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Telomere Maintenance and Survival in *Saccharomyces cerevisiae* in the Absence of Telomerase and *RAD52*

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ABSTRACT

Telomeres are essential features of linear genomes that are crucial for chromosome stability. Telomeric DNA is usually replenished by telomerase. Deletion of genes encoding telomerase components leads to telomere attrition with each cycle of DNA replication, eventually causing cell senescence or death. In the *Saccharomyces cerevisiae* strain W303, telomerase-null populations bypass senescence and, unless *EXO1* is also deleted, this survival is *RAD52* dependent. Unexpectedly, we found that the *S. cerevisiae* strain S288C could survive the removal of *RAD52* and telomerase at a low frequency without additional gene deletions. These *RAD52*-independent survivors were propagated stably and exhibited a telomere organization typical of recombination between telomeric DNA tracts, and in diploids behaved as a multigenic trait. The polymerase-δ subunit Pol32 was dispensable for the maintenance of *RAD52*-independent survivors. The incidence of this rare escape was not affected by deletion of other genes necessary for *RAD52*-dependent survival, but correlated with initial telomere length. If W303 strains lacking telomerase and *RAD52* first underwent telomere elongation, rare colonies could then bypass senescence. We suggest that longer telomeres provide a more proficient substrate for a novel telomere maintenance mechanism that does not rely on telomerase, *RAD52*, or *POL32*.

TELOMERES, the ends of eukaryotic chromosomes, are crucial for genome stability and the complete replication of genetic information encoded on linear chromosomes (reviewed in Chakhparonian and Wellinger 2003). The distal portion of *Saccharomyces cerevisiae* chromosomes is composed of ~350–500 bp of degenerate repeat sequences [$(TG_{1-3})_n$] (Walmsley *et al.* 1984), with a G-rich single-stranded 3′ extension that varies in length during the cell cycle (Wellinger *et al.* 1993; Larrivee *et al.* 2004). This organization is essential for the binding of telomere-specific proteins and ensures that the telomere is not repaired as a double-strand break (d'Adda di Fagagna 2008). Subtelomeres contain two distinct types of repeats (Chan and Tye 1983a,b). Tandem arrays of up to four Y′ elements are embedded between telomeric TG_{1-3} repeat tracts on half to two-thirds of yeast telomeres. The Y′ element contains a unique *Xho*I recognition site; digestion of genomic DNA with this enzyme yields a characteristic terminal restriction fragment (TRF) of ~1.2 kbp in wild-type cells. The other subtelomeric feature, the core X element, is found on all yeast chromosomes and is separated from Y′ sequences by telomeric TG_{1-3} repeats. Together, these regions contribute to the stability, replication, and maintenance of yeast telomeres.

Due to the semiconservative nature of DNA replication, telomeres shorten with each replication cycle (as first predicted by Watson 1972 and Olovnikov 1973). In the absence of telomerase, a reverse transcriptase that replenishes telomeric sequences, telomere shortening can lead to a critically short telomere length after several cell divisions. When a subset of telomeres reaches this so-called "critical" threshold, the cell enters a nonproliferative state termed senescence (Lundblad and Szostak 1989; Lundblad and Blackburn 1993; Lendvay *et al.* 1996). Deletion of any of the genes encoding telomerase subunits leads to an **e**ver **s**horter **t**elomere (EST) phenotype and cell death within 60–80 generations (Lendvay *et al.* 1996). However, a small subset of cells in the arrested population is able to maintain viability by replenishing telomere DNA via a recombination-based mechanism (Lundblad and Blackburn 1993; Le *et al.* 1999; Teng and Zakian 1999; Chen *et al.* 2001). These

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so-called "survivors" are not always stably propagated and telomeres may continue to shorten over time, with subsequent lengthening when telomeres become very short (Teng *et al.* 2000).

The generation of survivors almost always depends on *RAD52*-dependent homologous recombination. The two broad classes of survivors that have been described to date (type I and type II) differ in the sequence amplified at chromosome ends and the proteins required for recombination (Lundblad and Blackburn 1993; Le *et al.* 1999; Teng and Zakian 1999; Chen *et al.* 2001). In type I survivors, telomere maintenance involves amplification of subtelomeric Y′ sequences and acquisition of Y′ sequences on all telomeres (Lundblad and Blackburn 1993; Le *et al.* 1999), and survival depends on Rad51, Rad52, Rad54, Rad55, and Rad57 (Le *et al.* 1999; Teng and Zakian 1999). The telomeric DNA exhibits a characteristic *Xho*I terminal restriction fragment (TRF) distribution, although of a smaller size than in wild-type cells due to shorter TG_{1-3} telomeric DNA tracts. Chromosomes of type I survivors are longer than in wild-type cells, likely due to the amplification of the Y′ element, and appear heterogeneous when analyzed by pulsed-field gel electrophoresis (Liti and Louis 2003). In type II survivors, telomeres are maintained by TG_{1-3} amplification (Lundblad and Blackburn 1993; Le *et al.* 1999; Teng and Zakian 1999; Teng *et al.* 2000), which depends on Rad52, the MRX complex (Mre11, Rad50, and Xrs2), Sgs1, and Rad59 (Le *et al.* 1999; Teng *et al.* 2000; Chen *et al.* 2001; Huang *et al.* 2001; Johnson *et al.* 2001; Tsukamoto *et al.* 2001). When digested with *Xho*I, telomeric DNA exhibits discrete fragments of various sizes due to the amplification and propagation of differing telomere lengths on each chromosome end. Type I and type II telomere maintenance pathways appear to be closely related to the break-induced replication mechanism (BIR) that repairs chromosomal double-strand breaks (DSBs) (reviewed in McEachern and Haber 2006). The replication protein Pol32 is required for recovery of both types of survivors, suggesting that these pathways may depend on recombination-dependent DNA replication (Lydeard *et al.* 2007). Cells may survive via changes in telomeric DNA structure that permit recombination, rather than the accumulation of extragenic suppressors (reviewed in McEachern and Haber 2006).

The precise frequency of survivor generation has not been accurately determined, although frequencies of one survivor in $\leq 10^4$ cells/generation have been reported (Lundblad and Blackburn 1993; McEachern and Haber 2006). This low frequency suggests that the ability to survive is not determined by a single genetic locus. For example, Makovets *et al.* (2008) used a mating-based analysis to demonstrate that a survivor haploid could exert dominance over a senescent haploid. This finding confirms that the acquisition of extragenic suppressors is unnecessary for telomerase-independent survival (Lundblad and Blackburn 1993). Also, Zubko and Lydall showed that the survival of *cdc13-1* cells at 36° segregates as a multigenic trait; however, the presence of suppressor mutations has not been ruled out (Zubko and Lydall 2006).

Previously, survivors in *S. cerevisiae* had not been recovered in cells lacking telomerase and *RAD52* unless *EXO1* or *SGS1* was also absent (Maringele and Lydall 2004; Lee *et al.* 2008). Chromosomes in *exo1*Δ survivors are linear but have lost telomeric and subtelomeric sequences, resulting in atypical chromosome sizes. These survivors also exhibit large inverted and duplicated repeats (palindromes) at chromosome ends, which probably originate from small inverted repeats (Maringele and Lydall 2004; Lee *et al.* 2008).

Here, we report that the *S. cerevisiae* S288C strain can survive, at a low frequency, in the absence of telomerase and *RAD52*, without any other known genetic alterations. These *RAD52*-independent survivors could be propagated for several generations and exhibited a type II-like (*e.g.*, telomeric DNA amplification) pattern. Furthermore, the propensity to escape senescence appeared to depend on telomere length. Telomere elongation in telomerase- and *RAD52*-deficient strains increased the frequency of survival and even permitted survival in a W303 *est2*Δ *rad52*Δ strain (which under normal circumstances undergoes rapid senescence in the absence of telomerase and *RAD52*). *RAD52*-independent survivors arose in the absence of genes known to affect *RAD52*-dependent cell survival, and, in diploids, survival behaved as a multigenic trait.

MATERIALS AND METHODS

Yeast strains: W303 *MAT***a** and *MAT*α strains were obtained from M. Tyers (MT234, *MAT*α *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 URA3 GAL+ psi+ ssd1-d2 rad5-535*; and MT235, *MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3 GAL+ psi+ ssd1-d2 rad5-535*). S288C *MAT***a** and *MAT*α strains were obtained from M. Tyers (BY4741, *MAT***a** *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ; and BY4742, *MAT*α *his3*Δ *leu2*Δ *lys2*Δ *ura3*Δ).

Yeast manipulations: Replacement of the *Kan*^{r} cassette for selection of ORF deletion strains was performed in either haploid or diploid cells, using a PCR-based replacement protocol as described in Brachmann *et al.* (1998) and Longtine *et al.* (1998), followed by selection on appropriate medium. Gene disruption was confirmed by PCR, enzymatic digestion of the PCR products, and Southern blot analysis. Genomic DNA of a *tlc1*Δ strain was a gift from D. Durocher. Transformation of yeast was performed according to the lithium acetate method (Gietz and Woods 2006). For the telomere elongation experiments, W303 or S288C heterozygous diploids (*est2*Δ*/EST2 rad52*Δ*/RAD52* or *tlc1*Δ*/TLC1 rad52*Δ*/RAD52*) were transformed with the plasmid pVL1107 (Leu$^+$) encoding a Cdc13-Est2 fusion protein [obtained from D. Durocher, originally a gift of V. Lundblad (Evans and Lundblad 1999)]. After dissection and identification, the appropriate haploid was propagated in the absence of leucine for the indicated number of passages. Following telomere elongation, colonies that had lost the plasmid after growth in rich media were identified. Standard genetic procedures were

used for sporulation of diploids, microdissection of asci, and identification of haploids (Guthrie and Fink 1991).

Senescence assays on plates were performed according to Maringele and Lydall (2004) and Lebel *et al.* (2006). Briefly, cells were isolated from freshly dissected tetrads (passage 1) and propagated on YPD plates. After incubation for 4 days at 30°, single colonies were picked from the plate (passage 2) and repropagated on YPD plates to obtain passage 3, etc. This procedure was performed for a total of six serial propagations (the equivalent of 140 generations). Plates of each passage were stored at 4° until all propagations were completed. Growth over six serial propagations (24 days of serial growth for each genotype) was represented by a "summary senescence" plate (as shown in Figures 1, 2, 4, 6, and 7): an isolated colony from each of the individual propagation plates was repropagated onto a sector of a single YPD plate and incubated at 30° for 4 days.

To approximate the frequency of escape from senescence, at least 50 colonies were propagated on YPD plates for 4 days at 30°. After 3 passages, the majority of colonies had ceased proliferating and only *RAD52*-independent survivors were recovered. Surviving colonies were propagated stably for >10 serial passages. To generate type II survivors, freshly dissected *est2*Δ haploid cells were serially diluted in liquid media to a density of 10^5 cells/ml every 24 hr for 10 days. *RAD52* was subsequently deleted in type II survivor cells by mating with *rad52*Δ cells, followed by dissection and selection of *est2*Δ *rad52*Δ spores. Senescence assays were performed (following 50 individual colonies per experiment) as described above.

