Sgs1 RecQ Helicase Inhibits Survival of Saccharomyces cerevisiae Cells Lacking Telomerase and Homologous Recombination*§

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In yeast telomerase mutants, the Sgs1 RecQ helicase slows the rate of senescence and also facilitates the appearance of certain types of survivors of critical telomere shortening via mechanisms dependent on Rad52-dependent homologous recombination (HR). Here we describe a third function for Sgs1 in telomerase-deficient cells, inhibition of survivors that grow independent of Rad52. Unlike tlc1 rad52 double mutants, which do not form survivors of telomere dysfunction, tlc1 rad52 sgs1 triple mutants readily generated survivors. After emerging from growth crisis, the triple mutants progressively lost telomeric and subtelomeric sequences, yet grew for more than 1 year. Analysis of cloned chromosome termini and of copy number changes of loci genome-wide using tiling arrays revealed terminal deletions extending up to 57 kb, as well as changes in Ty retrotransposon copy numbers. Amplification of the remaining terminal sequences generated large palindromes at some chromosome termini. Sgs1 helicase activity but not checkpoint function was essential for inhibiting the appearance of the survivors, and the continued absence of Sgs1 was required for the growth of the established survivors. Thus, in addition to facilitating the maintenance of telomere repeat sequences via HR-dependent mechanisms, a RecQ helicase can prevent the adoption of HR-independent mechanisms that stabilize chromosome termini without the use of natural telomere sequences. This provides a novel mechanism by which RecQ helicas may help maintain genome integrity and thus prevent age-related diseases and cancer.

The linear chromosomes of eukaryotic cells require specialized structures to protect their ends from exonucleolytic degradation and to prevent them from engaging the checkpoint and repair activities that normally respond to DNA double strand breaks. These functions are termed “capping” and are mediated by telomeres, which are typically G-rich repeated sequences bound by specialized proteins that cooperate to protect the chromosome terminus (1, 2). Telomeres are susceptible to shortening by a variety of mechanisms, including the inability of the lagging strand replication machinery to fully copy DNA ends (3), exonucleolytic degradation (4, 5), oxidative damage (6), and recombination-mediated loss events (7). When telomeres become critically shortened, they become uncapped and thus elicit DNA damage responses (8, 9).

In many organisms, including mammals and Saccharomyces cerevisiae, telomere shortening can be countered by the action of telomerase, which catalyzes the synthesis of new telomere repeats at the 3′ end of the telomere (10). A second type of telomere maintenance mechanism, known as alternative lengthening of telomeres, relies on the HR machinery to enable shortened telomeres to invade other telomere sequences thus providing a template for synthesis of new telomeres by conventional DNA polymerases (11). In humans, telomerase expression is restricted to certain cell types, including the progenitors of highly proliferative tissues. Telomeres thus shorten in many tissues with age, which is thought to inhibit the proliferation of cancer cells by leading to apoptosis or permanent cell cycle arrest (cell senescence) once telomeres become critically short (12). Indeed, incipient cancer cells that bypass this barrier typically either activate telomerase expression or use alternative lengthening of telomeres to maintain their telomere lengths. In S. cerevisiae, telomerase is expressed constitutively, but the genetic inactivation of telomerase leads to telomere shortening and senescence of the culture; examples include est2 or tlc1 deletion mutants, which lack the catalytic component or template RNA of telomerase, respectively (13, 14). Rare cells (~1 in 105) escape from senescence to form HR-dependent survivors (15). Two types of such survivors form, type I and type II, involving amplification primarily of subtelomeric Y′ elements or telomere repeat sequences, respectively. The reliance of both types of survivors on HR was demonstrated by their dependence on Rad52, which is essential for most types of HR in yeast: fewer than 1 in 104 tlc1 rad52 mutants form survivors (15, 16).

Maringele and Lydall (17) made the remarkable observation that deletion of EXO1, which encodes a 5′–3′-exonuclease

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2 and Figs. 1–8.
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**TABLE 1**

S. cerevisiae strains used in this study

| Strain | Genotype | Ref. or source |
|--------|----------|---------------|
| yB1133 | MATα/a, hοΔ hmlΔ:ADE1 hmrΔ:ADE1 ade1 ura3-52, leu2-3,112, lys5, TLC1/tlc1Δ:kanMX, SGS1/sgs1Δ:hisG-URA3 | 21 |
| yB161  | yB1133 SGS1/sgs1Δ:hisG | 28 |
| yB158  | yB1133 RAD52/rad52Δ::LEU2 | 27 |
| yB115  | yB1133 RAD52/rad52Δ::LEU2 | This study |
| yB113b | yB1133 RAD52/rad52Δ::LEU2 | This study |
| yJL15  | yB1133 RAD52/rad52Δ::LEU2 | This study |
| yJL18  | yB1133 RAD52/rad52Δ::LEU2 | This study |
| yJL68  | MATα/a, hοΔ hmlΔ:ADE1 hmrΔ:ADE1 ade1 ura3-52, leu2-3,112, lys5, SGS1/sgs1Δ:hisG-URA3, RAD52/rad52Δ::LEU2 | 28 |
| yJL69  | yJL68 EST2/est2Δ:kanMX | This study |
| yJL72  | MATα/a, hοΔ hmlΔ:ADE1 hmrΔ:ADE1 ade1 ura3-52, leu2-3,112, lys5, SGS1/sgs1Δ:hisG-URA3, RAD52/rad52Δ::LEU2 | This study |
| yJL76  | yJL72 EST2/est2Δ:kanMX | This study |
| yMA76  | yB1161 SGS1/sgs1Δ::URA3 leu2/LEU2-SGS1* | 28 |
| yMA78  | yB1161 SGS1/sgs1Δ::URA3 leu2/LEU2-SGS1-hd* | This study |
| yMA97  | yB1161 SGS1/sgs1Δ::URA3 leu2/LEU2-SGS1-hd* | 28 |
| yB158  | yMA78 RAD52/rad52Δ::LYS5 | This study |
| yJL67  | yMA97 RAD52/rad52Δ::LYS5 | This study |
| yJL87  | yMA76 RAD52/rad52Δ::LYS5 | This study |
| yJL92  | yMA76 RAD52/rad52Δ::LYS5 | This study |

