mutant compared to the single *sgs1*Δ (Figure 5A). This was accompanied by increased Rad53 phosphorylation induced by 0.03% MMS in *uls1*Δ *sgs1*Δ compared to *sgs1*Δ suggesting the involvement of checkpoint pathway in the observed phenomena (Figure 5B).

Increased mitotic recombination rate is one of major phenotypes of *sgs1*Δ mutant so we sought to determine if deletion of *ULS1* had any effect on this trait. We measured the rate of recombination by monitoring loss of the *ADE2* and *CAN1* marker genes in the rDNA array and the *URA3* gene located at the *SUP4*-**o** locus surrounded by several δ repeats (Figure 5C). As previously found (49), deletion of *SGS1* conferred high level of recombination at both sites. Interestingly, frequency of recombination was 2-fold decreased in *uls1*Δ *sgs1*Δ

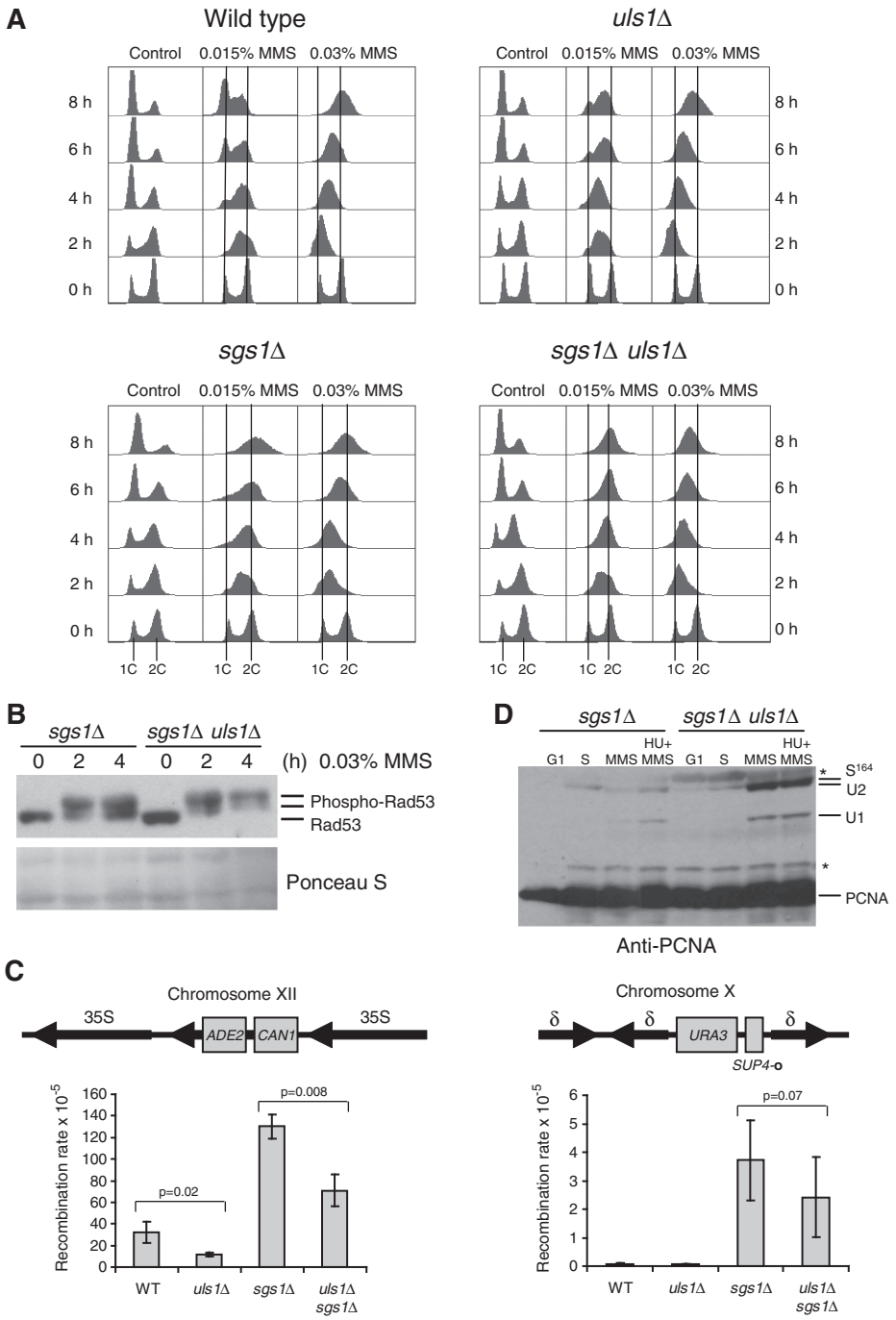


**Figure 5.** The *uls1*Δ mutation attenuates *sgs1*Δ defects in DNA damage response. (**A**) Flow cytometry analysis of DNA content in asynchronous cells treated with MMS revealed accumulation of *sgs1*Δ *uls1*Δ in S phase. (**B**) Deletion of *ULS1* upregulates checkpoint activation in *sgs1*Δ. Analysis of Rad53 phosphorylation in response to MMS exposure was performed as described in Figure 2. (**C**) Mutation of *ULS1* decreases recombination rate in both wild-type and *sgs1*Δ. Recombination rates were calculated by measuring either the number of cells resistant to FOA and thus exhibiting the loss of *URA3* marker from the *SUP4*-**o** locus or the number of cells resistant to canavanine and forming red colonies on YPD plates as a result of recombination between rDNA repeats leading to the simultaneous loss of *ADE2* and *CAN1* marker genes. All experiments were repeated six times with three independent clones each time. Standard deviations are indicated. (**D**) High increase of PCNA mono- and polyubiquitination in *sgs1*Δ after deletion of *ULS1*. Analysis of PCNA modifications was conducted as described in Figure 2.

and *uls1*Δ strains at the rRNA locus. The effect at the *SUP4*-**o** locus was not statistically significant due to high variability of data between experiments in our hands but we reproducibly observed decrease in recombination frequency in *uls1*Δ *sgs1*Δ cells compared to the *sgs1*Δ single mutant. Since hyperrecombination phenotype of *sgs1*Δ results from DNA replication-associated defects this strengthens our conclusion that Uls1 is involved in the Sgs1 pathway for dealing with replication stress. We should emphasize, however, that even though deletion of