Liquid growth assays were performed according to Chen *et al.* (2001) with modifications. Briefly, at least three colonies per genotype were isolated from freshly dissected tetrads and grown in YPD to saturation ($1–2 \times 10^8$ cells/ml). Every 24 or 48 hr, cell density was measured using a hemacytometer. The culture was then diluted with fresh liquid YPD to a density of 10^5 cells/ml. Cells were harvested and genomic DNA was extracted for telomere length analysis.

To mate a *RAD52*-independent survivor with a freshly dissected haploid (presenescent), cells of opposite mating types were mixed on a YPD plate and incubated for 6–8 hr at 30°. Diploids were selected for the presence of all deletion markers on appropriate media and confirmed as described above.

Telomere Southern blot: Genomic DNA was isolated, digested with *Xho*I, resolved through a 0.75% w/v agarose gel in 1× TBE, and transferred onto a nylon membrane. The membrane was hybridized to a radiolabeled yeast telomeric oligo (5′-CACACCCACACCCACACC-3′) to detect terminal restriction fragments (TRFs) (Lebel *et al.* 2006). As a loading control, a 1.76-kbp fragment of the *CDC15* locus was amplified as described in Foster *et al.* (2006) and Zubko and Lydall (2006), labeled, and used to probe genomic DNA digested with *Xho*I to reveal a single hybridization fragment at ∼3 kbp.

In-gel hybridization: The assay was performed as described in Zubko and Lydall (2006) with minor modifications. Genomic DNA was digested with *Xho*I for 4 hr and then incubated at 65° for 20 min. Radiolabeled and purified oligo (5′-CACACCCACACCCACACC-3′) (100,000 cpm) was added to the DNA and incubated at 37° for 15 min followed by 30 min on ice. Samples were subjected to electrophoresis through 0.75% w/v agarose in 0.5× TBE overnight at 30 V. The gel was dried and exposed to a phosphorimager screen (Molecular Dynamics). To detect double-stranded telomere DNA, the same samples were resolved on an agarose gel and transferred to a membrane under denaturing conditions, as above. Where indicated, genomic DNA was treated with exonuclease I (New England Biolabs, Beverly, MA) according to the manufacturer's instructions and as described previously (Wellinger *et al.* 1993). Samples were spotted onto nylon membrane using a vacuum apparatus, or digested with *Xho*I and subjected to electrophoresis as described above, and then probed with strand-specific ssDNA probes [CA rich, as above, or 5′-$(GGTGTG)_3$-3′]. To normalize the ssDNA signal to total telomere DNA signal, the nylon membrane or gel was subsequently denatured and reprobed with either the CA-rich or the GT-rich ssDNA oligonucleotide.

RESULTS

Expected senescence phenotype and telomeric DNA arrangement in W303 and S288C: Previous studies have reported that survivors could not be generated in W303 and closely related derivative strains in the absence of both *RAD52* and telomerase (Le *et al.* 1999; Teng and Zakian 1999; Chen *et al.* 2001; Maringele and Lydall 2004; Larrivee and Wellinger 2006; Wen *et al.* 2006; Zubko and Lydall 2006). We chose a different strain background, S288C [BY4741 and isogenic strain BY4742, for which genomewide, individual ORF deletions are available for all nonessential genes (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html)], to conduct a high-throughput, liquid-based genetic screen to isolate gene deletions, like *exoI*Δ, that promote survival in the absence of *RAD52* and telomerase (E. Rosonina, L. Maringele, D. Lydall and L. A. Harrington, unpublished results) (Cook *et al.* 2008). We discovered that S288C *est2*Δ *rad52*Δ or S288C *tlc1*Δ *rad52*Δ cells could survive regardless of the third gene deletion, suggesting that the genetic requirements for survival in the absence of telomerase might differ between S288C and W303.

To compare the senescence of S288C and W303 strains side by side, we constructed *de novo* mutant heterozygous diploids, verified their genotypes, and isolated haploids. As expected, *est2*Δ or *tlc1*Δ W303 haploids underwent senescence at approximately passage 2 (40–60 generations), and survivors were observed only when *RAD52* was present (Figure 1 and data not shown). In most cases, a similar result was obtained for the S288C strain, except that senescence was marginally delayed relative to the W303 strain (Figure 1), perhaps due to the slightly longer average initial telomere length in S288C (Figure 1J). In *rad52*Δ S288C colonies, TRFs were slightly longer than in wild-type strains, as previously reported (Chang *et al.* 2007) (Figure 1J). We also examined the vitality of W303 and S288C colonies in liquid media by serial dilution of cells to 10^5 cells/ml every 24 hr (Figure 1, K and L). While wild-type and *rad52*Δ cells from each strain reached a density $>10^8$ cells/ml every 24 hr, *est2*Δ *rad52*Δ cells from both strains exhibited decreased growth potential early during the experiment and failed to recover. Consistent with the growth on plates, S288C *est2*Δ or *tlc1*Δ (with or without *RAD52*) reached a growth crisis later than their W303 counterparts; however, in the presence of *RAD52*, recovery from crisis progressed with no observable decrease in growth rate (Figure 1, K and L).

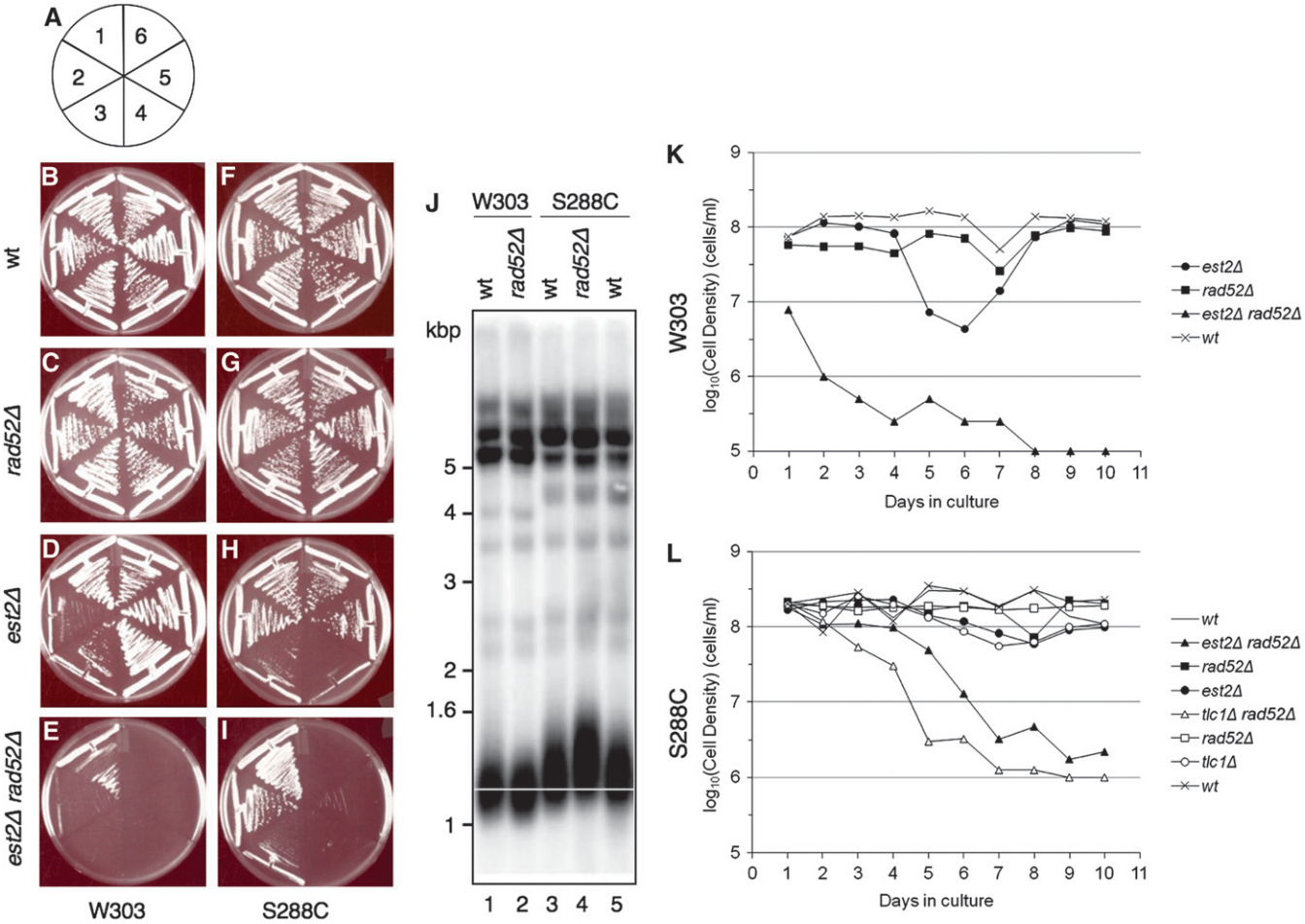

Figure 1.—The majority of haploid colonies generated from W303 *est2*::*NAT/EST2 rad52*::*URA3/RAD52* and S288C *est2*::*NAT/EST2 rad52*::*URA3/RAD52* strains exhibit an expected senescent phenotype. (A) Schematic of the senescence assays on plates, from passage 1 to 6 (see materials and methods). Senescence phenotypes for the haploid colonies (genotypes indicated at left) resulting from the sporulation of W303 (B–E) and S288C (F–I) diploids are shown. (J) Wild-type S288C telomeres are longer than W303 telomeres. Genomic DNA was digested with *Xho*I and the membrane was hybridized to a telomeric probe. (K and L) W303 *est2*Δ *rad52*Δ (K) and S288C *tlc1*Δ *rad52*Δ and *est2*Δ *rad52*Δ (L) lose viability in liquid growth assays. Cells were picked from a fresh dissection plate, inoculated into YPD media, and grown to saturation (1–2 × 10^8 cells/ml). Every 24 hr, cell density was measured using a hemacytometer, and the culture was diluted to 10^5 cells/ml.

Rare escape from senescence in S288C *est2*Δ *rad52*Δ or *tlc1*Δ *rad52*Δ strains: Consistent with our observations from the genetic screen, rare S288C *est2*Δ *rad52*Δ and *tlc1*Δ *rad52*Δ colonies escaped senescence. Of 50 individual S288C *est2*Δ *rad52*Δ or *tlc1*Δ *rad52*Δ colonies (derived from heterozygous diploids) propagated on plates every 4 days, 2–5 colonies consistently sustained growth after passage 6 (Figure 2, B–D), while the majority of colonies became senescent at approximately passage 3. Four to 10% of colonies survived regardless of the telomerase gene deleted (*i.e.*, e*st2*Δ *rad52*Δ or *tlc1*Δ *rad52*Δ) and the result was reproducible over many experiments (total $n > 250$ colonies). The escape from senescence was not due to a reversion of the *rad52* locus (supporting information, Figure S1) and was not observed in W303 *est2*Δ *rad52*Δ or *tlc1*Δ *rad52*Δ strains (total $n > 250$ colonies). Thus, in contrast to strains lacking telomerase that depend on *RAD52* for survival (*e.g.*, type I or type II survivors), S288C telomerase-deficient cells could survive independently of *RAD52*.