The conserved RecQ family of DNA helicases function to maintain genome stability and thus help stave off age-related diseases, including cancer. As part of this function, several RecQ helicases play important roles in telomere biology (18, 19). These include the mammalian WRN and BLM proteins, which are deficient in the human Werner progeroid and the Bloom cancer predisposition syndromes as well as S. cerevisiae Sgs1. For example, WRN and BLM localize, in part, to telomeres (20–22). Moreover, mammalian cells lacking WRN or BLM and telomerase, and yeast cells lacking Sgs1 and telomerase, experience elevated frequencies of telomere loss events that accelerate cellular senescence (21, 23–27). These events have been linked to defects in lagging strand replication of telomeres in Werner mutant cells and to defects in the resolution of replication-associated telomere recombination events in tcl1 sgs1 yeast mutants (25, 27). Furthermore, HR-based telomere recombination in mammalian alternative lengthening of telomere cells or in yeast type II survivors are regulated by WRN and BLM or Sgs1, respectively (21, 28–33). Sgs1 functions together with Rad52 in its two roles of preventing rapid senescence and in facilitating type II survivor growth (21, 27–29, 33).

Here we report that Sgs1 can play a novel, third type of role in events related to the maintenance of chromosome termini. We find that like Exo1, Sgs1 inhibits the survival of cells lacking telomerase and Rad52. This inhibition involves the helicase but not S-phase checkpoint function of Sgs1, and operates during the initial formation of survivors as well as in mature survivors. Like tcl1 rad52 exo1 survivors, tcl1 rad52 sgs1 survivors form large palindromes at some chromosome termini. In addition, we find that Ty1 element levels are increased in these survivors, which might reflect a role for these elements in the survivor pathway.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Senescence Assays**—All yeast strains were derived from JKM111 (34) (Table 1) and were cultured in standard YPAD media at 30 °C. All engineered mutations are deletions, except for rad52, which was generated via disruption using pSM20; for simplicity the Δ symbol has been dropped from all allele names except rad52Δ to distinguish it from the rad52 disruption allele. Other deletions were generated as described previously (27, 28). Liquid senescence assays were performed as described (21), inoculating 2 million cells per 5 ml of culture at the start of each day of growth. Once survivors had emerged, cells were passaged in liquid by serially inoculating 100 µl of culture into 5 ml of fresh medium each week. Senescence assays were also performed on YPAD agar plates where single colonies (containing ~2 million cells) were serially struck out on new plates; survivors were propagated by serially streaking single colonies on plates every 1–2 weeks. Haploid spores derived from the diploids listed in Table 1 are named with their parental diploid followed by a hyphen and a spore identifier (e.g. yJL76-10B); in some cases senescing lineages were subcloned, and these were distinguished by further identifiers (e.g. yJL17-15C, n15, and yJL17-15C, j15). Triple mutant cells at various days after the loss of telomerase were also grown on SC-ARG plates containing 0.004% canavanine to test for loss of CAN1; wild-type and can1 strains were used as controls.

**Plasmids and Transformation of Survivors**—The pSGS1-hygro plasmid was generated by replacing the Sbf1-NgoMIV fragment of the URA3 gene in pSGS1F2 (35) with a hygromycin resistance gene (derived from a NotI fragment of pAG32) flanked by engineered Sbf1 and NgoMIV sites generated by PCR. The corresponding control plasmid (pRS-hygro) was made by replacing the same fragment of pRS416. The activity of pSGS1-hygro was confirmed via rescue of the sensitivity of sgs1 mutants to methanol sulfonate. Transformation was performed using a standard LiAc method (36), except that prior to heat shock, DMSO was added to transformation mixtures at 10% final concentration. Cells were allowed to grow overnight...
in YPAD before plating on YPAD medium containing 250 µg/ml hygromycin.

Genomic DNA Isolation, Gel Electrophoresis, and Southern Analysis—Genomic DNA from dense cultures was purified by a standard glass-bead disruption protocol (36). XhoI-digested DNA was separated on 1% agarose gels, blotted onto Hybond-XL membrane, and hybridized to a 784-bp PCR-amplified Y′ telomere fragment (21). DNA plugs for contour-clamped homogeneous electric field (CHEF) gel electrophoresis were prepared according to Ref. 37 and the Bio-Rad CHEF-DR II system instruction manual. CHEF was performed using a Bio-Rad CHEF-DRII apparatus at 6 V/cm, with a 60-s switch time for 15 h, followed by a 90-s switch time for 9 h. Gels were stained with ethidium bromide and imaged prior to blotting on Hybond-XL membranes. Blots were probed using TG1–3 repeats of telomeres (27), telomere Y′ fragments (21), and PCR-generated fragments of RAD51 (AGCAGTAGCAT-ATGCTCCAGAAAGGAGATGGTGTTGGTG-GTCGCA). All blots were visualized and quantified using a Molecular Dynamics PhosphorImager (GE Healthcare).

Cloning of Chromosome Ends—Genomic DNA from dense cultures was purified using a Qiagen Genomic DNA purification kit. Cytidines were added to termini using terminal deoxynucleotidyltransferase (TdT), essentially as described hereafter as “triple mutant survivors” or simply “survivors”) formed survivors following senescence (Fig. 1A). These findings indicate that any possible residual RAD52 function in cells lacking telomerase and Rad52. This indicated that although Sgs1 functions with Rad52 during senescence and during one type of survivor formation, it can also affect events leading to Rad52-independent (and thus HR-independent) survivor growth. Several tlc1 rad52 sgs1 and est2 rad52 sgs1 triple mutant strains, which were germinated from spores derived from diploids heterozygous for the various mutations (27), formed survivors following senescence (Fig. 1A and Table 2). In all, 8 of 24 such cultures formed survivors (which we refer to hereafter as “triple mutant survivors” or simply “survivors”) compared with none of 21 mutants lacking telomerase and Rad52 (p < 0.005 by Fisher’s exact test). The liquid senescence assays involved inoculation of 2 × 10⁶ cells at the start of each day of growth, and thus sgs1 deletion increased the rate of survivor formation ~100-fold, from fewer than 1 in 10⁶ in cells lacking telomerase and Rad52 to ~1 in 3 × 10⁶ in cells also lacking Sgs1. Our initial experiments used a disruption allele of RAD52, but the same results were obtained with a full deletion allele, indicating that any possible residual RAD52 function in the disruption allele did not contribute to the survival (Fig. 1A and Table 2; also note that all sgs1, tlc1, and est2 mutant alleles are deletions). In almost all cases, after an initial growth crisis at senescence and the formation of survivors, the cells underwent a second growth crisis, but then recovered and continued to replicate at a steady rate thereafter (Fig. 1A). The exception was strain yIL69-2D, which did not recover from the second crisis (data not shown). Established survivors were propagated for over 500 days of continuous culture in liquid medium and also on solid medium. Survivors have a slow population doubling rate, attributable primarily to slowed progression through the cell cycle rather than an increased frequency of permanent cycle arrest, as indicated by near normal colony-forming efficiency in spot assays (Fig. 1B). These findings indicate that the absence of Sgs1 somehow enables the formation or maintenance of Rad52-independent survivors of telomerase inactivation.