*ULS1* can rescue many of *sgs1*Δ mutant phenotypes, however the suppression does not reach the wild-type level, so there are other aspects of Sgs1 activity which are clearly Uls1-independent.

Having established that in the *uls1*Δ mutant the level of ubiquitinated PCNA is significantly increased, we hypothesize that channeling of replication-associated DNA damage repair into PRR pathway in the absence of Uls1 may be responsible for the suppression of *sgs1*Δ sensitivity to HU and MMS. Accordingly, the analysis of PCNA modifications revealed that the *sgs1*Δ mutant exhibited low level of ubiquitinated PCNA in the presence of MMS, while the *uls1*Δ *sgs1*Δ double mutant was characterized by accumulation of mono- and polyubiquitinated forms of PCNA (Figure 5D) suggesting that indeed in these cells repair of replication-associated DNA damage is channeled into PRR. We conclude that this, together with increased checkpoint activation in the double mutant providing additional time to cope with DNA damage, contributes to the observed rescue of *sgs1*Δ DNA damage sensitivity by concomitant *ULS1* deletion.

## DISCUSSION

Uls1 belongs to the Swi2–Snf2 family of DNA-dependent ATPases whose other members like the RING-finger E3 ubiquitin ligase Rad5 (59), to which it shows a high degree of homology (40), or the chromatin remodeling complex Ino80 (64) are implicated in DNA repair and replication fork processing, so we sought to determine if Uls1 fulfills a similar role. In this study, we found that the strain deleted for *ULS1* does not show any sensitivity to DNA damaging agents suggesting that Uls1 has no role in DNA damage repair. However, the mutant proceeds slower than wild-type through S phase and exhibits delayed completion of DNA replication after MMS treatment as seen on the PFGE gels. Interestingly, deletion of *ULS1* leads to synthetic growth defect and dramatic elongation of S phase in *rad52*Δ background under standard conditions. *uls1*Δ *rad52*Δ mutant accumulated in G2–M phase of cell cycle with increased number of cells exhibiting aberrant morphology and flow cytometry profiles, indicative of the defects in S phase progression and chromosome segregation. Slow growth of *rad52*Δ mutant results from accumulation of ssDNA lesions and failure to repair spontaneous DSB arising infrequently in wild-type during replication (33). Together these results strongly point to the role of Uls1 protein in dealing with replication stress.