S288C *est2*Δ *rad52*Δ or *tlc1*Δ *rad52*Δ survivors arise after extensive telomere loss: Once established, *RAD52*-independent survivors could be propagated for many generations (Figure 2, B–D). Prior to senescence, telomeres in the *est2*Δ *rad52*Δ or *tlc1*Δ *rad52*Δ haploids were shorter in length than telomeres in the preceding heterozygous diploid and were also shorter than in wild-type, *est2*Δ, or *rad52*Δ haploid colonies (Figure 2E, lanes 1 and 2, and data not shown). Further, telomeres shortened with every passage until the cell population reached a growth crisis (Figure 2B, passage 3), which resulted in a low yield of genomic DNA at these particular passages [Figure 2, E (lane 4) and F (lanes 3 and 10)]. When the population regained growth potential [Figure 2, B (passage 4), E (lanes 5–11), and F (lanes 4–7 and 11–14)], the telomere pattern was similar

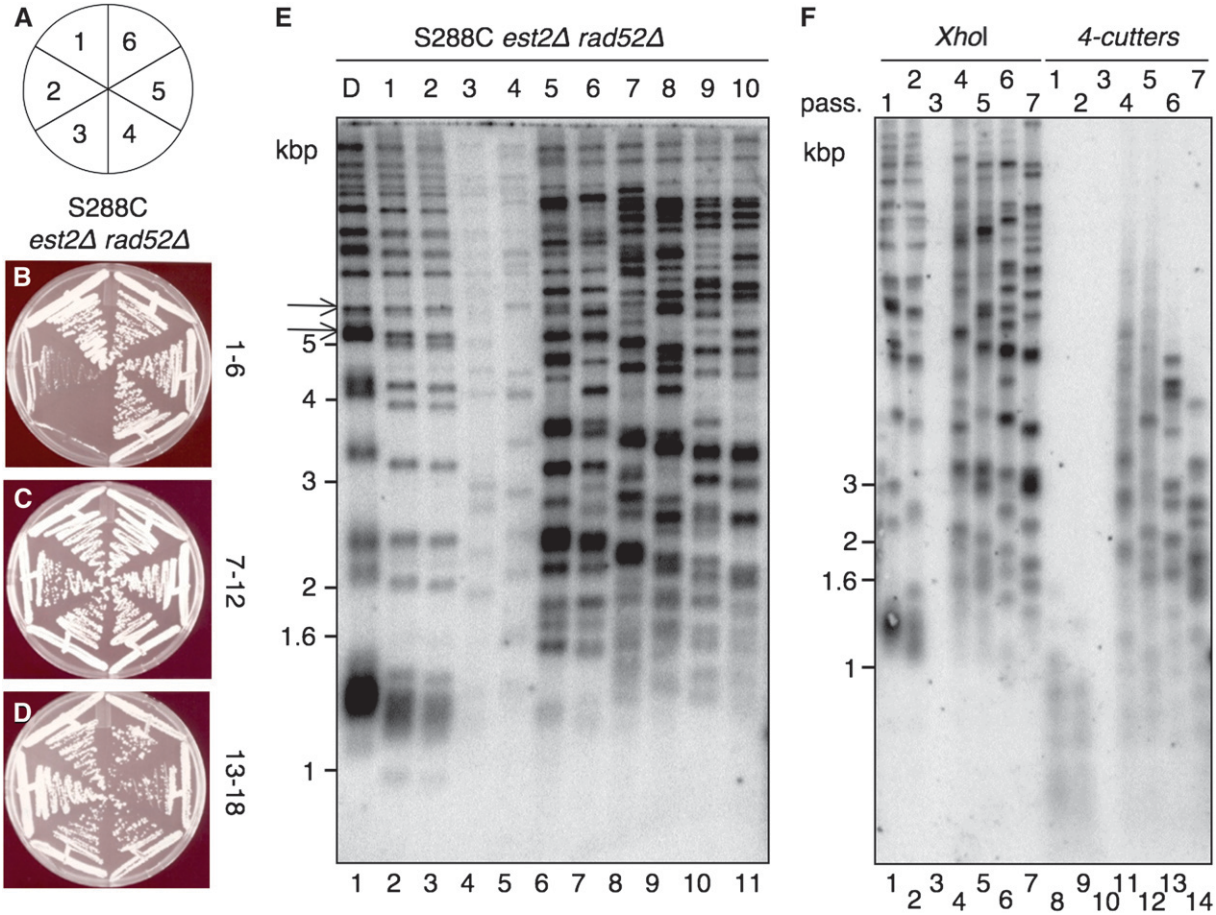

FIGURE 2.—Rare S288C *est2*Δ *rad52*Δ colonies continue to proliferate and exhibit a terminal restriction fragment (TRF) pattern typical of telomeric tract recombination. (A) Schematic of the senescence assays on plates, from passage 1 to 6 (see details in MATERIALS AND METHODS). (B–D) Typical S288C *est2*Δ *rad52*Δ survivors (or *tlc1*Δ *rad52*Δ; data not shown) can be propagated for several generations. (E) Telomere Southern blot on genomic DNA isolated from single S288C *est2::NAT rad52::KAN* colonies from passages 1–10 (20–200 generations). DNA was digested with *Xho*I and the membrane was hybridized to a telomeric probe. Lane 1, diploid S288C *est2::NAT/EST2 rad52::KAN/RAD52* (D); lanes 2–11, haploid S288C *est2*Δ *rad52*Δ at increasing passages. Black arrows at left indicate Y′ elements. (F) Telomere Southern blot of S288C *est2*Δ *rad52*Δ survivors at increasing passages. Genomic DNA was digested with either *Xho*I (lanes 1–7) or a mix of *Alu*I, *Hin*fI, *Hae*III, and *Msp*I (lanes 8–14), and the membrane was hybridized to a telomeric probe. For each panel, marker sizes are indicated at left in kilobase pairs.

to that of *RAD52*-dependent, type II survivors, *i.e.*, multiple discrete fragments from 1 to 6 kbp representing telomeres containing a variable number of TG_{1-3} repeats (TENG *et al.* 2000). Little or no amplification of the Y′ element, which is typical of type I survivors, was observed (Figure 2E and data not shown). The telomeric DNA recombination in *RAD52*-independent survivors was further confirmed by digestion of genomic DNA with a mixture of restriction endonucleases that recognize 4-bp sequences, to which long, nonpalindromic TG_{1-3} tracts would be resistant (WEN *et al.* 2006). Indeed, genomic DNA from *RAD52*-independent survivors yielded a telomeric pattern characteristic of long TG_{1-3} tracts (Figure 2F, lanes 11–14). We extracted genomic DNA after growth of *RAD52*-independent survivors in liquid media for <24 hr and also detected a TRF pattern indicative only of telomeric DNA amplification. It has been previously noted that *RAD52*-dependent type II survivors (telomeric DNA amplification) possess a growth advantage over type I survivors (Y′ amplification) in liquid culture (TENG and ZAKIAN 1999).

Generation of *RAD52*-independent survivors in S288C *est2*Δ *rad52*Δ or *tlc1*Δ *rad52*Δ in serial dilution growth assays: Mindful of the possible bias of enrichment of type II, *RAD52*-dependent survivors in liquid culture, we extended the growth period between serial dilutions from 24 (Figure 1, K and L) to 48 hr (Figure 3, A and B). We examined the viability of three individual haploid colonies that originated from the same heterozygous diploid (Figure 3, A and B, and data not shown). Dilution every 48 hr nearly abrogated the transient decrease in population doubling time for *est2*Δ and *tlc1*Δ colonies (Figure 3, A and B). In this particular experiment, one of three S288C *tlc1*Δ *rad52*Δ colonies regained growth potential at day 14, concomitant with a TRF pattern reminiscent of recombination between telomeric DNA [Figure 3, B (open circles) and C (lanes 17–19)]. In addition, one of the three S288C *est2*Δ

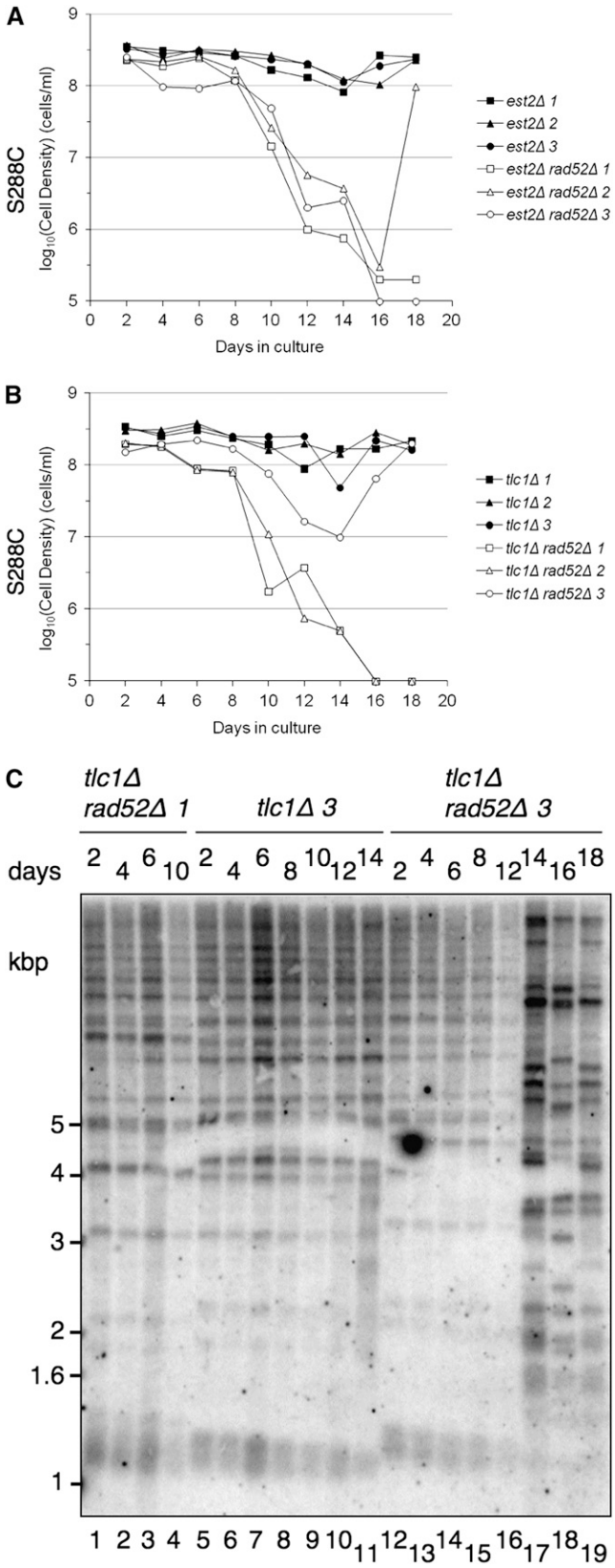

FIGURE 3.—A subset of S288C *est2*Δ *rad52*Δ or *tlc1*Δ *rad52*Δ cells escapes senescence in liquid culture and exhibits telomeric tract recombination. (A and B) Cells were isolated from a fresh dissection plate (isolates 1–3), inoculated into YPD media, grown to saturation (1–2 × 10^8 cells/ml), and diluted to 10^5 cells/ml every 48 hr. At each serial dilution, genomic DNA was extracted for telomere analysis. (C) Telomere Southern blot of S288C haploids with the indicated genotype, as shown in B, during serial propagation in liquid culture every 2 days. Genomic DNA was isolated from cells and digested with *Xho*I. The membrane was hybridized to a telomeric probe. Marker sizes are indicated at left in kilobase pairs.