Deletion of EOX1 has been shown to enable a similar bypass of the survivor block in cells lacking telomerase and Rad52 (17). However, we were unable to determine whether sgs1 and exo1 deletions stimulate bypass via the same or different pathways,
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TABLE 2
Survivor frequencies in triple mutant liquid cultures

| Parental diploid strain | Genotype         | No. of survivors/ No. of senescing cultures |
|-------------------------|------------------|---------------------------------------------|
| yJL13b                  | tlc1 sgs1 rad52  | 0/5                                         |
| yJL17                   | tlc1 sgs1 rad52  | 2/5                                         |
| yJL69                   | est2 sgs1 rad52  | 2/5                                         |
| yJL4                    | tlc1 sgs1 rad52Δ | 1/2                                         |
| yJL5                    | tlc1 sgs1 rad52Δ | 0/2                                         |
| yJL76                   | est2 sgs1 rad52Δ | 2/6                                         |
| Total triple mutants    |                  | 8/24                                        |
| Total telomerase single mutants |                  | 23/23                                       |
| Total telomerase sgs1 double mutants |                  | 24/24                                       |
| Total telomerase rad52 double mutants |                  | 0/21                                        |

because tlc1 rad52 sgs1 exo1 quadruple mutants were not viable (data not shown).

Telomeric and Subtelomeric Sequences Are Eroded in Triple Mutant Survivors—To characterize the chromosome ends of the survivors, we first performed Southern analysis on Xhol-digested genomic DNA from cultures spanning the first 20 days after spore germination (population doubling (PD) 35–100). In wild-type yeast, most telomeres contain one to four tandem copies of the subtelomeric Y′ element, and Xhol cleaves Y′ DNA 1-kb internal the start of telomere repeats (Fig. 2A). Thus the termini of Y′-containing telomeres appear as a smear at −1.35 kb in wild-type cells on blots probed with a Y′ probe located terminal to the Xhol site, and tandemly repeated Y′ elements appear as 5.7- and 6.2-kb bands (the two bands correspond to short and long forms of Y′ element present in varying frequencies at different chromosome ends) (Fig. 2B). As expected, telomeres shortened with cell division in the triple mutants (Fig. 2B). However, shortening continued beyond the initial growth crisis at senescence (approximately PD 55), the initial formation of survivors, the second growth crisis (approximately PD 70), and the formation of established survivors. No discrete changes were apparent in the bulk telomere patterns at any of these transitions. Erosion beyond the terminal Xhol site caused a gradual loss of full-length Y′ element bands and a smearing of these Y′ bands to lower molecular weights. This was confirmed by examining blots of genomic DNA digested with Drai, which cuts near the centromere-proximal end of Y′ elements and thus delayed the appearance of the downward smear (data not shown). RAD52-dependent HR is required for the generation of type I and type II survivors in telomerase mutants (e.g. Fig. 2B, tlc1 cells at PD 70 showing a mixed type I/II survivor pattern), and so it was not surprising that telomere structures indicative of either of these survivor types were absent in the triple mutant survivors. Thus the triple mutant survivors do not appear to maintain telomeric and subtelomeric repeat sequences using the HR-based mechanisms employed by survivors of simple telomerase inactivation.

We next used Southern analysis of chromosomes separated using CHEF gel electrophoresis to examine overall chromo-
some structure and the extent of end resection at later population doublings. Remarkably, most or all of the chromosome ends in all six of the triple mutant survivors analyzed eventually lost both telomere TG1–3 repeats and Y′ elements (Fig. 3, A–C, and supplemental Figs. 1 and 2). Because circular DNA does not migrate through CHEF gels, and because no TG1–3 or Y′ DNA accumulated in the sample wells, circularization of chromosomes containing these sequences did not explain their absence from the chromosomal bands that migrated into the gel. Therefore, resection of chromosome ends apparently progresses through the telomere repeats and subtelomeric Y′ elements of most chromosomes in the survivors.

Consistent with a gradual loss of chromosome ends, when CHEF gel blots were probed specifically for the left or right arm of chromosome V, using probes for the MCM3 and RAD51 loci, respectively, the results using each probe indicated that the chromosome shortened progressively for approximately the first 100–150 days of growth in some strains (e.g. yJL76-10B; Fig. 3, D and E, and supplemental Fig. 3). However, in other strains, chromosome V actually increased in size (e.g. yJL69-3C, supplemental Fig. 1). Although it is possible that fusion of chromosome V to other chromosome arms explained such increases in size, we were unable to obtain evidence for this. First, in every case, the left and right arms of chromosome V appeared to remain connected to each other because the MCM3 and RAD51 probes hybridized to the same chromosomal bands on blots of CHEF gels (Fig. 3 and supplemental Figs. 1–3), indicating that translocations, if present, did not involve an isolated arm of chromosome V. Second, when chromosome V bands of increased size were isolated from CHEF gels and then their chromosome termini were identified via PCR amplification, cloning, and sequencing (see below), all such clones proved to originate from chromosome V itself (data not shown). Therefore, the increased size of chromosome V was apparently not explained by its fusion to a different chromosome end. Although there are several other possible explanations for the increased chromosome size, the alternative we favor, based on analyses described below, is that large palindromes have formed at chromosome termini in the survivors.

