

# Evidence that the *S.cerevisiae* Sgs1 protein facilitates recombinational repair of telomeres during senescence

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## ABSTRACT

**RecQ DNA helicases, including yeast Sgs1p and the human Werner and Bloom syndrome proteins, participate in telomere biology, but the underlying mechanisms are not fully understood. Here, we explore the protein sequences and genetic interactors of Sgs1p that function to slow the senescence of telomerase (*tlc1*) mutants. We find that the S-phase checkpoint function of Sgs1p is dispensable for preventing rapid senescence, but that Sgs1p sequences required for homologous recombination, including the helicase domain and topoisomerase III interaction domain, are essential. *sgs1* and *rad52* mutations are epistatic during senescence, indicating that Sgs1p participates in a *RAD52*-dependent recombinational pathway of telomere maintenance. Several mutations that are synthetically lethal with *sgs1* mutation and which individually lead to genome instability, including *mus81*, *srs2*, *rrm3*, *slx1* and *top1*, do not speed the senescence of *tlc1* mutants, indicating that the rapid senescence of *sgs1 tlc1* mutants is not caused by generic genome instability. However, mutations in *SLX5* or *SLX8*, which encode proteins that function together in a complex that is required for viability in *sgs1* mutants, do speed the senescence of *tlc1* mutants. These observations further define roles for RecQ helicases and related proteins in telomere maintenance.**

## INTRODUCTION

*Saccharomyces cerevisiae* Sgs1p is a member of the RecQ family of 3'–5' DNA helicases (1–4). Human RecQ helicases include WRN, BLM and RTS, which are deficient in the Werner, Bloom and Rothmund-Thomson syndromes, respectively (5–7). These are recessive disorders characterized by genome instability, cancer predisposition and—particularly in the case of Werner syndrome—by premature features of aging (8–11). RecQ-family proteins maintain the genome through several mechanisms (3,4,12). For example, Sgs1p stabilizes stalled replication forks and facilitates the proper resumption of DNA synthesis from stalled forks (13–19). Sgs1p inhibits crossover events during homologous recombination (HR) (20), and certain phenotypes in *sgs1* mutants can be suppressed by deletion of genes in the *RAD52* epistasis group, indicating that Sgs1p normally prevents inappropriate recombination or facilitates resolution of recombination intermediates (21,22). Sgs1p, similar to WRN and BLM, can unwind a variety of DNA structures that may be important during replication or recombination, including replication fork-like substrates, Holliday junctions and G-quadruplexes (23–27). Sgs1p also functions in parallel with Rad24p to ensure a complete S-phase checkpoint response to DNA damage (28). Failure of these activities in *sgs1* mutants leads to aberrant recombination in repeated sequences, chromosome loss, gross chromosomal rearrangements, unequal sister chromatid exchange, sensitivity to methyl methanesulfonate (MMS) and hydroxyurea (HU), as well as to defects in ribosomal DNA replication and to the premature cessation of budding by yeast mother cells (1,13,15,29–32).

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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Sgs1p also participates in telomere function. Yeast telomeres are maintained naturally in part through the actions of telomerase, which is expressed constitutively (33,34). Telomerase can be inactivated genetically, for example by deleting *TLC1*, the gene encoding the RNA template component of the enzyme (35). Telomeres shorten with cell division in *tlc1* mutants and this leads eventually to induction of a DNA damage response and G2/M cell cycle arrest, a process termed senescence (33,36–38). Rare cells escape senescence and these survivors use *RAD52*-dependent recombination mechanisms to maintain their telomeres (39–41). There are two such survivor mechanisms, type I involving the amplification primarily of subtelomeric Y' elements along with telomere repeat DNA and type II involving primarily the amplification of the telomere repeat DNA alone. Although deletion of *SGS1* does not affect steady state telomere length in cells containing telomerase, mutants lacking both *SGS1* and telomerase senesce more rapidly than mutants lacking telomerase alone, and only type I survivors emerge in the absence of *SGS1* (29,36,42,43). Thus, Sgs1p slows senescence and is required for initiation of the type II survivor recombination mechanism. Recent findings also support a role for the mammalian WRN and BLM proteins in telomere function. For example, each protein interacts physically and functionally with the telomere chromatin protein TRF2 (44,45), which itself plays a critical role in maintaining telomeres in a protected or 'capped' state (46). The capacity of purified TRF2 to catalyze T-loop assembly is thought to be related to this capping function (47), and therefore demonstrations that the WRN helicase and exonuclease activities process telomeric T-loop structures *in vitro* support the idea that WRN and TRF2 cooperate to maintain telomeres (48,49). Moreover, Werner mutant cells suffer from an elevated level of S-phase-dependent telomere loss (50,51). Furthermore, BLM overexpression lengthens telomeres in cells that use recombination to maintain their telomeres (52), and *Wrn* and *Blm* mutations each synergize with telomerase (*Terc*) mutation to accelerate telomere dysfunction and cause pathology in mice (53,54). Our yeast model provides a relatively simple system in which to probe mechanisms by which RecQ helicases participate in telomere function.

The rapid senescence of *tlc1 sgs1* mutants is due, at least in part, to a synergistic interaction between the mutations that renders the cells more prone to arrest at a given average extent of telomere shortening (36). This observation might be explained by a role for Sgs1p in processes that are parallel to or downstream from telomere defects and thus affect the sensitivity of cells to telomere dysfunction. One such possibility is that rapid senescence might result from an elevated level of global genome instability caused by *sgs1* mutation, which could sum with telomere dysfunction to yield an overall DNA damage signal that leads to premature cell cycle arrest. A second such possibility is that rapid senescence might instead be due to loss of *SGS1*-dependent intra-S-phase checkpoint function, which may in turn preclude normal repair of telomere damage and thus cause premature telomere dysfunction. Alternatively, the premature arrest of *tlc1 sgs1* mutants at longer mean telomere lengths might reflect a direct role for Sgs1p in maintaining telomere integrity, perhaps via recombinational repair of shortened telomeres. Its requirement for type II survivor formation is consistent with a role for Sgs1p

in recombination involving the G-rich telomere sequences (36,42,43). One dysfunctional telomere is sufficient to cause cell cycle arrest (55), and so only a rare telomere might require recombinational repair, thus explaining the premature arrest of *tlc1 sgs1* cells at longer mean telomere lengths. Such defects could be repaired by telomerase, when present, and obviate a requirement for Sgs1p, thus explaining the normal telomere lengths and absence of G2/M arrest in *TLC1 sgs1* cells (36).