*rad52*Δ colonies regained growth potential at day 18 and also exhibited a similar TRF pattern (Figure 3A, open triangles and data not shown). In contrast, W303 *est2*Δ *rad52*Δ colonies failed to escape senescence when diluted every 48 hr (data not shown). Thus, the propagation of S288C *est2*Δ *rad52*Δ or *tlc1*Δ *rad52*Δ (but not W303 *est2*Δ *rad52*Δ or *tlc1*Δ *rad52*Δ) cells every 48 hr in liquid culture allowed the emergence of *RAD52*-independent survivors with a TRF pattern suggestive of telomeric DNA recombination (*e.g.*, type II).

The similar TRF pattern between S288C *RAD52*-independent survivors and type II survivors in other strain backgrounds prompted us to test whether the propagation of S288C *est2*Δ cells in liquid culture followed by deletion of *RAD52* (see MATERIALS AND METHODS) would affect the incidence of *RAD52*-independent survival. We noted that S288C *est2*Δ *rad52*Δ colonies survived at an increased frequency if S288C *est2*Δ cells had been propagated in culture to generate type II survivors prior to deletion of *RAD52*, compared with removal of both genes simultaneously ($p<0.01$). This result is in accord with a recent finding by GRANDIN and CHARBONNEAU (2009) that the generation of type II survivors permits survival upon the subsequent deletion of *RAD52* (see *Note added in revision*).

Telomere length and escape from senescence: We hypothesized that the slightly longer telomeres present in S288C compared to W303 (Figure 1J) might facilitate the emergence of *RAD52*-independent survivors. To test this prediction, we elongated telomeres in both S288C and W303 strains by transforming cells with a plasmid encoding a Cdc13-Est2 fusion protein [pVL1107; see MATERIALS AND METHODS (EVANS and LUNDBLAD 1999)]. Strains were propagated on selective media to ensure plasmid retention, and the TRF pattern of each strain was analyzed over several passages (Figure 4A). To compare strains with similar characteristics, (*i.e.*, transformed with pVL1107 and nearly equivalent average telomere length), we selected S288C passage 2 (Figure 4A, lane 1) and W303 passage 15 cells (Figure 4A, lane 8) for further study. Note that each strain possessed longer telomeres than wild-type W303 or S288C strains. The strains containing elongated ("EL") telomeres were propagated in rich liquid media for 12 hr and then plated on YPD media to allow loss of the plasmid ("−pVL1107"), which was confirmed by lack of growth on media lacking leucine. Fifty colonies containing elongated telomeres ($W303^{EL}$ *est2*Δ *rad52*$\Delta^{-pVL1107}$ and $S288C^{EL}$ *est2*Δ *rad52*$\Delta^{-pVL1107}$) were serially propagated

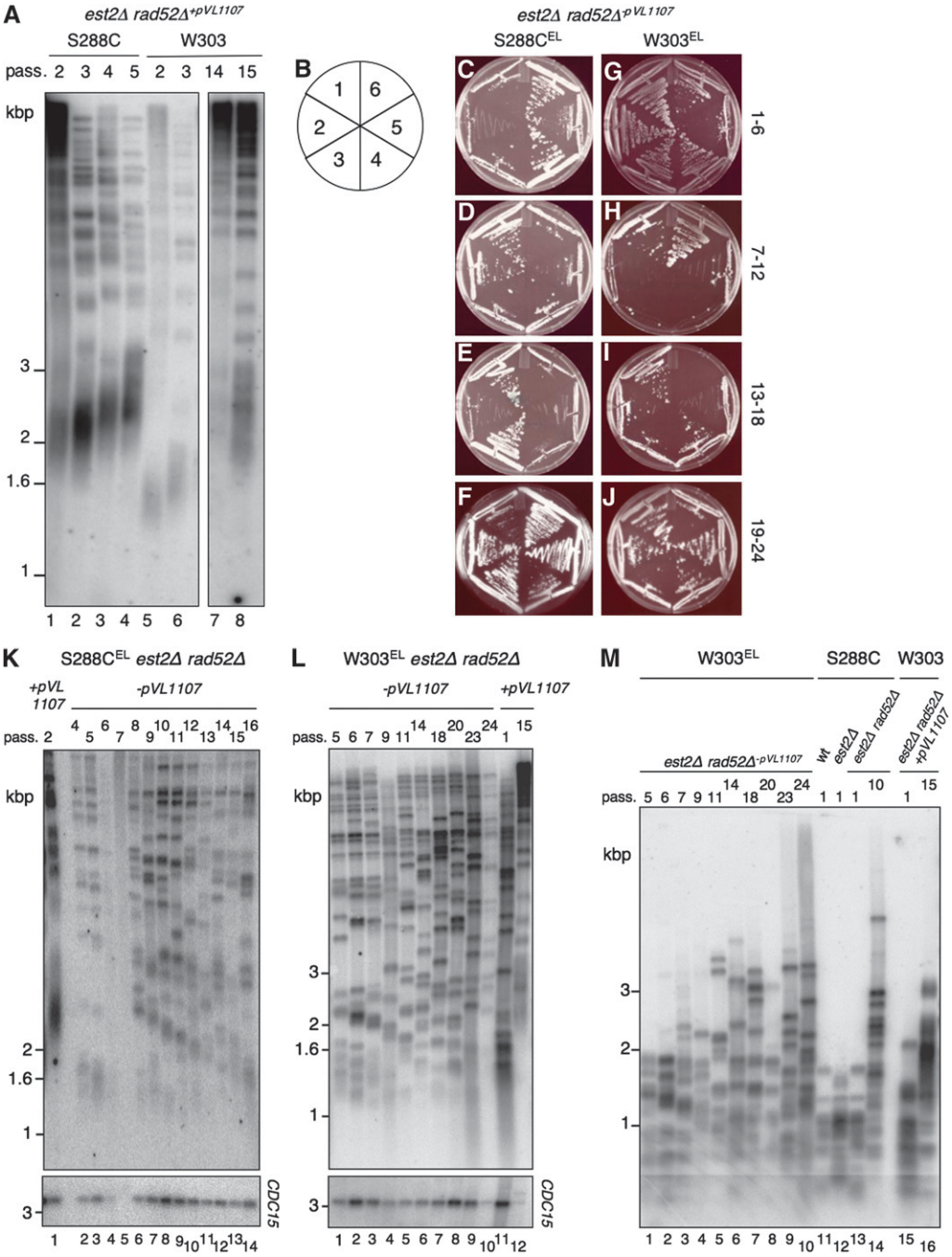

FIGURE 4.—Telomere length correlates with the incidence of escape from senescence. (A) Telomere elongation in S288C *est2*Δ *rad52*Δ and W303 *est2*Δ *rad52*Δ cells containing a plasmid encoding a Cdc13-Est2 fusion protein (pVL1107): telomere Southern blot on genomic DNA from cells containing pVL1107 propagated for several passages (pass.; every 2 days) on SD−LEU plates. Genomic DNA was digested with *Xho*I. The membrane was hybridized to a telomeric probe. All DNA samples were analyzed on one gel; W303 passages 4–13 were omitted from the image. (B) Schematic summary of the senescence assays on plates (see MATERIALS AND METHODS). Summary senescence assays of cells with elongated telomeres (EL) following removal of pVL1107 are shown: S288C^{EL} *est2*Δ *rad52*Δ$^{-pVL1107}$ (C–F) and W303^{EL} *est2*Δ *rad52*Δ$^{-pVL1107}$ (G–J). Telomere Southern blot on genomic DNA isolated from S288C^{EL} *est2*Δ *rad52*Δ$^{-pVL1107}$ (K) or W303^{EL} *est2*Δ *rad52*Δ$^{-pVL1107}$ (L) cells is shown. DNA was digested with *Xho*I and the membrane was hybridized to a telomeric probe. (K) Lane 1, S288C *est2*Δ *rad52*Δ after 2 passages with pVL1107 (passage at which plasmid was removed); lanes 2–14, S288C^{EL} *est2*Δ *rad52*Δ at increasing passages after plasmid loss. Bottom panel: blot was stripped and rehybridized to a *CDC15* probe as a loading control. Note the underrepresentation of *CDC15* in lanes 4 and 5 due to poor growth at these passages. (L) Lanes 11 and 12, W303 *est2*Δ *rad52*Δ after 1 and 15 (passage at which plasmid was removed) passages with pVL1107, respectively. Lanes 1–10, W303^{EL} *est2*Δ *rad52*Δ at increasing passages after plasmid loss. Bottom panel: blot was stripped and rehybridized to a *CDC15* probe as a loading control. The telomeric signal intensity in lanes 10 and 12 should be interpreted in light of the underrepresentation of *CDC15*. (M) Southern blot of genomic DNA digested with a mixture of the restriction endonucleases (*Alu*I, *Hin*fI, *Hae*III, and *Msp*I). The membrane was hybridized to a telomeric probe. Lanes 1–10, W303^{EL} *est2*Δ *rad52*Δ at increasing passages after plasmid loss; lanes 11–13, S288C (wt, *est2*Δ, and *est2*Δ *rad52*Δ, each at passage 1); lane 14, S288C *est2*Δ *rad52*Δ survivor at passage 10; lanes 15 and 16, W303 *est2*Δ *rad52*Δ$^{+pVL1107}$ at passages 1 and 15 (pVL1107 was removed at passage 15). For each panel, marker sizes are indicated at left in kilobase pairs.

on plates every 4 days. Telomere elongation delayed the onset of senescence in both strains (Figure 4, C–J). In S288C^{EL} *est2*Δ *rad52*Δ cells, a higher percentage of colonies sustained growth than prior to telomere elongation (Figure 4, C–F, and Figure S2). Notably, in contrast to W303 without elongated telomeres, telomere elongation in W303 *est2*Δ *rad52*Δ now allowed some cells to sustain growth for >15 passages (Figure 4, G–J, and Figure S2). In addition, the TRF pattern in both strains showed evidence of TG_{1-3} signal amplifica-