To obtain estimates of the rate and extent of chromosome end-resection using a complementary approach, we tested for loss of CAN1, located 32 kb from the left end of chromosome V. There are no essential genes between the telomere and CAN1, and its loss enables growth on media containing canavanine. Triple mutant survivors became canavanine-resistant ~30 days after loss of telomerase, SGS1, and RAD52 (Fig. 3F). Given an approximate population doubling time of 10 h in the survivors, this implies that roughly 45 bp of DNA are lost per population doubling.

Direct Cloning and Mapping of Survivor Chromosome Termini Reveal Palindrome Formation—To further characterize the chromosome ends of survivors, we cloned chromosome termini from three mature survivor strains (>390 days after sporulation) using TdT-mediated poly(C)-tailing of ends, digestion with AluI or RsaI, and amplification by anchor-bubble PCR (supplemental Fig. 4). Sequences from the clones were mapped to the yeast genome using BLAST. Thirty seven clones yielded interpretable sequence that included the expected anchor-bubble ends, restriction site, and poly(C) tail. Their locations are described in supplemental Table 1. Of these, eight (22% of the set of 37) corresponded to sequences within Ty1 elements, which are LTR-type retrotransposons (41). There was no obvious pattern among the clones for the locations of the poly(C)-tailed end within the Ty1 sequences. An additional small clone was entirely contained within Ty1 LTR sequence, and thus it might reflect a cloned fragment of a full Ty1 element or might instead reflect a δ element, which is an isolated Ty1 LTR generated by deletional HR between the LTRs at each end of a genomic Ty1 element. There are 32 Ty1 elements in the S288c reference strain of S. cerevisiae (42), and because each is 5.9 kb in length, full-length Ty1 DNA composes ~1.5% of genomic DNA. The number of Ty1 elements in the JKM strain background used in this study has not been determined, but even if it were twice that of S288c, the large number of cloned Ty1 sequences would still be unexpectedly high (p < 0.03 by Fisher’s exact test). Also of note, five other clones mapped to tandemly

FIGURE 3. Analysis of chromosomes in early triple mutant survivors. A, ethidium bromide-stained CHEF gel of two triple mutant survivors (yJL76-10B and -33D, est2 sgs1 rad52) at various time points after the loss of telomerase. The number of days since sporulation and growth in liquid culture are indicated; 25 days is equal to ~125 PD after sporulation. Wild-type and rad52 mutant controls and the yeast marker from New England Biolabs are indicated. A blot of the CHEF gel was probed (sequentially, with stripping between rounds) for telomere TG1–3 repeats (B), Y′ elements (C), RAD51 (chromosome V-R, 351 kb from right end) (D), and MCM3 (chromosome V-L, 87 kb from left end) (E). F, loss of the CAN1 function in triple mutant survivors. CAN1 is 32 kb from the left telomere of chromosome V, and when lost enables cells to grow in the presence of canavanine. Survivors were restruck serially on YPAD plates. Gray and black bars indicate the inability or ability, respectively, for cells at the indicated time points to grow on plates containing canavanine.

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repeated sequences, two within TIR1, three within the ribosomal DNA array, and one within MUC1.

Of the 28 non-Ty1 clones, all but three (in ADH4, GLIT1, or VMR1) mapped to sites centromeric to essential loci. This was unexpected, because end-resection of chromosome termini in viable cells must stop prior to the most telomeric essential locus. Although complex genomic rearrangements might have moved sequences that are naturally centromeric to new locations between chromosome ends and the most telomeric essential loci, thus giving the appearance of resection past essential loci, there are two more likely explanations. The first explanation is that some of the examples represent DNA ends that were cloned because they are in regions prone to DNA breakage in survivors, or they were broken in dying cells or during DNA isolation. The potential contribution of such events is supported by the increased levels of DNA in survivors that runs at a lower molecular weight than that of the smallest chromosome on CHEF gels (e.g. Fig. 3, A–E), and by the presence of a mitochondrial DNA clone among the set. The second explanation is that large palindromic duplications may have formed at chromosome termini (which we confirm below), and that the cloned termini derive from sequences telomeric to a palindrome center; loss of sequences telomeric to the center of a palindrome would only delete the dispensable copies of essential loci and leave intact the essential loci that are present centromeric to the center of the palindrome (see Fig. 7, steps 6* and 7*).

Closer examination of four of the clones provided clear evidence for the formation of terminal palindromes. The first clone, from yJL76-10B and obtained at 392 days post-telomerase loss, mapped within the 5' region of the SCW4 ORF, 41 kb from the end of chromosome VII. (Fig. 4A). The C-tailed end was oriented toward the centromere, opposite to that expected for a simple terminal deletion. SCW4 is a nonessential gene that is located immediately centromere-proximal to PXR1, the most telomeric essential gene on the chromosome arm (Fig. 4A). Because the cloned end was opposite to the telomeric orientation of the native SCW4 locus, this raised the possibility that a large palindrome had formed at the chromosome end, similar to those described by Maringele and Lydall (17) in tlc1 rad52 exo1 mutant survivors. To test this, we probed blots of genomic DNA digested with a PspXI, PmlI, Apal, or MluI with a fragment near the telomeric end of PXR1 (Fig. 4A). A Southern blot-based comparison of digested DNA from wild-type and early passage survivors to later passage survivors (Fig. 4B) indeed showed that clone yJL76-10B had formed a large palindrome on chromosome VII, centered within the nonessential YOR1 gene. This configuration maintained the essential PXR1 locus. Furthermore, the half-sized bands present in all the samples from the mature survivors also indicate the presence of a large palindrome, because they are the expected products of cruciform structures that could be generated by such a palindrome. Similar half-sized bands were observed in tlc1 rad52 exo1 survivors by Maringele and Lydall (17) at chromosomes bearing terminal palindromes. The bands could arise from cleavage of cruciform structures in vivo by a Holliday junction (HJ) resolvase (because the junction region of a cruciform structure is identical to that of an HJ), or alternatively by restriction endonuclease cleavage in vitro of cruciform structures that have extruded the symmetrically spaced restriction sites into the hairpin arms (Fig. 4C).