Here, we present evidence that neither loss of the *SGS1*-dependent intra-S-phase checkpoint nor increased global genome instability explains the rapid senescence of *tlc1 sgs1* mutants. Our findings rather support a role for Sgs1p in the recombinational repair of telomeres.

## MATERIALS AND METHODS

### Yeast strains

All yeasts were grown at 30°C using standard yeast media and techniques (56). All strains are described in Table 1 and were isogenic derivatives of JKM111 (57). The *sgs1* deletion in YBJ133 is a null allele (58) but retains the first 481 nt of the open reading frame (ORF) which might generate an N-terminal Sgs1p fragment that, although inert itself, could conceivably function in *trans* with other Sgs1p derivatives (59). To avoid such potential *trans* complementation from the various *SGS1* derivatives that were examined, the entire *SGS1* ORF except for the start and stop codons was deleted by PCR-mediated gene disruption to generate YMA75b. *rad52::LEU2* was generated with pSM20 (58). The *top1*, *mus81*, *slx1*, *slx5*, *slx8*, *est2*, *rrm3* and *srs2* mutations were

**Table 1.** *S.cerevisiae* strains used in this study

Strain	Genotype	Reference or source
YBJ133	MATa $\alpha$ , $\Delta$ ho $\Delta$ hml::ADE1 $\Delta$ hmr::ADE1 <i>ade1 ura3-52</i> , <i>leu2-3,112, lys5, TLC1/<math>\Delta</math>tlc1::kanMX</i> , <i>SGS1/<math>\Delta</math>sgs1::hisG-URA3</i>	(36)
YJL13b	YBJ133 <i>RAD52/rad52::LEU2</i>	This study
YMA64	YBJ133 <i>TOP1/<math>\Delta</math>top1::LEU2</i>	This study
YBJ161	YBJ133 <i>SGS1/<math>\Delta</math>sgs1::hisG'</i>	This study
YMA75b	YBJ161 <i>SGS1/<math>\Delta</math>sgs1::URA3</i>	This study
YMA76	YMA75b <i>leu2/LEU2::SGS1*</i>	This study
YMA77	YMA75b <i>leu2/LEU2::SGS1-hd*</i>	This study
YMA79	YMA75b <i>leu2/LEU2::SGS1-<math>\Delta</math>N50*</i>	This study
YMA90	YMA75b <i>leu2/LEU2::SGS1-<math>\Delta</math>N795*</i>	This study
YMA97	YMA75b <i>leu2/LEU2::SGS1-<math>\Delta</math>C200*</i>	This study
YMA99	YMA75b <i>leu2/LEU2::pRS405</i>	This study
YBJ216	YMA75b <i>leu2/LEU2::TOP3-SGS1-<math>\Delta</math>N106*</i>	This study
YMA102	YBJ133 <i>MUS81/<math>\Delta</math>mus81::HygB</i>	This study
YMA104	YBJ133 <i>SLX8/<math>\Delta</math>slx8::HygB</i>	This study
YMA105	YBJ133 <i>TOP3/<math>\Delta</math>top3::HygB</i>	This study
YMA107	YBJ133 <i>SLX5/<math>\Delta</math>slx5::LEU2</i>	This study
YMA109	YBJ133 <i>SLX1/<math>\Delta</math>slx1::LEU2</i>	This study
YJL22	MATa $\alpha$ , $\Delta$ ho $\Delta$ hml::ADE1 $\Delta$ hmr::ADE1 <i>ade1 ura3-52</i> , <i>leu2-3,112, lys5, TLC1/<math>\Delta</math>tlc1::kanMX</i>	This study
YJL42	YJL22 <i>RRM3/<math>\Delta</math>rrm3::HygB</i>	This study
YJL44	YJL22 <i>SRS2/<math>\Delta</math>srs2::HygB</i>	This study
YJL68	MATa $\alpha$ , $\Delta$ ho $\Delta$ hml::ADE1 $\Delta$ hmr::ADE1 <i>ade1 ura3-52</i> , <i>leu2-3,112, lys5, SGS1/<math>\Delta</math>sgs1::hisG-URA3</i> , <i>RAD52/rad52::LEU2</i>	This study
YJL69	YJL68 <i>EST2/<math>\Delta</math>est2::kanMX</i>	This study

complete PCR-mediated ORF deletions, using pRS405, pUG6 (kanR) or pAG32 (hphMX4) as templates for marker gene amplification (60). The *top3* deletion extended from nucleotides 10 to 1666 of the ORF, to avoid disruption of the overlapping ORFs *YLR235C* and *YLR236C*. Deletions were confirmed by PCR amplification of fragments that crossed both mutation breakpoints. Oligonucleotide sequences are available on request.

### Plasmids

The *SGS1* deletion and point mutation derivatives (pJM526, pJM511, pJM531, pJM512 and pRL1; kindly provided by S. J. Brill) were cloned downstream a 0.15 kb *SGS1* promoter fragment in pRS405, as described previously (59). The *TOP3-SGS1-ΔN106* allele was generated by PCR amplification of the *TOP3* ORF from pRK500 (kindly provided by J. Wang) with incorporation of NdeI and NcoI sites at the 5' and 3' ends, respectively, and insertion of NdeI and NcoI digested product into NdeI and partially-NcoI digested pJM531; this yielded a fusion of the full *TOP3* ORF to the *SGS1* ORF beginning at amino acid 107, in a fashion similar to that described previously (61). For all *SGS1* derivatives, BstEII-digested plasmid DNA was transformed into yeast cells, and proper integration of a single copy of each *SGS1*\* mutant at the *LEU2* locus was confirmed by Southern blot analysis of SacI-digested genomic DNA probed with pRS405.

### Liquid senescence assays

To ensure that senescence comparisons were made between strains that inherited similar telomeres, senescence assays were performed on the haploid spore products of diploids that were heterozygous for *tlc1* and the various test mutations. Liquid senescence assays were performed as described previously (36), starting with spore products from reference plates (estimated population doubling of 30). At the start of each day of growth,  $2 \times 10^6$  cells were inoculated into 5 ml of YPD followed by growth for 22 h, and the process was repeated until survivors emerged. Cells numbers were determined with a Coulter counter, and population doubling calculated as  $\log_2(\text{final cell number}/\text{starting cell number})$ . Each genotype was represented by three to six independent spore products, and the mean and standard errors of measurements are shown in figures.

### Spot growth assays

Cells were grown overnight on YPD plates and were suspended in sterile water at an  $OD_{600}$  of 3.0. Cells were spotted at this concentration and at four serial 10-fold dilutions on YPAD plates and were grown at 30°C for 60 h.