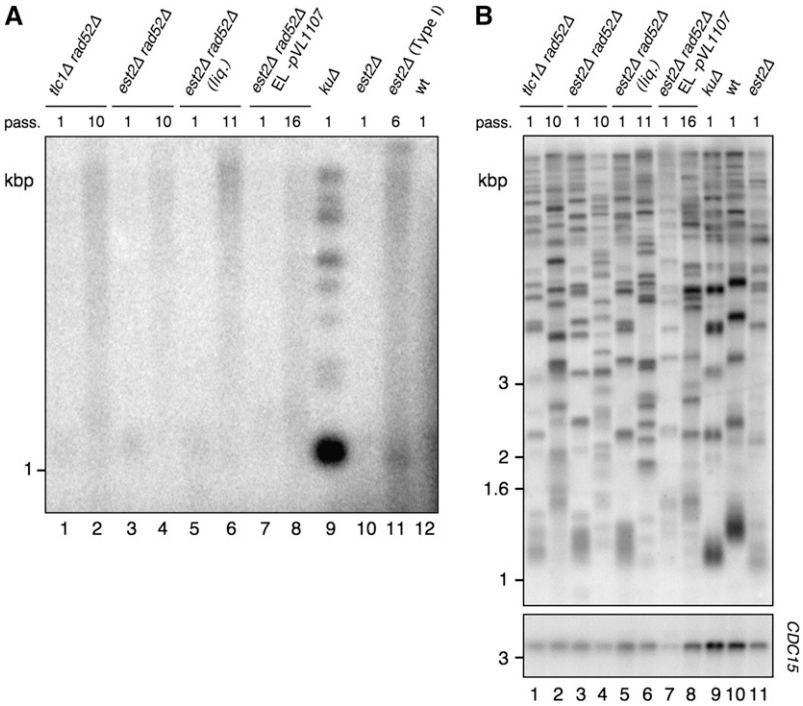

Figure 5.—*RAD52*-independent survivors exhibit an increased G-rich, single-stranded telomere signal. (A) Native in-gel analysis of genomic DNA isolated from cells of the indicated genotype and passage (pass.), grown either on plates or in liquid media (liq.). DNA was digested with *Xho*I and the membrane was hybridized to a radiolabeled C-rich oligonucleotide (5′-CACACCCACACCCACACC-3′). (B) Denaturing Southern blot of the same samples as in A (but not the same gel, as the gel in A was resolved in the presence of radiolabeled probe). DNA was digested with *Xho*I and the membrane was hybridized to the radiolabeled C-rich oligonucleotide. Marker sizes are indicated at left in kilobase pairs. Bottom panel: blot was stripped and rehybridized to a *CDC15* probe as a loading control. Note that the genomic DNA in lane 7 is slightly underrepresented relative to other samples.

tion and resistance to restriction endonuclease digestion (Figure 4, K–M, and Figure S2). The ability of W303 *est2*Δ *rad52*Δ cells with elongated telomeres to sustain growth after many passages is consistent with a *RAD52*-independent telomere maintenance mechanism. These data suggest that telomere length itself may promote survival in S288C and W303.

Single-strand G-rich extensions in S288C *RAD52*-independent survivors: The occurrence of TRF patterns reminiscent of telomeric DNA recombination (type II survival) suggested that *RAD52*-independent survivors might similarly possess tracts of single-stranded, G-rich telomere DNA. Native in-gel analysis of *RAD52*-independent survivors revealed the presence of ssDNA capable of hybridization to a ^{32}P-labeled CA-rich oligonucleotide that was not observed in the same strains prior to escape from senescence (Figure 5A, compare lanes 1 and 2, 3 and 4, etc.) or when incubated with a ^{32}P-labeled GT-rich oligonucleotide (*e.g.*, to detect C-rich ssDNA; data not shown). As controls, S288C *est2*Δ type I survivors (exhibiting Y′ amplification) and *ku70*Δ cells exhibited distinct G-rich ssDNA patterns typical for these strain backgrounds (Figure 5A and data not shown) (Gravel *et al.* 1998; Polotnianka *et al.* 1998). However, unlike *ku70*Δ cells, whose ssDNA signal is sensitive to *Escherichia coli* Exonuclease I (ExoI) and thus specific to the telomere 3′ terminus (Wellinger *et al.* 1993), the ssDNA signal observed in late passage *tlc1*Δ *rad52*Δ or *est2*Δ *rad52*Δ cells was ExoI-resistant (Figure S3). The presence of DNA in all lanes (including those lacking an overhang signal) was confirmed by Southern blotting of the corresponding denatured samples (Figure 5B and Figure S3; note that A and B represent different gels). Thus, S288C *RAD52*-independent survivors possessed a G-rich, telomeric ssDNA signal that differed in appearance and nature from the ssDNA signal observed in other types of telomerase-independent survivors and *ku70*Δ cells.

The *RAD52*-independent survivor phenotype shows complex penetrance in diploids: Makovets *et al.* (2008) recently demonstrated that *RAD52*-dependent (type I or II) survival in diploids created by mating two telomerase-deficient haploids exhibited dominance over senescence, and the ability of haploid progeny to survive could be inherited in a non-Mendelian (*i.e.*, multigenic) manner. To examine the viability of various diploids created by mating *RAD52*-independent survivor haploids, we created three different S288C diploid strains: (1) two S288C *est2*Δ *rad52*Δ survivors mated together (survivor × survivor), (2) an *est2*Δ *rad52*Δ survivor mated to a presenescent *est2*Δ *rad52*Δ colony (freshly isolated from a heterozygous diploid) (survivor × presenescent), and (3) two presenescent *est2*Δ *rad52*Δ colonies mated together (presenescent × presenescent). We examined the survival of each diploid strain upon serial propagation of 50 isolated colonies. If the ability to generate *RAD52*-independent survivors were the result of a single dominant, extragenic suppressor mutation, then all diploids of a "survivor × presenescent" cross would be expected to survive. If the suppressor were recessive, then no survivor × presenescent strains should survive. None of the diploids obtained by mating two presenescent *est2*Δ *rad52*Δ colonies (by analyzing 50 independent diploids on plates or by inoculating 30,000 diploid cells in liquid culture) emerged as *RAD52*-independent survivors (Figure 6B, panels 7 and 8, and Figure S4). However, diploids obtained by mating a *RAD52*-independent survi-

vor with a presenescent *est2*Δ *rad52*Δ colony exhibited an ~50% incidence of prolonged survival, with some colonies undergoing senescence (Figure 6B, panel 4) and others sustaining growth (Figure 6B, panels 5 and 6, and Figure S4). Finally, all diploids generated by mating two *RAD52*-independent haploid survivors continued to grow beyond 11 passages (Figure 6B, panels 1–3, and Figure S4). These *RAD52*-independent survivors also exhibited a TRF pattern indicative of telomeric tract recombination (Figure 6, C and D). Therefore, unlike Makovets *et al.*, who observed dominance of type I or type II survival upon mating a *RAD52*-independent survivor to a presenescent haploid, the viability of diploids created by mating a *RAD52*-independent survivor to a presenescent haploid was neither dominant nor recessive. The *RAD52*-independent survivor phenotype thus suggests complex multigenic mechanisms that may share similarities with other survivor phenotypes (Zubko and Lydall 2006; Makovets *et al.* 2008).

Possible genes or pathways involved in the generation of *RAD52*-independent survivors: Genes involved in the generation of *RAD52*-dependent survivors include *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *SGS1*, *XRS2*, and *MRE11* (Le *et al.* 1999; Teng and Zakian 1999; Chen *et al.* 2001). To examine the possibility that *RAD52*-independent survivors might nonetheless depend on the function of one of these genes, we constructed triple-mutant haploid strains (*est2*Δ *rad52*Δ *xxx*Δ). Deletion of any of these genes did not affect the incidence of *RAD52*-independent survivors in W303 *est2*Δ *rad52*Δ or S288C *est2*Δ *rad52*Δ backgrounds ($n = 50$ for each strain) (Table 1). *RAD5*, which is nonfunctional in W303 (Fan *et al.* 1996) and regulates strain-specific responses to DNA damage (Demogines *et al.* 2008), has been implicated in tolerance to DNA damage and replication fork reversal (Blastyak *et al.* 2007; Klein 2007). Thus, we examined the influence of *RAD5* upon the generation and maintenance of *RAD52*-independent survivors in W303 compared to S288C. Deletion of *RAD5* in S288C, either before or after the establishment of *RAD52*-independent survivors, had no influence on the frequency of viable *est2*Δ *rad52*Δ colonies ($P > 0.05$, data not shown). In addition, the presence of wild-type *RAD5* in W303 did not permit the generation of *RAD52*-independent survivors (L. Maringele, unpublished data).

We also examined candidate genes involved in the regulation of chromatin structure or DNA replication (*DNL4*, *GCN5*, *HTA1*, *HTA2*, *POL32*, and *SIR2*) for a potential role in the generation of *RAD52*-independent survivors and analyzed all eight possible haploid genotypes from the appropriate triple-heterozygous diploids (Table 1 and Figure 7B). With the exception of *POL32*, deletion of these genes did not alter the viability or incidence of *RAD52*-independent survivors in *est2*Δ *rad52*Δ *xxx*Δ strains ($n = 50$ for each strain).

The polymerase-δ subunit *POL32* has been implicated in break-induced replication and telomerase-independent telomere maintenance (Lydeard *et al.* 2007), and *pol32*Δ strains exhibit TRFs slightly longer than in wild-type strains (Askree *et al.* 2004; Gatbonton *et al.* 2006). *POL32* exhibited a synthetic genetic interaction with *RAD52* since both *rad52*Δ *pol32*Δ and *est2*Δ *rad52*Δ *pol32*Δ colonies remained small after microdissection (data not shown). However, when serially passaged on plates, *rad52*Δ *pol32*Δ colonies gained growth potential and became viable to a similar extent as other colonies, whereas *est2*Δ *rad52*Δ *pol32*Δ microcolonies were unable to sustain growth (Figure 7B).

Since this outcome did not allow us to assess a role for *POL32* in the emergence of *RAD52*-independent survivors, we examined whether *POL32* is required for the continued viability of *RAD52*-independent survivors. Diploids created by crossing an established *RAD52*-independent haploid survivor (*tlc1*Δ *rad52*Δ, passage 9) with a *pol32*Δ single-mutant haploid were sporulated and tested for viability. The resulting *tlc1*Δ *rad52*Δ *pol32*Δ haploids exhibited heterogeneous colony sizes (data not shown). Serial propagation was possible in *tlc1*Δ *rad52*Δ *pol32*Δ cells arising from a colony with an initial size comparable to a *tlc1*Δ *rad52*Δ colony, whereas *tlc1*Δ *rad52*Δ *pol32*Δ cells arising from a microcolony failed to sustain growth (Figure 7C and data not shown). TRF analysis of viable *tlc1*Δ *rad52*Δ *pol32*Δ cells revealed a telomeric DNA pattern reminiscent of telomere tract recombination and "type II" survivors (Figure 7D). We were unable to obtain viable *est2*Δ *rad52*Δ *pol32*Δ haploids using the same mating procedure (0/100 colonies; data not shown). Taken together, these data suggest that *POL32* is not strictly required for the maintenance of the *RAD52*-independent survivor phenotype. The synthetic lethal interaction of *RAD52*, *POL32*, and *EST2* or *TLC1* did not allow us to determine whether *POL32* may be important for the emergence of *RAD52*-independent survivors.