FIGURE 4. Mapping a palindrome at a survivor chromosome terminus. A, top, map shows the region 41 kb from the right telomere of chromosome VII in wild-type cells, with the location and orientation of a cloned fragment from mature yJL76-10B survivors in SCW4, which was amplified by TdT-mediated poly(C) tailing and anchor-bubble PCR. The locations of the restriction enzyme cleavage sites, the predicted fragment sizes, and the probe used for Southern analysis (bar with asterisk) are shown. Essential and nonessential loci are shaded or open, respectively. Bottom, predicted fragment sizes for a large palindrome centered in YOR1, which correspond closely to those observed in survivors. B, genomic DNA was purified from yJL76-10B at the indicated days after sporulation, or wild-type cells, digested with the indicated restriction enzymes and Southern blotted. The half-sized bands, which begin to appear as palindromes form by day 206 but are most apparent at day 429, are circled. C, potential mechanisms of half-sized band formation. i, cruciform structures branch migrate to the point where restriction enzyme digestion yields half-size hairpin products; ii, cruciform structures are cleaved by a Holliday junction resolvase in vivo.

A second clone, amplified from yJL17-15C at 484 days post-telomerase loss, mapped to the MSH3 ORF, 37.4 kb from the end of chromosome III (supplemental Fig. 5). The orientation
of the amplified fragment also suggested that a palindrome might have formed at this locus. Indeed, Southern blotting of digested DNA showed patterns consistent with a palindrome, including half-bands. As in the first example, this configuration maintains the most telomeric essential gene on the chromosome arm, which is CDC39. Similar results were obtained for a third clone, obtained from yJL76-10B at 392 days, which formed a large palindrome that encompassed the essential KRR1 locus and centered near the centromeric end of HML on chromosome III-L (data not shown). Examination of a fourth clone, in MUC1 on chromosome IX-R and also obtained from yJL76-10B at 392 days, did not reveal clear evidence of a palindrome center within 25 kb of MUC1. However, subsequent tiling array analysis (see below) showed that the terminal deletion (which would localize to the presumptive palindrome center) extended 40 kb toward the centromere from MUC1 and thus explained why it was not observed by Southern analysis. The analyses of these four clones support the bona fide location of the cloned fragments near chromosome termini and demonstrate that at least some chromosome termini in the triple mutant survivors exist as large palindromes.

**Tiling Array Analysis of Survivors**—To confirm our findings and to gain a broader perspective on the genomic organization of the triple mutant survivors, we used tiling microarrays to examine the differences in copy numbers among genomic regions in strain yJL76-10B at two different time points after telomerase loss as follows: day 5 (presenescent) and day 504 (mature survivors). The array contains ~2.6 million 25-mer overlapping probes with ~5-nucleotide spacing that correspond to the top (i.e. Watson) strand of the yeast genome (43). Chromosome plots that represent the difference in copy number of chromosomal regions between the survivors and their presenescent precursors are shown in Fig. 5 and supplemental Fig. 7. As expected, we found losses of sequences at all ends, including all telomeric repeats and subtelomeric Y′ and X sequences. The extent of each terminal deletion, the location of the most telomeric essential ORF, and the distance between the two are described in supplemental Table 2. Also as expected, deletions never extended as far as the most telomeric essential ORF at any chromosome end. The deletions extended to an average distance of 17 kb from the most telomeric essential ORF, but came as close as 156 bp in the case of YPD1 on chromosome 4L. Consistent with the canavanine resistance measurements, the chromosome 5L terminal deletion extended to the middle of the CAF1 locus. Also of interest, there was a rough but significant (p < 0.02) inverse relationship between the size of the terminal deletion and the distance from the deletion breakpoint to the first essential ORF (supplemental Fig. 6). This implies that large deletions might be limited by encroachment upon essential ORFs but that smaller deletions are limited by some other factor, e.g. the ability of the sequences to form a stable terminal structure.

Even more striking than the terminal deletions, there were amplifications of large contiguous segments of the chromosome extending inward from the terminal deletions, consistent with palindrome formation, at 19 of the 32 chromosome ends. In several cases these extended beyond 100 kb in length (e.g. chromosome arms IIIIR and XIIIR). Of the ends without obvious palindromes, nine showed no evidence of sequence amplification (e.g. chromosomes VR and VIIIIR) and so apparently maintain their ends in the absence of palindrome formation. This might reflect a terminal hairpin structure, as described further under “Discussion.” Also of note, we expected that centromeres would be excluded from palindromes so that dicentric chromosomes would be avoided. This proved true except for chromosome I, where centromeric sequences are located in the duplicated sequences on the right arm (supplemental Fig. 7). We do not know how this chromosome is stably maintained, although it is possible that one of the centromeres has been inactivated (44).

The tiling array analysis was performed on the same survivor clone (yJL76-10B) as that used for the Southern analysis of the palindromes in the vicinities of YOR1 and HML; as expected, the deleted regions of chromosomes VII-R and III-L in the array analysis corresponded to the center of the palindrome in YOR1
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and to a site just centromeric to HML, respectively. Furthermore, even though the clones used for the two analyses were different, the deleted region of chromosome IIIIR obtained from tiling array analysis of yJL76-10B was very close (~1.6 kb) to the center of the palindromic observed on this chromosome arm in yJL17-15C, and in both cases the essential CDC39 gene was present. This implies that breakpoints and palindromes might form in similar regions in different clones.