### Telomere Southern analysis

Southern analysis was performed essentially as described previously (36). Briefly, purified genomic DNA was digested with XhoI, separated by electrophoresis in 1% agarose gels, transferred to Hybond-XL membranes, and probed and washed at high stringency with a cloned 256 bp fragment of *S.cerevisiae* telomere repeat DNA (BamHI–MluI fragment of pJL8-50, Julia Y. Lee and F. Brad Johnson, manuscript in preparation) or with a 784 bp *Y'* fragment distal to the XhoI site (36). *tlc1 rad51* type II survivor DNA was from (36).

## RESULTS

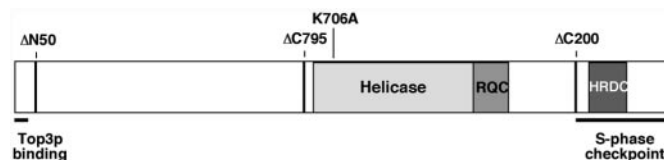
### The C-terminus of Sgs1p, required for intra-S-phase checkpoint function, is not required to rescue rapid senescence

To begin to dissect how Sgs1p slows the rate of senescence of telomerase (*tlc1*) mutants, we tested different deletion and point mutation alleles of *SGS1* (Figure 1) for their ability to rescue the rapid senescence of *tlc1 sgs1* mutants. The derivative alleles, designated with an asterisk, were placed downstream of the *SGS1* promoter and integrated at the *LEU2* locus (59). Diploids heterozygous for *tlc1* and *sgs1* mutations and an \* allele were sporulated, and the rates of senescence of haploid products lacking telomerase were determined using liquid senescence assays. We first confirmed that wild type *SGS1* sequences integrated at *LEU2* (denoted *SGS1*\*) could rescue an *sgs1* mutation during senescence. *tlc1 sgs1* mutants carrying *SGS1*\* senesced at a rate equivalent to *tlc1* mutants (Figure 2A), while a vector control lacking all *SGS1* sequences integrated at *LEU2* failed to have any effect (Figure 2B). Furthermore, *tlc1* mutants carrying *SGS1*\* senesced at the same rate as *tlc1* mutants, indicating that *SGS1*\* did not have effects beyond the rescue of the *sgs1* mutation. Thus, integrated *SGS1*\* can function in the senescence assay in the same fashion as the native *SGS1* locus.

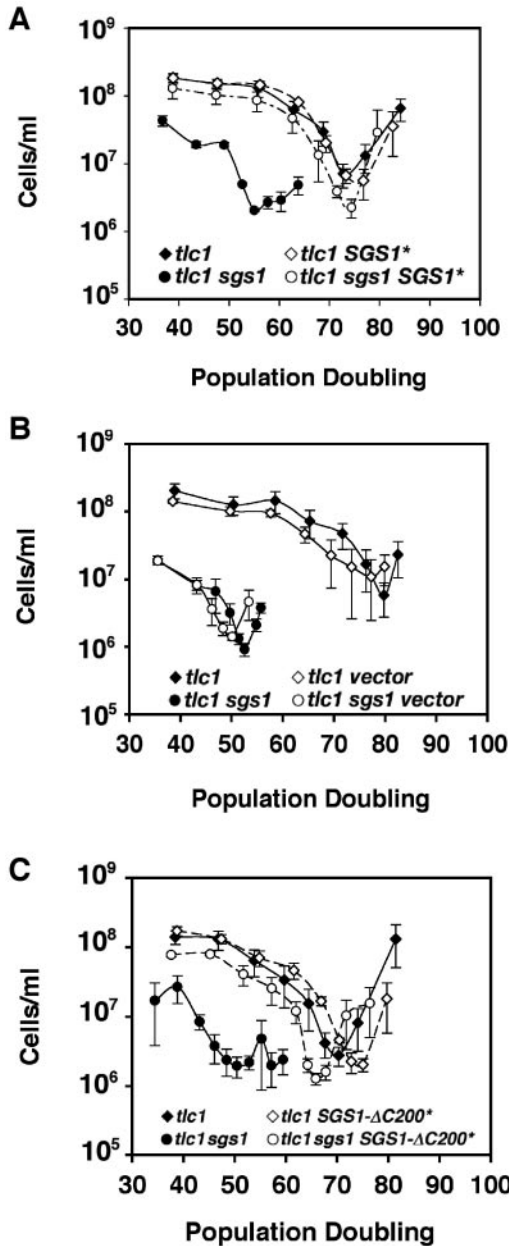
The C-terminal 200 amino acids of Sgs1p have been shown to be essential for the intra-S-phase checkpoint activity of the protein (28). We therefore tested the ability of *SGS1-ΔC200*\*, which encode an Sgs1p derivative lacking these amino acids, to rescue rapid senescence. Similar to *SGS1*\*, *SGS1-ΔC200*\* slowed the rate of senescence to that observed in *tlc1* mutants (Figure 2C). Therefore, the intra-S-phase checkpoint function of *SGS1* does not appear to be required to prevent rapid senescence.

### Sgs1p slows senescence through an HR-dependent pathway

Because the checkpoint function of *SGS1* appeared not to be involved in slowing senescence, we considered the possibility that the homologous (or homeologous, in the case of the imperfect yeast telomere repeats) recombination (HR) functions of *SGS1* might instead play a role. *RAD52* is required for nearly all HR reactions in yeast (62), and so we tested for a possible epistatic relationship between *sgs1* and *rad52*



**Figure 1.** Map of the 1447 amino acid Sgs1p protein. Indicated by shaded boxes are the helicase domain, the RQC domain (a C-terminal extension from the helicase domain of shared homology among RecQ-family helicases, which is involved in protein–protein interactions) and the HRDC domain (helicase/RNase D C-terminal domain, which appears to be involved in DNA binding). The locations of the N- and C-terminal deletion breakpoints used in this study are indicated by thick vertical lines and labels above the protein, as is the helicase-deficient alanine substitution mutant, K706A. Sequences containing amino acids that are essential for the binding of Sgs1p to Top3p or for the intra-S-phase checkpoint function of Sgs1p are indicated by horizontal bars below the protein.