DISCUSSION

The generation of survivors in telomerase-negative yeast has been well described in the literature. In all cases, the requisite pathways were *RAD52* dependent with the exception of PAL survivors, which can be generated only in cells lacking *EXO1* or *SGS1* (Lundblad and Blackburn 1993; Le *et al.* 1999; Teng and Zakian 1999; Teng *et al.* 2000; Chen *et al.* 2001; Maringele and Lydall 2004; Lee *et al.* 2008). In this study, we describe an ability of *S. cerevisiae* to escape senescence in the absence of both telomerase and *RAD52*. We speculate that the phenomenon had not been characterized fully until now because of its rarity and because most studies have been carried out in W303 or other strain backgrounds in which the survivors would normally be *RAD52* dependent.

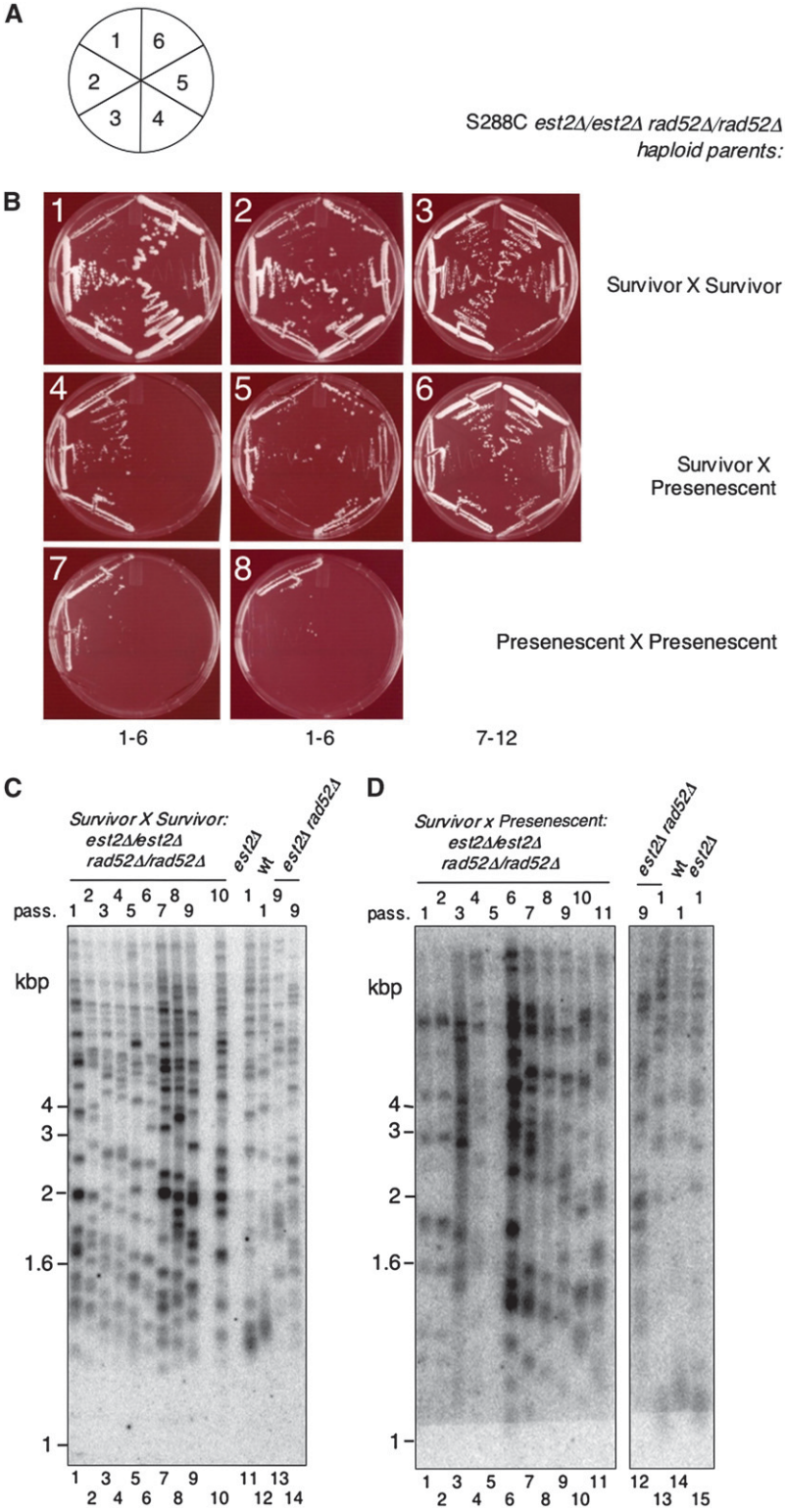

FIGURE 6.—*RAD52*-independent survival in diploids generated from *RAD52*-independent haploid survivors. Freshly dissected S288C *est2*Δ *rad52*Δ haploids ("presenescent") and S288C *est2*Δ *rad52*Δ haploid survivors ("survivor") were mated to generate *est2*Δ*::est2*Δ *rad52*Δ*::rad52*Δ diploid strains. (A) Schematic of the senescence assays on plates, from passage 1 to 6 (see MATERIALS AND METHODS). (B) Summary senescence assays of representative diploids grown on plates for the indicated number of passages. B1 and B2, two representative viable diploids from a survivor × survivor cross (passages 1–6), which all survive later passages (*e.g.*, B2–B3). B4 and B5, representative diploids from a survivor × presenescent cross, some of which do not sustain growth beyond 3 passages (*e.g.*, B4), while others remain viable beyond 12 passages (*e.g.*, B5–B6). B7 and B8, two representative diploids from presenescent × presenescent crosses (no diploids sustain growth; therefore there are no data for passages 7–12). See also Figure S4. (C) Telomere Southern blot of genomic DNA. Two S288C *est2*Δ *rad52*Δ haploid survivors at passage 9 (lanes 13 and 14) were mated to produce an *est2::NAT/est2::NAT rad52::URA3/rad52::KAN* diploid (lanes 1–10, at indicated passages). Lanes 11 and 12, S288C *est2*Δ and wild-type haploids. DNA was digested with *Xho*I and the membrane was hybridized to a telomeric probe. (D) Telomere Southern blot of genomic DNA from a "survivor × presenescent" diploid that escaped senescence. An S288C *est2*Δ *rad52*Δ haploid survivor (passage 9; lane 12) was mated to a presenescent *est2*Δ *rad52*Δ haploid (passage 1; lane 13) to produce an *est2::NAT/est2::NAT rad52::URA3/rad52::KAN* diploid (survivor × presenescent) (lanes 1–11, at indicated passages). Lanes 14 and 15, S288C haploids at passage 1 (wt, *est2*Δ). DNA was digested with *Xho*I and the membrane was hybridized to a telomeric probe. All samples were analyzed on one gel; lanes between 11 and 12 were omitted. For each panel, marker sizes are indicated at left in kilobase pairs.

RAD52-independent survival in diploids behaved as a multigenic trait, similar to other *RAD52*-dependent survival phenotypes (ZUBKO and LYDALL 2006; MAKOVETS *et al.* 2008); however, targeted deletion of genes required for survival in the absence of telomerase and the presence of *RAD52* had no effect on *RAD52*-independent survival. Together with previous studies, which suggest that *RAD52*-dependent survivors do not absolutely require Cdc13 (LARRIVEE and WELLINGER 2006; PETREACA *et al.* 2006; ZUBKO and LYDALL 2006), our study bolsters the emerging evidence that alternate strategies exist to cap telomeres and permit cell viability even in the absence of telomerase and *RAD52*.

TABLE 1

The effect of a third gene deletion on the percentage of colonies that escape senescence in *est2*Δ *rad52*Δ strains

S288C (% survival)					W303 (% survival)		
	Recombination:		Chromatin/replication:			Recombination:	
*est2*Δ *rad52*Δ	*est2*Δ *rad52*Δ *xxx*Δ		*est2*Δ *rad52*Δ *xxx*Δ		*est2*Δ *rad52*Δ	*est2*Δ *rad52*Δ *xxx*Δ	
4–10	6	*rad50*	2	*dnl4*	0	0	*rad50*
	4	*rad51*	4	*gcn5*		0	*rad51*
	6	*rad54*	4	*hta1*		0	*rad54*
	4	*rad57*	4	*hta2*		0	*rad57*
	4	*rad59*	[a]	*pol32*		0	*rad59*
	4	*sgs1*	4	*sir2*		0	*sgs1*
	4	*xrs2*				0	*xrs2*

The percentage of *RAD52*-independent survivors recovered from each genotype is indicated. Diploids of the appropriate genotype were generated by deletion of the locus into diploid cells or mating of appropriate haploids, followed by sporulation, microdissection, and selection of the indicated haploids (*est2*Δ *rad52*Δ *xxx*Δ). The majority of S288C *est2*Δ *rad52*Δ and *est2*Δ *rad52*Δ *xxx*Δ colonies ceased proliferation after ~60 generations.

[a] The S288C *est2*Δ *rad52*Δ *pol32*Δ haploids that could not be propagated from the microdissection plate (20 generations). The same genes were deleted in W303 *est2*::*NAT/EST2 rad52*::*URA3/RAD52* diploids. No W303 *est2*Δ *rad52*Δ or *est2*Δ *rad52*Δ *xxx*Δ survivors emerged, and cell populations entered senescence at 40–60 generations.

RAD52-independent survivors exhibited a telomere DNA pattern indicative of amplification of telomeric repeats (*i.e.*, type II). We did not observe TRF properties consistent with amplification of subtelomeric DNA (Y′) or formation of terminal palindromes (Le *et al.* 1999; Chen *et al.* 2001; Maringele and Lydall 2004). It is possible that the TRF pattern in *RAD52*-independent survivors might comprise subtle mixtures of other TRF types or may represent a novel type of telomeric DNA amplification. One distinction between the TRF patterns of *RAD52*-independent and *RAD52*-dependent survivors was the apparent 3′–5′ exonuclease insensitivity of the G-rich ssDNA in the former, indicating that the G-rich signal is not a free 3′ overhang (Figure S3). This