In addition to the changes at the chromosome termini, there were increases and decreases in copy number at several small loci scattered throughout the chromosomes. One of the regions with decreased copy number corresponds precisely to the repeated ribosomal DNA loci on chromosome XII (supplemental Fig. 7). The remaining loci correspond almost entirely to Ty retrotransposons, including all known Ty1 and Ty2 elements (marked with * and # symbols, respectively, in Fig. 5 and supplemental Fig. 7). Remarkably, all of the Ty elements with increased copy number in the survivors are transcribed in the direction of the bottom (i.e. Crick) strand of the genome, whereas all of those having decreased copy number are in the opposite orientation. This observation can be explained, as detailed below (see supplemental Fig. 8), by a higher level of RNA-DNA Ty virus-like particles (VLPs) in mature survivors, but a lower level of chromosomal Ty elements, compared with presenescent cells. RNA transcribed from genomic Ty elements is reverse-transcribed in VLPs, and although it can eventually be converted to double-stranded DNA in fully mature particles, the majority of VLPs possess RNA-DNA hybrids that are the initial products of reverse transcription (45). Because the tiling array contains only Watson strand sequences and because DNA (and not RNA) strands are transcribed into probes for the tiling array, VLPs containing RNA-DNA hybrids can be detected only where the RNA is in the orientation of the Crick strand (see supplemental Fig. 8). Thus, higher levels of RNA-DNA Ty VLPs in survivors would yield the observed increased levels of Ty elements with transcripts equivalent to the Crick strand. Presenescent tlc1 mutants are known to accumulate very high levels of chromosomal Ty1 DNA that decline in RAD52-dependent tlc1 survivors (46) and thus the decreased level of Watson strand elements is most likely explained by lower levels of chromosomal Ty elements in the triple mutant survivors compared with presenescent cells (albeit still at levels higher than in non-senescing TCLI+ cells; see below). It is also conceivable that a reduced efficiency of conversion from RNA-DNA to full duplex DNA forms of Ty VLPs in survivors compared with presenescent cells might contribute to the apparent decline in Ty copy numbers, although we have not tested this possibility.

Analysis of Chromosomal Ty1 Copy Number Changes in Survivors—Given the enrichment for Ty1 elements in the set of clones obtained by TdT-mediated tailing and anchor bubble PCR of survivor DNA above, as well as the complexities of measuring changes in Ty1 copy numbers using the tiling array, we sought an additional measure of overall chromosomal Ty1 copy numbers in survivors. Chromosomes from triple mutants (strain yJL76-10B) at 11 different times ranging from 25 and 440 days after sporulation, and a rad52Δ sgs1 control, were separated on CHEF gels, blotted, and probed for Ty1. Ty1 intensities were quantified, using only signals falling between the smallest and largest wild-type chromosomes to minimize any contribution from degraded chromosomes. The blots were then probed for BAR1, which is located at a region of chromosome IX that is free of amplifications and deletions, and the Ty1 signals were normalized to the BAR1 signals. The ratio of the normalized Ty1 signals in the survivor samples to the normalized Ty1 signal of the control was determined for each time point and plotted in Fig. 6. The copy number of chromosomal Ty1 elements increased gradually in early survivors, peaking at twice that of wild-type cells at 110 days after sporulation, and then declined to a level ~50% greater than wild-type cells that remained stable past 400 days. These findings indicate that in comparison to non-senescing TCLI+ controls, triple mutant survivors accumulate an increased level of chromosomal Ty1 sequences.

Mapping of SGS1 Functions That Prevent Survivor Formation—Two key functional elements of the Sgs1 protein are the centrally located helicase domain and a C-terminal domain required for checkpoint responses to DNA damage in S-phase (47, 48). To gain further insight into mechanisms of survivor formation, we tested the importance of each of these domains in the prevention of survivor formation by Sgs1. Full-length SGS1, a helicase-deficient allele containing a K706A point mutation in the helicase domain (sgs1-hd), or a checkpoint-deficient allele lacking the last 200 amino acids of Sgs1 (sgs1-D200) were placed under the control of the SGS1 promoter and integrated at the LEU2 locus (49). tcl1 sgs1 rad52Δ cells containing these alleles were then tested for their capacity to form survivors. As expected, no survivors formed in triple mutants that expressed the intact Sgs1 protein (Table 3). Although it was conceivable that the checkpoint function of Sgs1 would play a role in cell cycle arrest related to telomere shortening, we found that the sgs1-D200 allele was as effective as full-length Sgs1 in preventing the formation of survivors. In contrast, 4 of 6 cultures that expressed Sgs1 with the mutant helicase domain formed survivors, similar to the frequency obtained in cells devoid of Sgs1. Control experiments showing that the sgs1-hd allele was able to rescue the slow growth of sgs1 top1 mutants confirmed that this allele expressed functional
protein (28). These results indicate that the helicase domain but not the checkpoint function of Sgs1 is required to prevent triple mutants from escaping senescence.

Sgs1 Interferes with the Maintenance of Established Survivors—Although Sgs1 prevents the formation of survivors, it was possible that, once formed, established survivors would become independent of Sgs1 action. This possibility was addressed by transforming established survivors with SGS1 under the control of its own promoter on an ARS/CEN plasmid. Although survivors could be transformed with a control vector, and sgs1 rad52Δ control cells were transformed efficiently with both the control vector and SGS1 plasmids, essentially no survivors containing the SGS1 plasmid were obtained. Survivors were transformed with the SGS1 plasmid at a frequency at least 500-fold lower than the vector control (Table 4), indicating that Sgs1 was toxic to the survivors. Therefore, even established survivors require the continued absence of Sgs1 to grow. This finding also makes it unlikely that sgs1 deletion facilitates survivor formation by simply increasing genome instability and thus indirectly enabling the accumulation of mutations that allow the survival of cells lacking telomerase and HR.

DISCUSSION

We have described our unexpected discovery that deletion of SGS1 facilitates the formation of survivors that maintain chromosome termini in the absence of telomerase and Rad52-dependent HR. This function of Sgs1 to inhibit Rad52-independent survivor growth is distinct from its previously described Rad52-dependent roles to slow senescence and facilitate the growth of type II survivors in cells lacking telomerase. The chromosomes in these telomerase and HR-independent survivors lack natural telomeric and subtelomeric repeat sequences. Instead, some termini have acquired large palindromic duplications extending for up to hundreds of kilobases, whereas others apparently have no such duplications. These findings are similar to an earlier report showing that deletion of EXO1 also enabled the formation of such survivors, which were also accompanied by palindrome formation at some chromosome termini (17). In addition, we have observed an increase in the chromosomal copy number of Ty1 retrotransposon elements in the survivors. As an aside, we note that a similar increase in Ty1 elements was not observed in the previous report that examined EXO1, but this may be because chromosome ends were not cloned directly in the study and because the microarrays used were of much lower resolution than those used in our study. Alternatively, as we describe below, exo1 deletion might not affect Ty1 elements and thus our findings might reflect a process specific to sgs1 deletion.