**Figure 2.** Full-length and  $\Delta C200$  forms of Sgs1p rescue the fast senescence of *tlc1 sgs1* mutants. Full-length *SGS1*, *SGS1- $\Delta C200$*  or control vector sequences were integrated at the *LEU2* locus of diploids heterozygous for *sgs1* and *tlc1* null mutations. *tlc1* and *tlc1 sgs1* spore products without or with the integrated *SGS1* alleles (indicated by an asterisk) were compared in liquid senescence assays (see Materials and Methods). The cumulative population doubling since spore germination, and the density of cells after 22 h of growth, from cultures inoculated with  $4 \times 10^5$  cells/ml are shown. For each time point, the mean and standard errors for at least three independent spore products for each genotype are indicated. For each experiment, filled diamonds indicate *tlc1*, filled circles indicate *tlc1 sgs1*, open diamonds indicate *tlc1* with an integrated \* allele, and open circles indicate *tlc1 sgs1* with an integrated \* allele, as shown. (A) Rescue by full-length integrated *SGS1\** (B) Lack of rescue by the pRS405 vector used to integrate the *SGS1\** alleles. (C) Rescue by *SGS1- $\Delta C200*$* , which encodes an Sgs1p derivative lacking the C-terminal 200 amino acids.

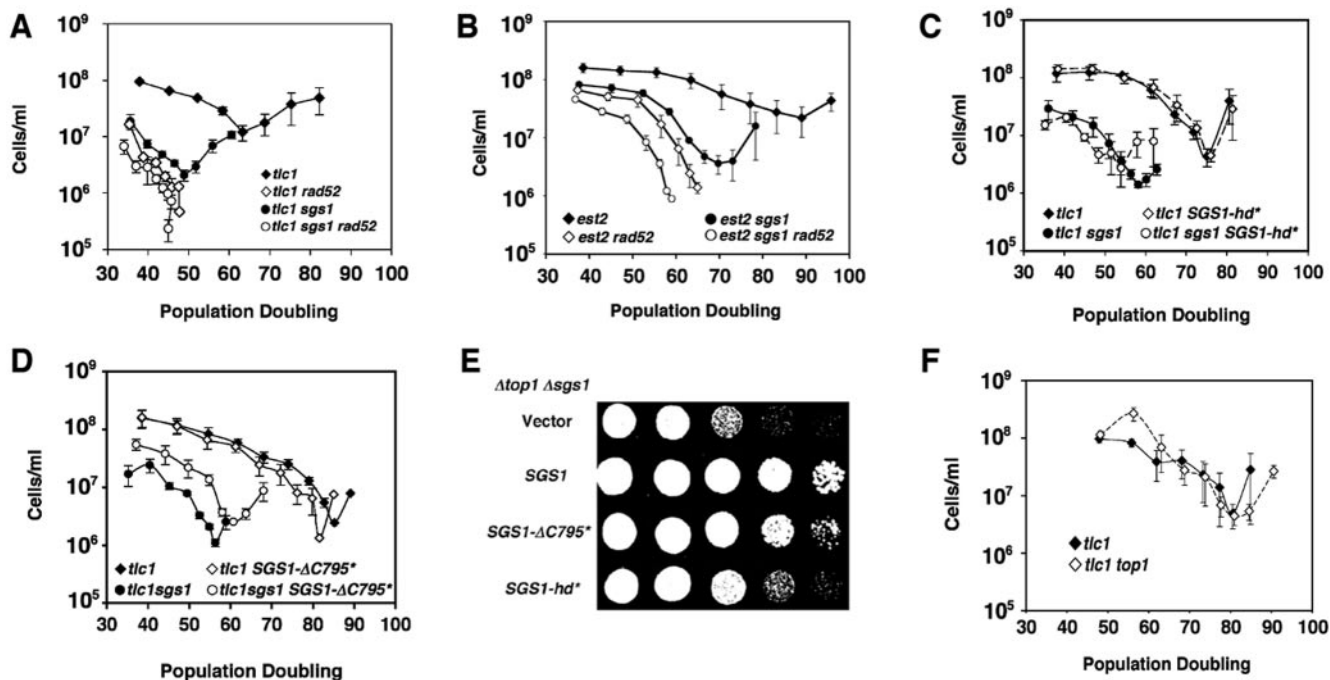
mutations in the absence of telomerase. As reported previously, *tlc1 rad52* mutants senesced rapidly (39,57), consistent with a role for HR in slowing senescence, and at a rate similar to *tlc1 sgs1* mutants (Figure 3A); also, as expected,

no survivors of senescence were formed in the absence of *RAD52* function. Although *tlc1 rad52 sgs1* mutants senesced slightly faster than *tlc1 rad52* or *tlc1 sgs1* mutants, the contribution of the *sgs1* or *rad52* mutation to the faster senescence of the triple mutant was far less than the contribution of either mutation to the rapid senescence in the double mutants, and thus the *rad52* and *sgs1* mutations are largely epistatic in our assay. This result was repeated twice using independent sets of spore products and was also observed when telomerase was inactivated via deletion of *EST2*, rather than *TLC1* (Figure 3B). *SGS1* therefore appears to function in a *RAD52*-dependent pathway during senescence. Furthermore, the helicase activity of Sgs1p, which is required for Sgs1p HR activities in other contexts (31,59), was necessary to prevent rapid senescence because the *SGS1-hd\** allele, which encodes a K706A mutant devoid of helicase activity, failed to rescue the rapid senescence of *tlc1 sgs1* mutants (Figure 3C). Similarly, the *SGS1- $\Delta C795*$*  allele, which encodes a protein lacking the helicase domain and C-terminus of Sgs1p, did not rescue rapid senescence (Figure 3D). These observations are consistent with a role for *SGS1* in HR during senescence.

To confirm that the failure of the *SGS1-hd\** and *SGS1- $\Delta C795*$*  alleles to rescue rapid senescence was not simply due to lack of their expression, we examined their activities in cells lacking topoisomerase I (*top1* mutants). Loss of *SGS1* function causes a slight decrease in growth rate, as does loss of *TOP1* function, but an *sgs1 top1* double mutant has a synthetic growth defect (63). As reported previously (59), the *SGS1-hd\** and *SGS1- $\Delta C795*$*  alleles partially and fully, respectively, restored the growth rate of *sgs1 top1* mutants to that of *top1* mutants (Figure 3E), confirming the activities of the alleles. Furthermore, because Sgs1p helicase activity is critical during senescence but less important for growth in *top1* mutants, different functions of *SGS1* are apparently involved in each case, and this in turn implies that *TOP1* function may not play a role during senescence. Indeed, *tlc1 top1* null mutants senesce at the same rate as *tlc1* mutants (Figure 3F). This is remarkable because, similar to *sgs1* deletion, *top1* deletion causes genome instability, particularly in the ribosomal DNA (64,65). Therefore, a decrease in genome stability, at least in a generic sense, is not sufficient to speed senescence. This point is explored more fully below.