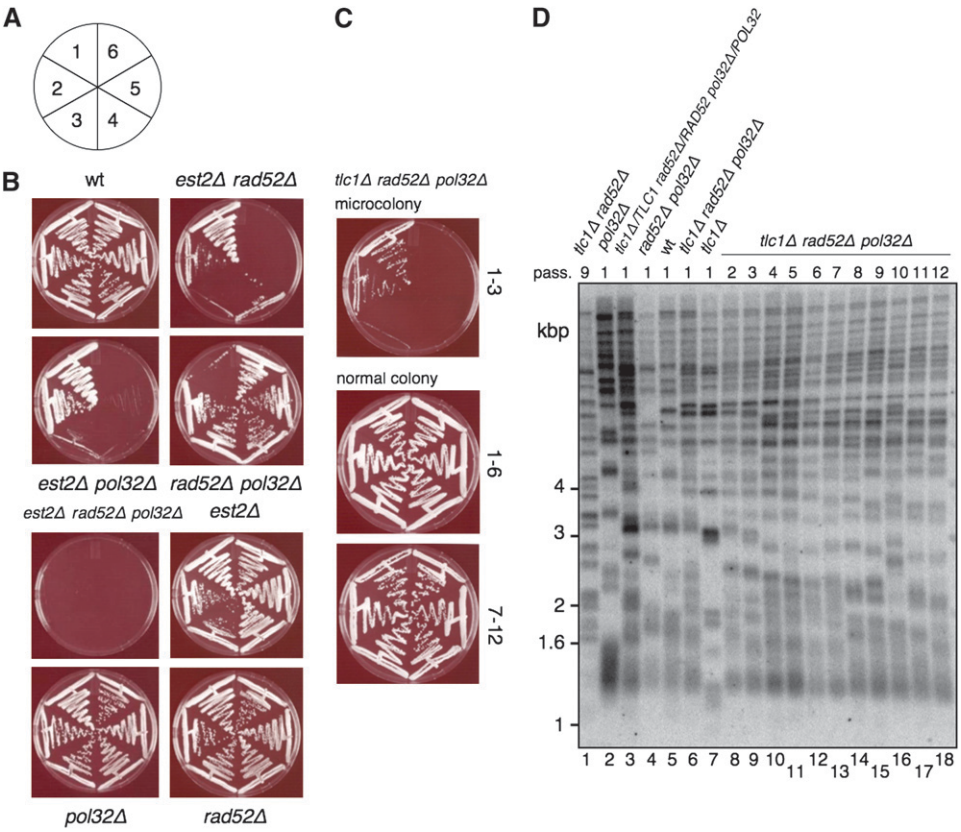

Figure 7.—Investigation of the potential role of *POL32* in *RAD52*-independent survival. (A) Schematic of the senescence assays on plates, from passages 1 to 6 (see materials and methods). (B) Summary senescence assays of the indicated genotype obtained from sporulation and microdissection of the diploid S288C *est2*::*NAT/EST2 rad52*::*URA3/RAD52 pol32*::*KAN/POL32*. (C) Summary plates of the senescence of *tlc1*Δ *rad52*Δ *pol32*Δ haploids obtained from the diploid generated by mating an S288C *tlc1*Δ *rad52*Δ survivor (at passage 9) to a haploid *pol32*Δ colony. Microcolonies could not be propagated for >3 passages (*e.g.*, top panel), whereas colonies of regular size could be propagated for at least 12 passages (middle and bottom panels). (D) Telomere Southern blot of DNA of a *tlc1*Δ *rad52*Δ *pol32*Δ viable haploid colony (lanes 6 and 8–18). This haploid was obtained from a diploid (lane 3) generated by mating an S288C *tlc1*Δ *rad52*Δ survivor (passage 9; lane 1) to a *pol32*Δ haploid (lane 2). Lanes 4–7, haploids derived from the heterozygous diploid in lane 3. DNA was digested with *Xho*I and the membrane was hybridized to a telomeric probe. Marker sizes are indicated at left in kilobase pairs.

ssDNA signal is specific to the G strand and is detected only after the emergence of survivors. Further analysis is required to determine the precise nature of the ssDNA; one possibility could be the presence of telomeric DNA circles (Larrivee and Wellinger 2006) or ssDNA regions within the telomeric DNA that might promote signal-strand annealing and recombination (reviewed in Lyndaker and Alani 2009).

Repetitive noncoding DNA, specialized proteins, and capping structures are important features of telomere integrity and maintenance in all species. Changes in the structure of telomeric DNA (facilitated by increased length) might be important for the generation of *RAD52*-independent survivors. A longer telomeric "seed" sequence, especially during early generations after telomerase loss, may facilitate survival through the creation of telomeric circles via intratelomeric recombination. The generation of telomeric circles, particularly in cells possessing long telomeres, has been documented in both yeast and humans (Bucholc *et al.* 2001; Wang *et al.* 2004; Lin *et al.* 2005; Muntoni and Reddel 2005; Williams *et al.* 2005; Pickett *et al.* 2009). For example, in *Kluyveromyces lactis* the roll-and-spread mechanism of DNA synthesis in telomerase-deficient and telomerase template-mutated strains does not absolutely require *RAD52* (McEachern and Blackburn 1996). It is possible that the G-rich telomeric ssDNA in *RAD52*-independent survivors (Figure 5, Figure S3) could be excised as a telomeric DNA circle, which could be integrated at a shorter telomere or extend a telomere via a similar rolling-circle mechanism. Elongated telomeres could then be used as a template for intertelomeric BIR to lengthen other telomeres (Natarajan and McEachern 2002; McEachern and Haber 2006). Similar events might explain the abrupt emergence of *RAD52*-dependent survivors harboring very long telomeric tracts in *S. cerevisiae* (Teng *et al.* 2000). Relatively few long terminal telomeric extensions might be sufficient to initiate this sequence of events.

If *RAD52*-independent survival were reliant on a roll-and-spread mechanism, telomere maintenance would presumably require the DNA replication machinery. Payen *et al.* (2008) suggest the involvement of Pol32, a nonessential subunit of DNA polymerase-δ, in segmental duplication promoting genomic instability through a *RAD52*-independent mechanism of template switching between microsatellites or microhomologous sequences. This new mechanism, named microhomology/microsatellite-induced replication (MMIR), differs from the known DNA double-strand repair pathways and occurs in the absence of homologous recombination and nonhomologous end-joining machineries (Payen *et al.* 2008). Although the synthetic lethality of the *est2*Δ *rad52*Δ *pol32*Δ and *tlc1*Δ *rad52*Δ *pol32*Δ gene deletions in S288C is notable, it did not allow us to test the requirement of *POL32* for the generation of *RAD52*-independent survivors. Our results nonetheless indicate that the Pol32 protein is not required for viability once a *RAD52*-independent survivor has been established. We have not ruled out that a subset of the population may require Pol32 when telomeres become critically short. Indeed, it was suggested that in the absence of mismatches between repeated sequences, not all segmental duplications require Pol32 (Payen *et al.* 2008). These Pol32-independent segmental duplications likely result from unequal crossing over between repeated sequences, as would be possible at yeast telomeres. Lundblad and colleagues also found that deletion of genes important for mismatch repair (*MSH2*, *MLH1*, *PMS1*) promotes the generation of survivors in the absence of telomerase (Rizki and Lundblad 2001).

The incidence of survival in diploids created by mating *RAD52*-independent survivors suggests a multigenic and potentially epigenetic pattern of inheritance. For example, the nearly 50% incidence of escape from senescence in diploids created by mating a *RAD52*-independent survivor with a presenescent *est2*Δ *rad52*Δ population argues against a simple recessive or dominant extragenic suppressor mutation. Interestingly, the incidence of escape from senescence in diploids created by mating two *est2*Δ *rad52*Δ haploid strains is less frequent than in an *est2*Δ *rad52*Δ haploid (Figure 6 and data not shown). Diploidy also reduces the incidence of *RAD52*-dependent (type II) survivors (Liti and Louis 2003).

When *RAD52*-independent survivors were propagated continuously on plates, not all colonies survived indefinitely. These observations suggest that the mechanism leading to telomere maintenance and escape from senescence is the exception and not the rule. Over time, the population is able to maintain telomeres and bypass senescence even without telomerase and *RAD52*. Like many cellular processes, there is an overriding selection for cell survival by whatever means possible. The fact that a means to survive exists in the absence of *RAD52* and telomerase suggests that multiple, redundant pathways have evolved to ensure telomere homeostasis even under the most extreme conditions. Uncovering the genetic pathways that allow telomerase- and homologous recombination-independent mechanisms of telomere maintenance should further our understanding of how some human tumors are able to bypass the reacquisition of telomerase activity during tumorigenesis (Muntoni and Reddel 2005; Muntoni *et al.* 2009).

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Note added in revision: During the revision of this manuscript, Grandin and Charbonneau (N. Grandin and M. Charbonneau, 2009, Telomerase- and Rad52-independent immortalization of budding yeast by an inherited-long-telomere pathway of telomeric repeat amplification. Mol. Cell. Biol. **29:** 965–985) showed that telomerase-deficient cells with long telomeres (*e.g.*, type II survivors) could survive the subsequent deletion of *RAD52*. They employed a different strain background (BF264a-15D), which could not survive the simultaneous deletion of telomerase and *RAD52*. These cell populations, referred to as interlengthening of telomeres (ILT), also exhibited amplification of telomeric repeats, similar to type II survivors. Dissimilar to our findings, ILT survivors relied on *MRE11* and *RAD50* for survival, and lengthening of telomeres by a different means (*rif2*Δ or introduction of a Cdc13-Est1 fusion protein) failed to promote survival. Thus, multiple mechanisms exist for *RAD52*-independent survival in the absence of telomerase, whose gene dependence may reflect the context in which longer telomeres are introduced.

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Supporting Information

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Telomere Maintenance and Survival in *Saccharomyces cerevisiae* in the Absence of Telomerase and *RAD52*

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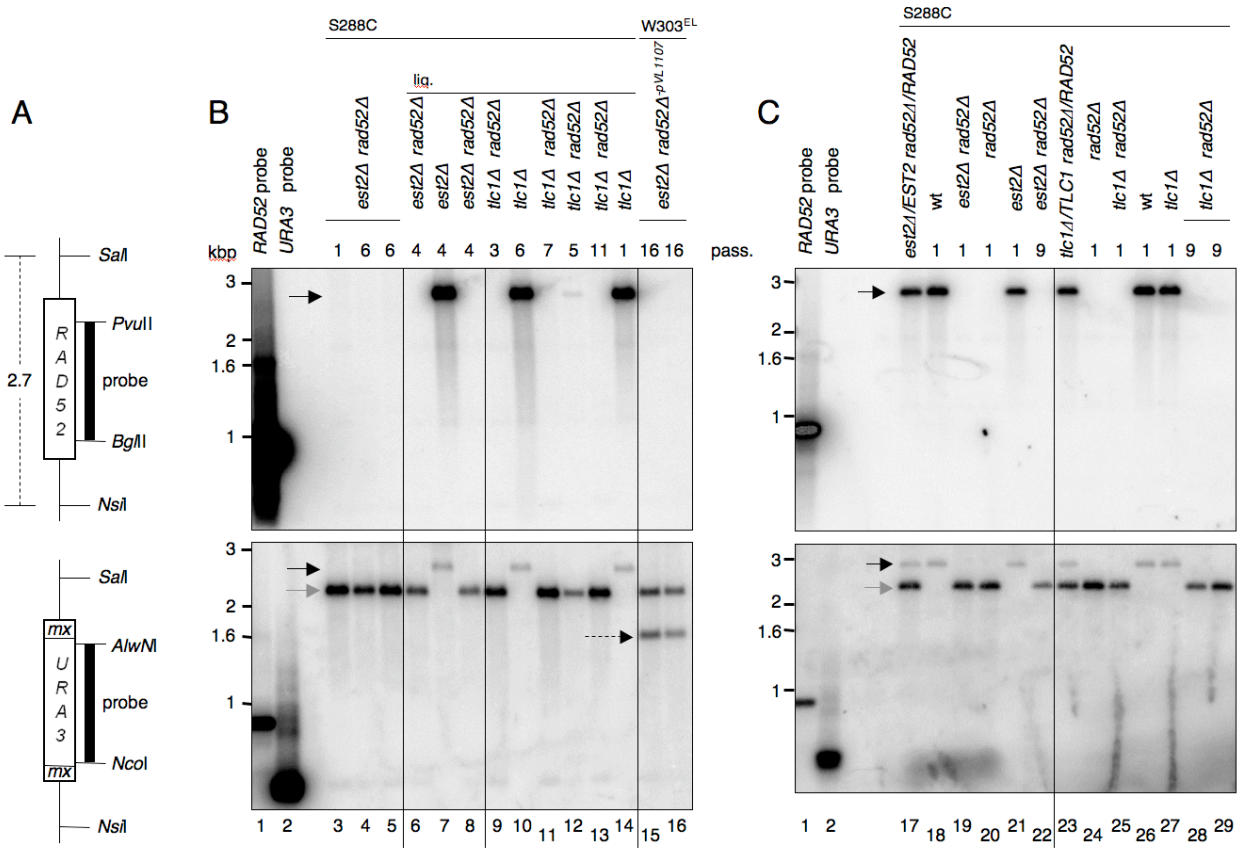