We next examined the effect of deleting *ULS1* in the background of several genes involved in replication fork metabolism and found no synergism with *mph1*Δ, *srs2*Δ, *rad5*Δ and *yen1*Δ, but demonstrated interesting genetic interactions with *mus81*Δ and *sgs1*Δ, both dependent on Uls1 ATPase activity as the *uls1-hd* strain behaved like the *uls1*Δ mutant. The *uls1*Δ *mus81*Δ double mutant was characterized by highly elevated sensitivity to MMS, HU and CPT, as well as a more pronounced G2–M arrest with a 3-fold increase in the number of cells with morphology defects as compared to the *mus81*Δ single mutant, suggesting that Uls1 and Mus81 endonuclease act in complementary pathways. The RecQ helicase Sgs1 also works in parallel to Mus81 in DNA replication and repair, and interestingly we observed partial suppression of HU and MMS sensitivity of *sgs1*Δ mutant by simultaneous deletion of *ULS1*. Surprisingly, this suppression was specific to Sgs1 function not shared with Rmi1–Top3 complex, as it was not visible in the double *uls1*Δ *rmi1*Δ nor in the *uls1*Δ *top3*Δ mutant. Deletion of *ULS1* in the *sgs1*Δ background not only rescues its sensitivity to HU and MMS but also considerably slows down S phase progression under MMS treatment as compared to the single *sgs1*Δ mutant, probably due to enhanced Rad53 phosphorylation observed in *uls1*Δ *sgs1*Δ. Recently it has been shown that in *S. pombe*, apart from canonical checkpoint genes, three additional genes, *mus81*$^{+}$, *rqh1*$^{+}$ (*SGS1*) and *sfr1*$^{+}$, were necessary to slow replication in the presence of MMS (65) but this ability was not correlated with MMS sensitivity of tested strains, the latter resulting rather from DNA damage repair defects in G2. Failure to slow replication may instead contribute to increased genomic instability as is seen in human patients lacking S phase DNA damage checkpoint (66), a phenotype also observed in both *mus81*Δ and *sgs1*Δ mutants. We found that deletion of *ULS1* partially complemented the hyperrecombination in *sgs1*Δ mutant at rRNA locus with only mild albeit consistent effect at *SUP4*-**o** indicating that slowing of S phase progression might have resulted in a decrease in genomic instability in the double mutant.

A model has been proposed by Fabre *et al.* (33) where, apart from its role in dissolution of recombination intermediates with Top3–Rmi1, Sgs1 would aid in replication fork progression through obstacles and regulate the restart of stalled forks by non-recombinogenic mechanisms to prevent initiation of HR. The function of Sgs1 with Top3 and Rmi1 in HR repair is separable from processing DNA replication intermediates and can be uncoupled by deleting a single aspartic acid residue (49). Also, even though the synthetic lethality of *sgs1*Δ *mus81*Δ double mutant can be suppressed by deletion of HR genes, suggesting that both proteins may act in non-redundant but overlapping pathways for the removal of toxic recombination intermediates, the generation times of such triple mutants are significantly higher than those of respective *rad* mutants. This would imply that Mus81 and/or Sgs1 also have roles that are independent of recombination (33). As described above *uls1*Δ *rad52*Δ mutant exhibits synthetic growth defect and the generation time of *sgs1*Δ *rad52*Δ double mutant is also increased relative to the single *rad52*Δ mutant (67–69), reinforcing the notion that Sgs1 and Uls1 might participate in the upstream events at replication forks. It has been shown that human BLM helicase by itself can mediate replication fork regression (70), dissociate D-loops (71) and promote disruption of inactive hRad51 filament (72) thus preventing the formation of toxic recombination intermediates and channeling replication associated structures away from HR. Given the conservation of function among RecQ helicases, Sgs1 may possess similar biochemical activities facilitated by Uls1. Alternatively, Sgs1 might

limit early Rad51-dependent recombination at the fork and thus suppress genome rearrangements, but have no role in Rad51-independent, but Rad52-dependent processes of fork restart, as has recently been shown for *S. pombe* Sgs1 homolog, Rqh1 (73). This activity would involve disassembling D-loops or Rad51 filaments but not dissolution of HJ.

Uls1 can thus act upstream of Sgs1, for example providing chromatin environment facilitating its activity related to regulation of replication fork progression, not Top3–Rmi1-dependent dissolution of recombination intermediates. In its absence other factors might take over and divert replication fork restart into Sgs1-independent pathways, such as error-free PRR, Mus81 or Esc2–Mph1 related HR repair (63), thus alleviating HU and MMS sensitivity and hyperrecombination phenotype of the *sgs1*Δ mutant strain. Interestingly, deletion of *ULS1* not only results in increased levels of PCNA polyubiquitination in a single mutant but also in the double *uls1*Δ *sgs1*Δ mutant, indicative of the channeling of replication associated lesions into PRR pathway, which could possibly contribute to the observed rescue of *sgs1*Δ phenotype described in this paper. It would be of great interest to determine in the future what are the roles of the chromatin remodeling and SUMO-dependent ubiquitin ligase activities in the phenotypes observed upon *ULS1* deletion.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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