### Sgs1p cooperates with Top3p to slow senescence

A derivative of Sgs1p lacking the N-terminal 50 amino acids, encoded by *SGS1- $\Delta N50*$* , was tested and found to be devoid of *SGS1* function in the senescence assay (Figure 4A). The deleted N-terminus includes residues that are essential for the functional and physical interaction of the protein with topoisomerase III (Top3p), a type I topoisomerase that cooperates with Sgs1p during homologous recombination (22,61,66–69). Indeed, this may be the only critical function of the N-terminus of Sgs1p because when the *TOP3* ORF is fused to a fragment of the *SGS1* ORF lacking the first 106 codons, the fusion product (Top3p- $\Delta N106$ Sgs1p) is able to rescue the recombination defects and sensitivity of *sgs1* mutants to MMS and HU (61). The failure of *SGS1- $\Delta N50*$*  to rescue rapid senescence therefore implies a role for *TOP3* during senescence. We investigated this possibility by first



**Figure 3.** Evidence that Sgs1p functions via recombination to slow senescence. Senescence rates were measured in the same fashion as Figure 2. (A) *rad52* and *sgs1* mutations are epistatic during senescence. Haploid spore products were derived from diploids heterozygous for *tlc1*, *sgs1* and *rad52* mutations. Curves for *tlc1* (filled diamonds), *tlc1 rad52* (open diamonds), *tlc1 sgs1* (filled circles) and *tlc1 sgs1 rad52* (open circles) mutants are shown. This result was repeated two additional times with independently derived spore products (data not shown). (B) *est2* and *tlc1* mutations are equivalent in the context of *sgs1* and *rad52* mutations. The experiment was the same as in (A), but with telomerase inactivation via *est2* rather than *tlc1* deletion (C) Sgs1p helicase activity is required to slow senescence. Diploids heterozygous for *tlc1* and *sgs1* mutations and with an integrated *SGS1-hd\** allele, which encodes the helicase-defective K706A point mutant, were sporulated and the senescence rates of *tlc1* (filled diamonds), *tlc1 sgs1* (filled circles), *tlc1 SGS1-hd\** (open diamonds) and *tlc1 sgs1 SGS1-hd\** haploid products were measured. (D) Same as (C), except that the *SGS1-ΔC795\** allele, which encodes a derivative lacking the C-terminal 795 amino acids of Sgs1p was used instead of *SGS1-hd\**. (E) *SGS1-hd\** and *SGS1-ΔC795\** encode active proteins. The ability of the \* alleles to rescue the synthetic slow growth of *sgs1 top1* mutants was tested by generating *top1* deletions in haploid cells with *sgs1* deletion and the indicated \* allele. Shown is growth of serial 10-fold dilutions of double mutants containing integrated vector, *SGS1\**, *SGS1-ΔC795\** or *SGS1-hd\** alleles, as indicated. (F) *top1* deletion does not speed senescence. Diploids heterozygous for *top1* and *tlc1* deletions were sporulated and senescence rates of *tlc1* (filled diamonds) and *tlc1 top1* (open diamonds) haploid spore products were measured.

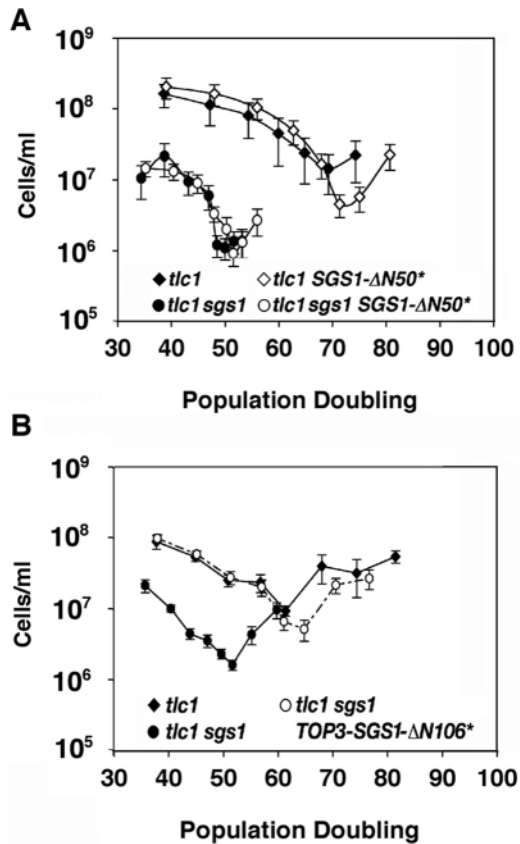
examining the rate of senescence of *tlc1 top3* null mutants. Haploid spores were derived from diploids with *TLC1/tlc1* and *TOP3/top3* mutations and *tlc1* and *tlc1 top3* products compared with each other. *top3* and *tlc1 top3* mutant spores germinated at very low frequency in our strain background. However, we obtained one *tlc1 top3* spore product, and this senesced rapidly, ~20 generations after germination (data not shown). Additional evidence for the importance of *TOP3* during senescence was provided by the ability of the *Top3p-ΔN106Sgs1p* fusion, encoded by *TOP3-SGS1-ΔN106\**, to rescue the rapid senescence of *tlc1 sgs1* mutants (Figure 4B). Because the fusion lacks all the residues from the N-terminus of Sgs1p that are absent in Sgs1p-ΔN50, this indicates that the inactivity of Sgs1p-ΔN50 was due to its inability to bind productively to Top3p. We conclude that Sgs1p and Top3p cooperate to slow the senescence of *tlc1* mutants.

#### Compared with most proteins that function with Sgs1p in other settings, Sgs1p plays a more important role during senescence

A number of mutations, referred to as 'slx' mutations, have been found to cause synthetic lethality (or extremely poor growth) in combination with *sgs1* null mutations (70–73).

Among these are null mutations in *SLX1*, *SLX5*, *MUS81*, *SRS2* and *RRM3*, as well as in *TOP1* as described above. Slx1p together with Slx4p forms an endonuclease complex that cleaves 5' flap structures and is required for normal replication of the ribosomal DNA and for resistance to MMS (13,70,74). Slx5p together with Slx8p forms a complex of undetermined biochemical activity, although the complex is known to promote resistance to HU (70). Mus81p complexes with Mms4p to form an endonuclease that cleaves 3' flap structures, D-loops and nicked Holliday junctions, and the complex appears to help rescue stalled replication forks (75–77). Srs2p is a 3'–5' DNA helicase that promotes non-crossover recombination, apparently by inhibiting Rad51p-mediated strand invasion, and that is also required for adaptation following DNA damage (20,78–80). Rrm3p is a 5'–3' DNA helicase that prevents replication fork stalling in non-nucleosomal chromatin regions, including telomeres and ribosomal DNA sequences (81–83). Similar to *SGS1*, each of the genes in this group is required for the maintenance of DNA stability throughout the genome.