Nature of Chromosome End Maintenance in Triple Mutant Survivors—How can our findings explain the maintenance of chromosome termini in the triple mutant survivors? First, it is possible that prior to resection into an essential locus, chromosome termini form covalently closed hairpins (Fig. 7, step 4), perhaps at small inverted repeats that base pair within themselves and are then sealed by ligase. This was hypothesized by Maringele and Lydall (17) for tcl1 rad52 exo1 survivors and is similar to mechanisms involved in palindrome formation related to the maintenance of Streptomyces linear plasmids and poxviral genomes (50, 51). Hairpin formation would serve to both cap the terminus and to solve the end replication problem, as proposed previously (52, 53). Replication around terminal hairpins would create two chromosomes in a palindromic arrangement and linked at the point of the original hairpins. This model is similar to those proposed to explain the genesis of palindromes formed in Tetrahymena ribosomal DNA, and in yeast and Chinese hamster ovary cell chromosomes when a double strand break occurs next to a short inverted repeat (54–56). Isomerization of the palindrome symmetry center to a cruciform structure, followed by cleavage by a HJ resolvase, would generate a nicked hairpin at each chromosome terminus that could be sealed by ligase to regenerate a perfect copy of the original end (Fig. 7, steps 5–7, and Fig. 4C). This process of replication around a hairpin, cruciform formation, and then cleavage to reform the hairpin could be carried out indefinitely without loss of sequences. Moreover, it provides a means by which both daughter cells could inherit chromosomes with a full complement of essential loci, in contrast to models proposed earlier to explain the growth of tcl1 rad52 exo1 mutants (17). This model predicts that HJ resolvase activity would be required for the growth of mature survivors. The Mus81-Mms4 complex cleaves HJ in vitro, and there is evidence that it also

### TABLE 3

| Parental diploid strain | Genotype | No. of survivors/No. of senescing cultures |
|-------------------------|----------|------------------------------------------|
| yJL92 tcl1 sgs1 Rad52Δ | SGS1a   | 0/5                                      |
| yJL87 tcl1 sgs1 Rad52Δ | SGS1-hΔ | 4/6                                      |
| yJL87 tcl1 sgs1 Rad52Δ | SGS1-ΔC200 | 0/5                                  |
| yJL92 tcl1 sgs1 Rad52Δ |          | 1/1                                      |
| yJL92 tcl1 SGS1 Rad52Δ |          | 0/1                                      |
| yJL92 tcl1 SGS1 Rad52Δ |          | 0/1                                      |
| yJL92 tcl1 sgs1 Rad52Δ |          | 1/2                                      |
| yJL92 tcl1 sgs1 Rad52Δ |          | 1/2                                      |

### TABLE 4

| Strain | Genotypea | prS-hydro (control), colony no. | pSGS1- hydro, colony no. | Days of growthb | Transformants/nmol of plasmid (control, pSGL) | Relative no. of transformants (control, pSGL) |
|--------|-----------|---------------------------------|--------------------------|-----------------|-----------------------------------------------|---------------------------------------------|
| yJL6-13B rad52Δ sgs1 | 67,644 | 72,028 | 2,2 | 2.9 × 10⁴, 3.1 × 10⁴ | 0.9 |
| yJL7-14A rad52Δ sgs1 | 108,606 | 107,729 | 2,2 | 4.6 × 10⁴, 4.6 × 10⁴ | 1.0 |
| yJL6-10B rad52Δ sgs1 est2 | 4,823 | 0 | 8,11 | 2.1 × 10⁴, <4.3 × 10⁴ | >4 × 10⁴ |
| yJL7-15C, n15 rad52Δ sgs1 tcl1 | 55,734 | 96 | 7,11 | 2.4 × 10⁴, 6.4 × 10⁴ | 5.8 × 10² |
| yJL7-15C, j15 rad52Δ sgs1 tcl1 | 4,717 | 0 | 8,11 | 2.0 × 10⁴, <4.3 × 10⁴ | >4 × 10⁴ |
| yJL6-3C rad52Δ sgs1 est2 | 105,244 | 181 | 4,11 | 4.4 × 10⁴, 2.2 × 10⁴ | 5.8 × 10² |

a All triple mutants are survivors that had all grown for more than 475 days since sporulation.

b Days of growth refer to the number of days that transformants were grown on plates prior to the enumeration of colony numbers.
perform this function in vivo, and it is thus a candidate for such an activity (57–59). Although mus81 and sgs1 mutations together cause synthetic lethality, this is suppressed by rad52 mutation (60), and so we have recently been able to determine that tlc1 rad52 sgs1 mus81 mutants generate survivors, indicating that Mus81 is dispensable for the initial stages of survivor formation. However, several more months of growth will be required before we can determine whether Mus81 is required for the growth of mature survivors, whose growth we predict will be the most dependent on HR resolvas because, as described above, terminal palindromes do not appear until more than 100 days after the initial formation of survivors. An alternative to the use of an HR resolvase for cruciform resolution is the use of pairs of a type I topoisomerase (i.e. Top1 or Top3), nicking at sites symmetrically (or approximately symmetrically) distributed around the cruciform center, as appears to occur at the telomeres of the Shope fibroma poxvirus (61).

Second, the origin of the large palindromes observed at some survivor chromosome termini could be explained by the mechanism just described if cruciform cleavage is not perfectly efficient, because an attempt to segregate unresolved dicentric chromosomes during mitosis could result in asymmetric breakage leading to one product with a deletion and the other with a palindromic duplication. The cell that inherits the deleted chromosome would presumably arrest because of loss of essential loci, but the chromosome with the duplication could then shorten to the center of symmetry or might be stabilized by a new hairpin formed prior to shortening back to the center. We note that this model does not explain the origin of the dicentric example of chromosome 1 (supplemental Fig. 7), and so some other event must have given rise to this rearrangement, e.g. a double strand break during DNA replication. Large palindromes are common in cancer cells and can provide a substrate for additional gene amplification via fusion-breakage-bridge cycles in cases where dicentric chromosomes are formed (56, 62). Perhaps the mechanisms that stabilize chromosome ends in our triple mutant survivors contribute to genome instability in cancer cells by both enabling palindrome formation and inhibiting checkpoint activation during breakage of dicentric chromosomes.