FIGURE S1.— Confirmation of *RAD52* deletion in *rad52Δ* strains. A) Upper: Schematic of the *RAD52* locus. Lower: Schematic of the *RAD52* locus disrupted by *URA3*. B-C) Genomic DNA was digested with *Nsi*I/*Sal*I to generate a 2.7 kbp fragment containing the *RAD52*/YML032C open reading frame. DNA was transferred onto a nylon membrane by Southern blotting and hybridized to a purified labeled probe generated by PCR-amplification of the *Bgl*II/*Pvu*II fragment of the *RAD52* locus (upper). The black arrow corresponds to the 2.7 kbp fragment (wt *RAD52* locus). The membrane was stripped and hybridized to a purified labeled probe generated by PCR-amplification of the *Nco*I/*Alw*NI fragment of the *URA3* disruption cassette (lower). The grey arrow corresponds to hybridization of the probe to the *rad52::URA3* locus. The dashed black arrow corresponds to hybridization of the probe to the *ura3-1* locus in the W303 strain. The black arrow (lower) corresponds to *RAD52* probe remaining on the membrane from the previous hybridization. Lane 1, *RAD52* PCR fragment; lane 2, *URA3* PCR fragment. Marker sizes are indicated at left in kbp. S288C or W303EL haploids were passaged on plates or in liquid (liq.) culture for the indicated number of passages (pass.) Lanes 4, 5, 11, 13, 15, 16, 22, 28, 29 represent long-term survivors. Lanes 17, 23 show the parental heterozygous diploids of haploids shown in lanes 18-22 and 23-29, respectively.

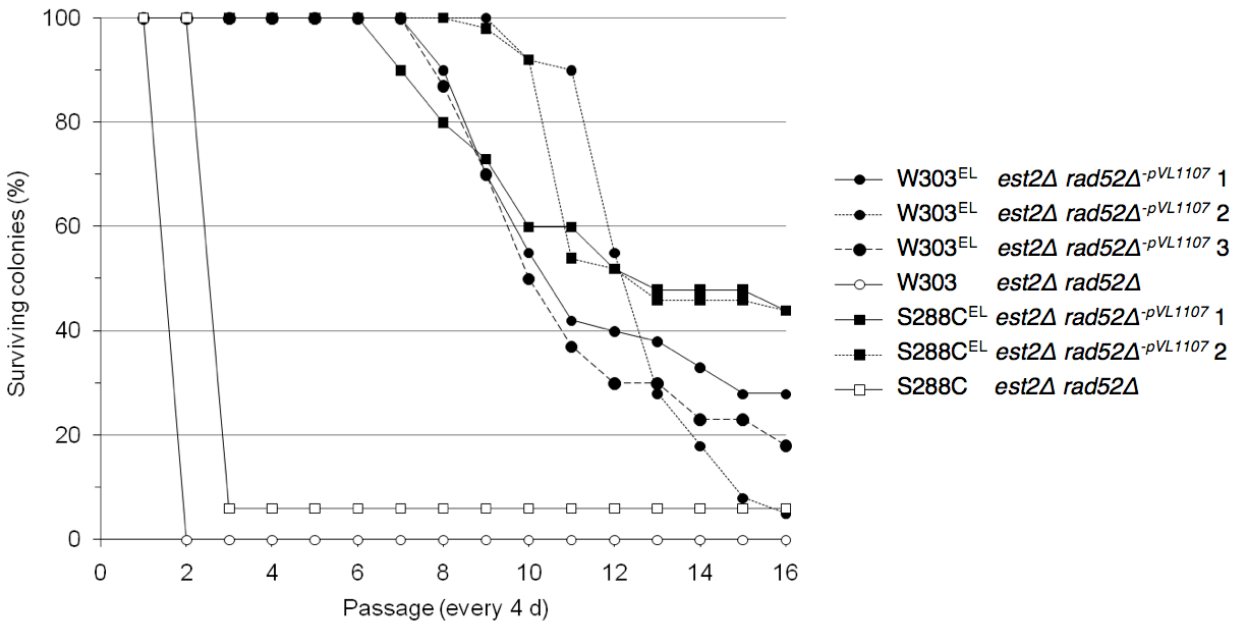

FIGURE S2.—Telomere elongation increases the incidence of long-term survival in W303 *est2Δ rad52Δ* and S288C *est2Δ rad52Δ* strains. W303 *est2Δ rad52Δ* and S288C *est2Δ rad52Δ* colonies were transformed with the plasmid pVL1107 to elongate telomeres ("EL"; Figure 4A), after which time the plasmid was removed ("-pVL1107") and loss of plasmid was confirmed. Fifty colonies of each background were serially streaked on YPD plates. Y-axis represents the percentage of colonies surviving at each passage after loss of the plasmid.

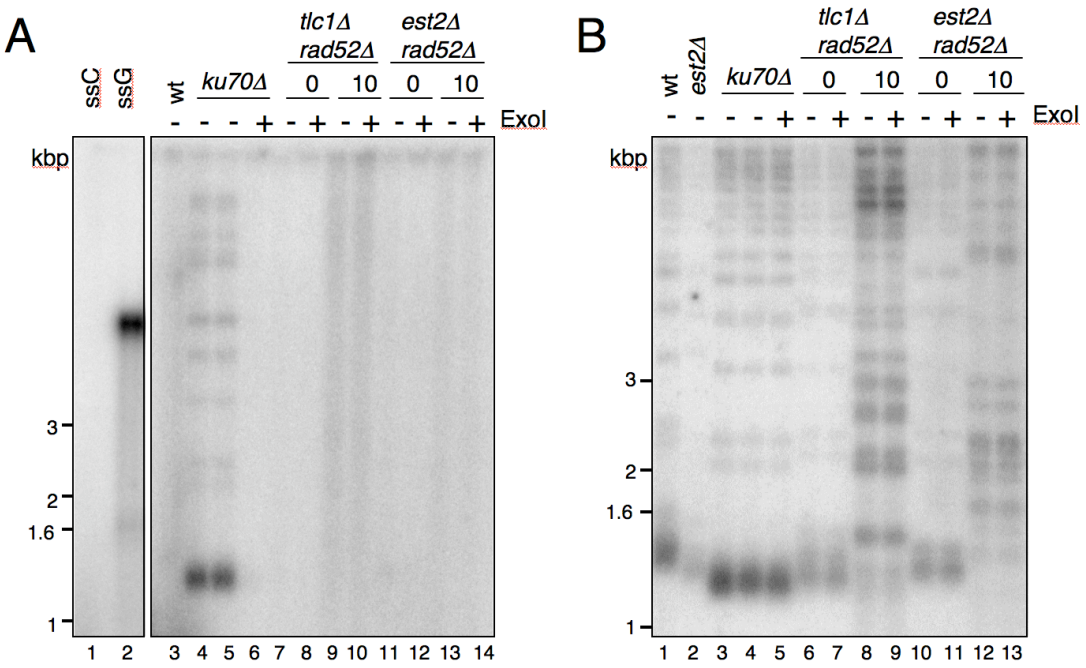

FIGURE S3.—Single-stranded G-rich DNA in *RAD52*-independent survivors is insensitive to *E. coli* Exonuclease I. (A) Native in-gel analysis of genomic DNA as indicated, digested with *Xho*I, and incubated with a radiolabelled CA-rich oligonucleotide prior to electrophoresis. Lanes 1, 2, C-rich and G-rich ssDNA controls, respectively; lanes 3-14, genomic DNA of the indicated genotype and passage (0 or 10, respectively), untreated (-) or treated (+) with ExoI prior to *Xho*I digestion. (B) DNA processed as in A, including a genomic DNA sample from early passage *est2D* cells (lane 2), was resolved on a separate agarose gel, transferred to nylon membrane, denatured, and probed with the same CA-rich probe as in A. At left, DNA markers in kilobase pairs (kbp).

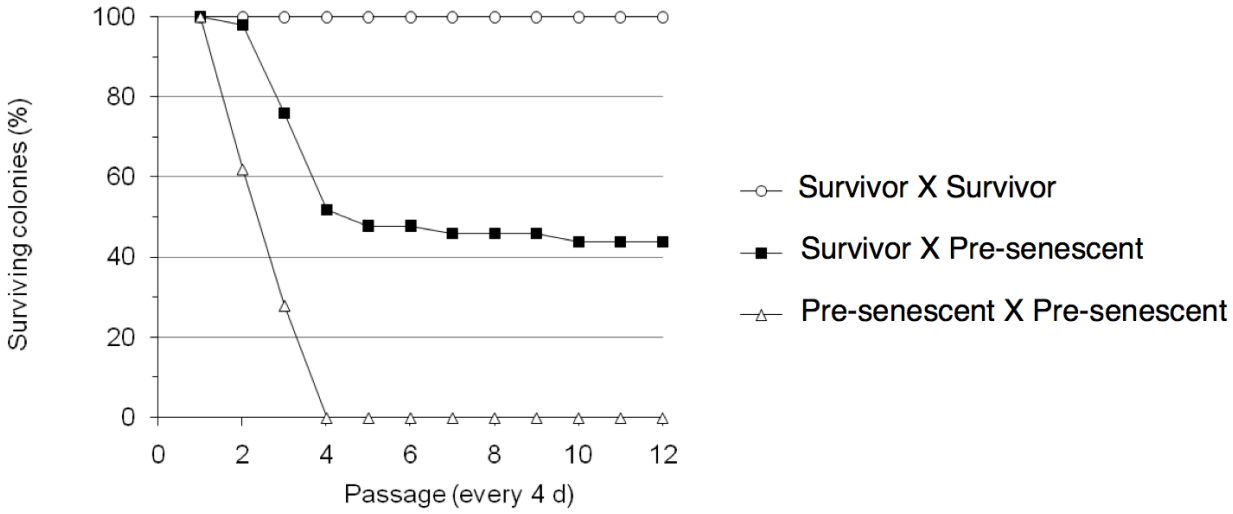

FIGURE S4.—In diploids, the *RAD52*-independent survivor phenotype appears as a complex, multi-genic trait. S288C *est2Δ rad52Δ* survivors (Survivors) or freshly dissected tetrads (Pre-senescent) were mated to generate *est2Δ/est2Δ rad52Δ/rad52* diploids. After confirmation of ploidy and genotype, 50 colonies of each diploid strain were serially propagated on YPD plates and monitored for survival.