A mutation that is synthetically lethal with *sgs1* may identify a gene that accomplishes an essential function through a pathway that is distinct from and parallel to an *SGS1*-dependent pathway. Thus, either *SGS1* or the gene in question can support a critical biochemical pathway at a level that



**Figure 4.** Sgs1p cooperates with Top3p to slow senescence. Strains were generated and senescence experiments were performed in the same fashion as in Figure 2. (A) *SGS1-ΔN50\**, which encodes a derivative lacking the N-terminal 50 amino acids of Sgs1p, does not rescue the fast senescence of *tlc1 sgs1* mutants. Senescence curves for *tlc1* (filled diamonds), *tlc1 sgs1* (filled circles), *tlc1 SGS1-ΔN50\** (open diamonds) and *tlc1 sgs1 SGS1-ΔN50\** (open circles) haploid spore products are indicated. (B) *TOP3-SGS1-ΔN106\**, which encodes Top3p fused to an Sgs1p derivative lacking the N-terminal 106 amino acids, rescues rapid senescence. Senescence curves for *tlc1* (filled diamonds), *tlc1 sgs1* (filled circles) and *tlc1 sgs1 TOP3-SGS1-ΔN106\** (open circles) haploid spore products are indicated.

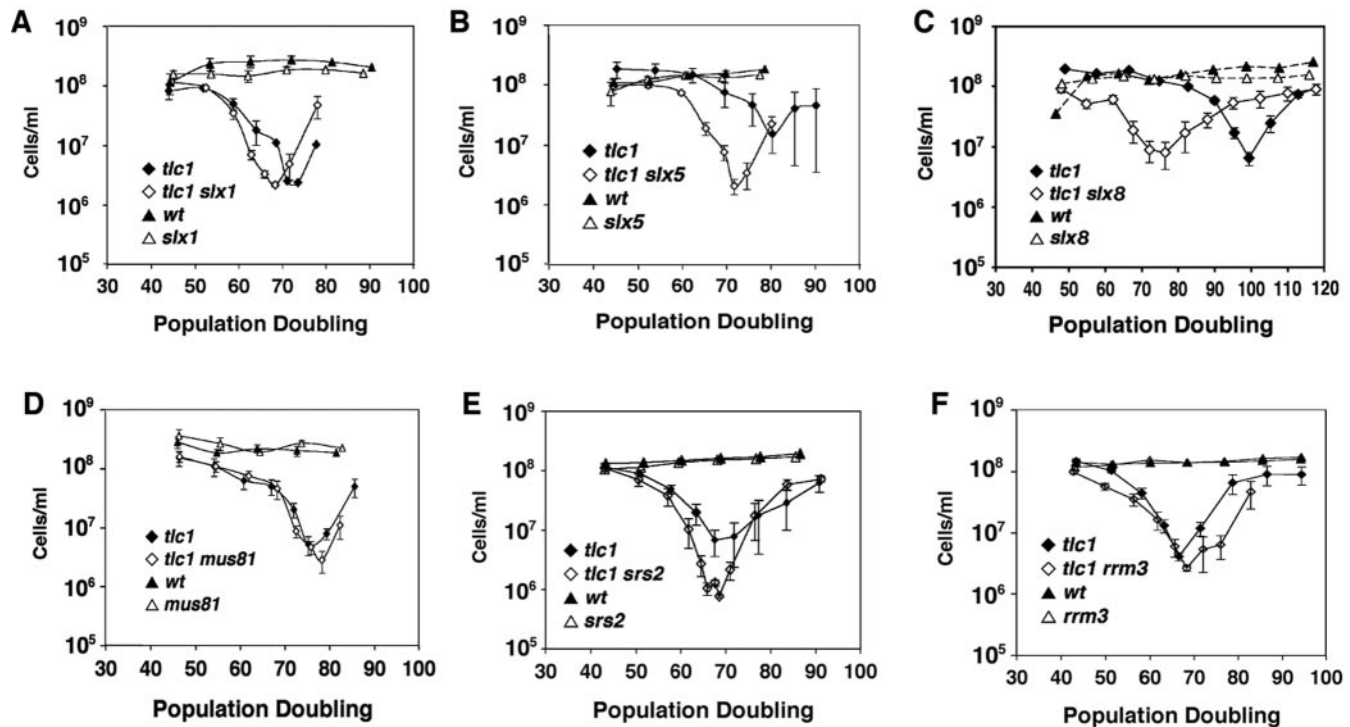
is sufficient for viability, but loss of both genes is lethal. Alternatively, the two genes may each contribute to the efficiency of a single pathway, so that loss of both genes slows the pathway to below a threshold needed for viability. By either model, some of the synthetic lethal genes might possibly function, like *SGS1*, to slow the senescence of *tlc1* mutants. We therefore compared rates of senescence in *tlc1* strains with *tlc1* strains also containing deletions of *SLX1*, *SLX5*, *MUS81*, *SRS2* or *RRM3* (Figure 5A, B, D–F). Remarkably, of all the mutations in this group only *slx5* had an appreciable effect on the rate of senescence, accelerating senescence by 10 population doublings (PD) (Figure 5A–F). To confirm the effect of the *slx5* mutation, we examined the effect of mutating *SLX8*, which encodes the partner of Slx5p. As expected, *tlc1 slx8* mutants senesced more rapidly than *tlc1* mutants, by ~20 PD (Figure 5C). Although *srs2* mutation did not speed senescence appreciably, it did delay by 2–3 days the formation of survivors and caused a concomitant depression of the minimum cell density prior to survivor formation (Figure 5E). We conclude that, with the exception of *SLX5/8*, none of

the genes we examined plays a role as important as *SGS1* to slow senescence. Furthermore, as detailed in the Discussion, the findings indicate that generalized genome instability is not the cause of rapid senescence in *tlc1 sgs1* mutants, but rather that Sgs1p plays a more specific role at telomeres or in response to telomere dysfunction.