Third, the increased level of Ty elements in triple mutant survivors, together with previous demonstrations that these elements can help repair DNA breaks (63), raises the possibility that Ty elements might play a role in stabilizing chromosome termini during the evolution of survivors. An increase in Ty1 transcription and retrotransposition during the senescence of tlc1 mutants has been observed previously (46, 64). Furthermore, Ty1-mediated transposition of subtelomeric Y’ elements has been found to be elevated in type I survivors of telomerase deletion, leading to the suggestion that this process might play a role in the generation of these Rad52-dependent survivors, although there is not yet any direct evidence for this (65). Ty elements might provide an even greater benefit for the Rad52-independent survivors studied here because, unlike Rad52-dependent survivors, the triple mutant survivors lose all telomere sequences and thus might have an increased need for end protection. In support of a role for Ty elements at chromosome termini, human L1 elements were found recently to retrotranspose efficiently to telomeres in Chinese hamster ovary cells, particularly when telomeres were uncapped by a dominant-negative form of TRF2, and also that organisms like Drosophila melanogaster use retrotransposons as a natural basis of telomere maintenance (66, 67). We note, however, that although the significantly increased presence of Ty1 elements in our collection of clones obtained from TdT-mediated tailing and anchor-bubble PCR suggests that the elements may lie at chromosome termini, it is also possible that some of the cloned elements derived from extrachromosomal VLP copies or from double-stranded DNA breaks, which have been found to occur at increased frequency at closely spaced pairs of Ty elements that are arranged in an inverted orientation (68). More work is required to understand the possible role of Ty elements in the survival of cells lacking telomerase and HR.

Role of sgs1 Deletion in Triple Mutant Survivors—How does deletion of SGS1 facilitate the formation and maintenance of survivors in cells lacking telomerase and HR? Our data argue that survivor formation is not caused by a diminished checkpoint response related to the established function of Sgs1 in the intra-S phase checkpoint (47), but rather relates to the helicase activity of the protein. We propose that after telomeres have become uncapped by shortening, and thus become accessible to exonucleases that include principally Exo1 (5), Sgs1 can unwind DNA secondary structures at chromosome termini that would otherwise impede resection by such exonucleases. This model can explain why deletion of either EXO1 or SGS1 is sufficient to stimulate the formation of survivors. The nature of the secondary structure removed by Sgs1 is unclear, but one candidate

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A. Chavez and F. B. Johnson, unpublished observations.
would be the short hairpins that, as hypothesized above, if not unwound could lead to a covalently closed terminal cap. Alternatively, Sgs1 might act at an earlier stage of end resection, perhaps by removing structures formed by the G-rich telomere repeats (e.g. G-quadruplexes) that could sterically block access of exonucleases (see Fig. 7). This idea is consistent with the findings that Sgs1 is a potent G-quadruplex unwinding helicase and that overexpression of Sgs1 reverses protection by the G-quadruplex binding protein Stm1 of telomeres that have lost the natural telomere capping protein Cdc13 (69, 70). Interestingly, the ataxia telangiectasia mutated kinase has recently been shown to inhibit the survival of lymphocytes bearing unrepaired double-stranded breaks induced by the RAG proteins during V(D)J recombination (71). The BLM helicase, a mammalian homologue of Sgs1, is an important target of ataxia telangiectasia mutated, and individuals with Bloom syndrome suffer from increased rates of hematological malignancies that are frequently associated with aberrant V(D)J recombination events (72, 73). We therefore speculate that, like Sgs1, BLM might play a role in inhibiting the survival of cells possessing unrepaired DNA breaks and thus inhibit chromosome rearrangements that promote tumorigenesis.

We found that Sgs1 not only inhibits the formation of survivors, but also inhibits the growth of mature survivors. Although it is possible that the same activity of Sgs1 explains its effects at both stages (e.g. the unwinding of hairpins prior to their covalent closure), another possibility is that Sgs1 inhibits some process unique to mature survivors. Several RecQ helicases, including Sgs1, have been shown to efficiently branch migrate Holliday junctions (74), and so it is possible that similar unwinding by Sgs1 of cruciform structures could interfere with their cleavage by HJ resolvases, as proposed in Fig. 7, and thus cause catastrophic levels of chromosome breakage or non-disjunction.

The inhibition of mature survivor growth by Sgs1 also indicates that the stimulatory effect of sgs1 deletion on survivor growth is not simply an indirect consequence of an elevated rate of bypass suppressor mutations caused by lack of Sgs1. However, it is still formally possible that such additional mutations are also involved in the survivor mechanism. Although we cannot rule it out, we do not favor a major role for such mutations because available data do not indicate that sgs1 deletion stimulates mutation rates in a rad52 background enough to explain the ~100-fold increase in rates of survivor formation. A principal effect of sgs1 deletion in normal cells is to increase inappropriate homologous recombination, and this increase is actually partially suppressed by rad52 deletion so that sgs1 deletion causes less than 10-fold increases in recombination (75). Furthermore, levels of unequal sister chromatid exchange and illegitimate plasmid recombination are increased less than 5–10-fold by sgs1 deletion in rad52 backgrounds and are lower than in wild-type cells, and levels of gross chromosomal rearrangements in a rad52 background are not increased by sgs1 deletion (76–78). Regardless of any potential role for such uncharacterized bypass mutations, it is clear that Sgs1 plays a direct role in inhibiting the growth of survivors in cells lacking telomerase and Rad52.

There are also connections between Sgs1 and Ty1 elements that, to the extent that Ty elements play a role in survivor formation (see above), might contribute to the enhanced survivor formation caused by sgs1 deletion. The level of Ty1 retrotransposition is enhanced ~50-fold by sgs1 deletion (79). This increase appears to involve enhanced recombination among extrachromosomal Ty1 elements, and although the majority of the increase depends on Rad52, there is a still a residual ~2-fold Rad52-independent stimulation of transposition caused by sgs1 deletion (79), consistent with earlier findings that elevated recombination in sgs1 mutants is partially Rad52-independent (75). Therefore, it is conceivable that increased Ty1 retrotransposition contributes to enhanced survival in mutants lacking telomerase, Rad52, and Sgs1.

The triple mutant survivors described here provide a system that should allow further insight into mechanisms by which RecQ helicases regulate the maintenance of chromosome termini and thus promote genome stability.

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