In addition to speeding the senescence of *tlc1* cells, *sgs1* mutation increases telomere shortening per population doubling and blocks the formation of type II survivors (36,42,43). We therefore asked if *slx8* mutation also had these other effects. We examined Y'-containing telomere length by Southern analysis (Figure 6A). *slx8* mutation did not affect steady-state telomere length in *TLC1+* cells. Comparison of telomere lengths for four *tlc1* mutants at days 1 and 4 of growth in liquid (mean PD 49 versus 75) and four *tlc1 slx8* mutants at 1 and 5 (mean PD 48 versus 71), yielded telomere shortening rates of  $2.3 \pm 0.12$  bp/PD for *tlc1* and  $4.0 \pm 0.62$  bp/PD for *tlc1 slx8* cells ( $P = 0.04$ ), indicating that telomeres shorten faster per population doubling in the absence of *SLX8* function (Figure 6A and data not shown; samples were from the cultures shown in Figure 5C). We next examined survivor type by Southern analysis of XhoI-digested genomic DNA. Type I survivors are typified by amplification of subtelomeric Y' elements of 5.2 and 6.7 kb in size, the presence of ~1 kb Y' fragments containing terminal telomere repeat DNA, and by the relative absence of telomere repeat-containing fragments between these two signals. Type II survivors appear as fragments of heterogeneous size that range in size from 1 to >10 kb and they usually have less amplification of Y' elements. In general, type I survivors predominate early in survivor formation, but because of their faster growth rate type II survivors eventually overtake liquid cultures (40). Three of four *tlc1* cultures formed type II survivors by the 17th day after loss of telomerase (PD 155–170) and all four were type II by day 22 (PD 200–215) (Figure 6B). In comparison, two of four *tlc1 slx8* cultures had clear type II character at day 17, but there was little further progression to type II by day 22, and one of the cultures (Figure 6B, G) actually developed more type I character. Thus, *SLX8* may facilitate the formation or growth of type II survivors, but, in contrast to *SGS1*, is not absolutely required for type II survivors to form.

## DISCUSSION

We have investigated possible mechanisms by which the Sgs1p helicase slows the senescence of *tlc1* mutants. Our findings are most compatible with a role for Sgs1p in HR-mediated repair of telomeres, rather than a role for the Sgs1p-dependent S-phase checkpoint or for a generalized role of Sgs1p in genome stability. In support of an HR role are (i) the epistatic relationship of *sgs1* and *rad52* mutations in telomerase mutants, (ii) a requirement for Sgs1p helicase activity and (iii) a role for Top3p. Helicase activity and cooperation with Top3p have been shown to be essential for all of the recombination functions of Sgs1p so far investigated (22,59,61,69,84). Furthermore, Rad52p is a component of a protein complex that includes Sgs1p, consistent with a role for Sgs1p in Rad52p-dependent HR (85). The higher rate of recombination among shortened telomeres in *Kluyveromyces lactis* (86), the direct observation of recombinant sequences

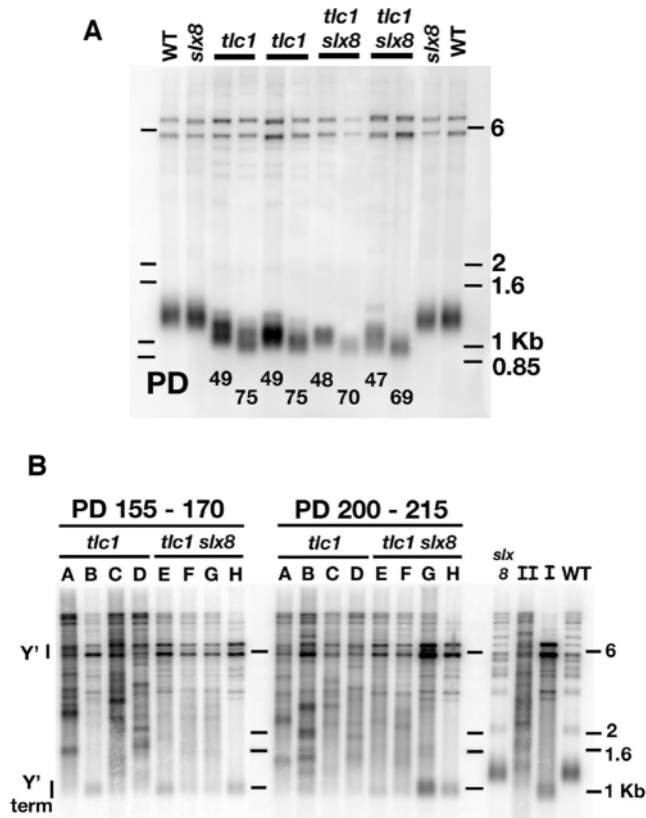


**Figure 5.** Effects of *sgs1* synthetic lethal mutations on senescence. The indicated synthetic lethal mutations were introduced individually into diploids heterozygous for *tlc1* deletion. Senescence rates of spore products were measured in the same fashion as Figure 2. In all cases, filled diamonds indicate *tlc1* mutants, open diamonds indicate *tlc1* plus the synthetic lethal mutation, and filled and open triangles indicate wild-type and the synthetic lethal mutant, respectively. Comparison of *tlc1* and *tlc1* with (A) *slx1* deletion, (B) *slx5* deletion, (C) *slx8* deletion, (D) *mus81* deletion, (E) *srs2* deletion and (F) *rrm3* deletion. Note the increased number of days (data points) spent in the nadir in *tlc1 srs2* mutants.

at the termini of ~6% of telomeres in *tlc1* mutants (87), and the rapid senescence of *tlc1* mutants also lacking *RAD52*, *RAD51*, *RAD54* or *RAD57* (57) support the idea that HR can contribute to telomere maintenance during senescence. We note that a different interpretation of the relationship between *RAD52* and *SGS1* in cells senescing due to telomerase inactivation was reported previously (43), where an additive effect of *rad52* and *sgs1* mutations on senescence was observed. However, in the report telomerase was inactivated in haploid strains already carrying the *rad52* and/or *sgs1* mutations, and it is thus possible that differences in telomere integrity in the different mutant backgrounds prior to telomerase inactivation contributed to senescence rates. Our experimental approach eliminates this confound by comparing haploid spore products derived from diploids that contain one wild-type allele for each mutation being studied and, further, compares cells with telomeres derived from the same diploid parent and is thus less subject to random fluctuations in steady state telomere length. We are therefore confident that *SGS1* and *RAD52* function in a less-than-additive, i.e. epistatic, fashion during the senescence of telomerase mutants.

A reasonable candidate for an HR-based mechanism of telomere repair is break-induced replication (BIR) (88–90). Such a BIR mechanism could, for example, involve invasion of a critically shortened telomere end into a longer telomere, which would then be used as a template for DNA synthesis to extend the shortened telomere. A role for the Sgs1p–Top3p complex in this context could be to facilitate progression during DNA synthesis, and thus resolution at the telomere

terminus, of a Holliday junction formed by such an invasion event. Particularly in a case where the telomere DNA was not free to rotate, as could be the case in telomeric chromatin, the helicase activity of Sgs1p could cooperate with the strand-passage activity of Top3p to enable separation of the newly synthesized strand from the template. This would be mechanistically similar to the branch migration and resolution of double Holliday junctions shown to be catalyzed by the BLM–TOP3 $\alpha$  complex (91), and may also be related to the stalling of DNA synthesis during HR that occurs in *Drosophila blm* mutants (92). A BIR mechanism has been proposed previously to underlie the formation and maintenance of survivors of telomerase deletion (57), and it is possible that BIR operates both during and after senescence. BIR is *RAD51*-independent when initiated from chromosomal sites cleaved by the HO-endonuclease, but proceeds by a *RAD51*-dependent mechanism when initiated from a transformed chromosomal fragment (88,90). Because *tlc1 rad51* mutants senesce rapidly [(57) and Q. Chen and F.B. Johnson, unpublished], Rad51p is also involved in slowing senescence and so any such telomere BIR mechanism would apparently be of the *RAD51*-dependent type. Consistent with the chromosome fragment assay reflecting a BIR process similar to that proposed to operate at telomeres during senescence, *RAD50* and *RAD59* are dispensable for BIR in this assay (despite being required for BIR in the HO-cleavage assay) and *tlc1 rad50* or *tlc1 rad59* mutants senesce at normal rates (57,90,93). Furthermore, although *SGS1* is not required for BIR at an internal chromosomal site cleaved by the HO endonuclease (94), it remains possible



**Figure 6.** Telomere shortening and survivor formation in *tlc1 slx8* cells. (A)  $Y'$ -containing telomere lengths from wild type, *slx8* and from two independent cultures each of *tlc1* and *tlc1 slx8* mutants during senescence were measured by digesting genomic DNA with XhoI and probing with  $Y'$  sequences distal to the cut site. The number of population doublings (PD) since the start of spore growth is indicated. (B) Survivor type in four *tlc1* (A–D) and four *tlc1 slx8* (E–H) cultures was examined by Southern analysis of XhoI-digested genomic DNA visualized with a telomere repeat probe. Samples are from days 17 (PD 155–170) and 22 (PD 200–215) after sporulation, and samples were obtained from the cultures shown in Figure 5C.  $Y'$  and  $Y'$  term indicate the position of tandemly-repeated  $Y'$  elements, and the terminal  $Y'$  fragment, respectively, that are most prominent in type I survivors. On the right, samples from *slx8*, pure type I (*tlc1 sgs1*), pure type II (*tlc1 rad51*) and wild-type cultures are provided for comparison, and the position of DNA markers is indicated with sizes in kilobases (kb).

that it is required for BIR from non-HO cleaved ends or there could be particular requirements for Sgs1p in recombination involving G-rich telomere repeats, for example the unwinding of G-quadruplexes.

We were surprised to find that so many different genes involved in maintenance of genome stability had no significant effect on senescence rates. These include five '*slx*' genes (*SLX1*, *MUS81*, *SRS2*, *RRM3* and *TOP1*) whose loss of function causes synthetic sickness or lethality in combination with *sgs1* mutation, and so which might be expected to have some functions similar to Sgs1p. Indeed, one such pair of genes, *SLX5* and *SLX8*, do have effects on the senescence rate (see below). Even though the *slx* genes have genetic relationships to *SGS1*, they clearly affect different classes of processes required for genome stability, based on their different genetic interactions with other factors, biochemical activities and mutant phenotypes. Thus, the five mutations sample the importance during senescence of several distinct

functions required for genome maintenance. Our findings are consistent with previous demonstrations that mutations in *RAD1* or *RAD10*, which are required for nucleotide excision repair and the single-strand annealing pathway of double-strand break repair, mutations in *MSH2*, which is required for mismatch repair, the *pol3-01* mutation, which leads to an elevated rate of point mutations, and mutations in *TEL1* or *MEC1*, which mediate DNA damage responses, do not speed the senescence of telomerase mutants (38,95). Together with our findings, these results indicate that induction of several different types of genome instability is not sufficient to speed senescence, and they further suggest that Sgs1p plays a relatively specialized role in telomere function.

In contrast to the other *sgs1*-synthetic lethal mutations we tested, *slx5* and *slx8* mutations sped the senescence of *tlc1* mutants. The molecular function of the Slx5p/8p complex is currently unknown, and so this finding does not yet provide any detailed mechanistic insight. However, it is interesting that the synthetic lethality of *sgs1 slx5* mutants is not suppressed by *rad52* mutation, unlike the synthetic lethality of *sgs1* with several other mutations including *mus81*, *srs2* and *rrm3* (17,18,21,22,76). This might reflect an involvement of Slx5p in a pathway for which HR is an essential component; the key role of HR in slowing senescence is consistent with an HR-related role of Slx5p being important during senescence. We also found that *SLX8* is not required for the formation of type II survivors of *tlc1* deletion, although their appearance appears to have been inhibited. This contrasts with an apparent absolute requirement for *SGS1* in type II survivor formation. However, we note that once formed, type II survivors no longer absolutely require the continued presence of *SGS1* (36), and so the functional differences between *SGS1* and *SLX8* in the type II mechanism may be subtle and reflect a particularly important role for *SGS1* during the initial stages of the formation of type II survivors.

Although *srs2* mutation did not speed the senescence of *tlc1* mutants, it did substantially delay the appearance of survivors. We do not yet understand the basis for this delay, but it might be connected with the requirement for *srs2* in the process of adaptation. Adaptation enables cells that have arrested their cell cycle in response to DNA damage to resume growth even prior to repair of the offending lesion. *srs2* mutants show impaired adaptation after incurring an irreparable double strand chromosomal break (78). Because critically shortened telomeres are interpreted by the cell as a form of DNA damage (37,38), the survivor mechanism might involve an adaptation-like response to enable the resumption of growth. Further experiments will be necessary to determine if other components of the adaptation response are required for the formation of survivors.

Recently, it was reported that telomere sequences that are the products of lagging-strand DNA synthesis are selectively lost in Werner mutant cells (51). This was attributed to a defect in the removal of G-quadruplexes from the replication template caused by the absence of WRN. Our findings are consistent with this possibility. However, our data suggest a role for Sgs1p in a HR-based pathway at telomeres, and because replication forks stall naturally in telomere repeat DNA (81,96), and because HR is critical for the restart of stalled replication forks (97), we also suggest an alternative that RecQ-family helicases might remove G-quadruplexes that



would otherwise interfere with HR-based rescue of stalled replication forks at telomeres.

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