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 by-pass 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 this survival segregated as a multi-epigenic trait. The polymerase delta 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 by-pass 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*.
INTRODUCTION

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 *S. cerevisiae* chromosomes is composed of approximately 350-500 bp of degenerate repeat sequences [abbreviated (TG\textsubscript{1-3})\textsubscript{n}] (WALMSLEY et al. 1984), with a G-rich single stranded 3’-extension that varies in length during the cell cycle (LARRIVEE et al. 2004; WELLINGER et al. 1993). 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; CHAN and TYE 1983b). Tandem arrays of up to four Y’ elements are embedded between telomeric TG\textsubscript{1-3} repeat tracts on half to two-thirds of yeast telomeres. The Y’ element contains a unique XhoI recognition site; digestion of genomic DNA with this enzyme yields a characteristic terminal restriction fragment (TRF) of approximately 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\textsubscript{1-3} repeats. Together, these regions contribute to the stability, replication, and maintenance of yeast telomeres.

Due to the semi-conservative nature of DNA replication, telomeres shorten with each replication cycle [as first predicted by (OLOVNIKOV 1973; WATSON 1972)]. 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 non-proliferative state termed senescence (LENDVAY et al. 1996; LUNDBLAD and BLACKBURN 1993; LUNDBLAD and SZOSTAK 1989).
Deletion of any of the genes encoding telomerase subunits leads to an ever shorter telomere (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 (CHEN et al. 2001; LE et al. 1999; LUNDBLAD and BLACKBURN 1993; TENG and ZAKIAN 1999). These 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 (CHEN et al. 2001; LE et al. 1999; LUNDBLAD and BLACKBURN 1993; TENG and ZAKIAN 1999). In type I survivors, telomere maintenance involves amplification of subtelomeric Y' sequences and acquisition of Y' sequences on all telomeres (LE et al. 1999; LUNDBLAD and BLACKBURN 1993), and survival depends on Rad51, Rad52, Rad54, Rad55 and Rad57 (LE et al. 1999; TENG and ZAKIAN 1999). The telomeric DNA exhibits a characteristic XhoI terminal restriction fragment (TRF) distribution, although of a smaller size than in wild type cells due to shorter TG₁₋₃ 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 pulse field gel electrophoresis (LITI and LOUIS 2003). In type II survivors, telomeres are maintained by TG₁₋₃ amplification (LE et al. 1999; LUNDBLAD and BLACKBURN 1993; TENG et al. 2000; TENG and ZAKIAN 1999), which depends on Rad52, the MRX complex (Mre11, Rad50 and Xrs2), Sgs1 and Rad59 (CHEN et al. 2001; HUANG et al. 2001; JOHNSON et al. 2001; LE et al. 1999; TENG et al. 2000; TSUKAMOTO et al. 2001). When digested with XhoI, 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) which 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 $10^4$ cells/generation or less 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. used a mating-based analysis to demonstrate that a ‘survivor’ haploid could exert dominance over a senescent haploid (Makovets et al. 2008). 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°C segregates as a multi-genic 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 EXOI was also absent (Maringele and Lydall 2004). Chromosomes in these 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).

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 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 *est2Δ rad52Δ* 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 the phenotype appeared to segregate as a multi-meric trait.

**MATERIALS AND METHODS**

**Yeast strains.** W303 MATα and MATa strains were obtained from M. Tyers (MT234, MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 URA3 GAL+ psi+ ssd1-d2 rad5-535 and MT235, MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3 GAL+ psi+ ssd1-d2 rad5-535). S288C MATa and MATα strains were obtained from M. Tyers (BY4741, MATα his3Δ leu2Δ met15Δ ura3Δ and BY4742, MATα his3Δ leu2Δ lys2Δ ura3Δ).

**Yeast Manipulations.** Replacement of the Kan' cassette for selection of ORF deletion strains was performed in either haploid or diploid cells using a PCR-based replacement protocol as described in (LONGTINE et al. 1998) and (BRACHMANN 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 tlc$\Delta$ 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$\Delta$/EST2 rad52$\Delta$/RAD52 or tlc1$\Delta$/TLC1 rad52$\Delta$/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 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 one) and propagated on YPD plates. After incubation for four days at 30°C, single colonies were picked from the plate (passage two) and re-propagated on YPD plates to obtain passage three, et cetera. This procedure was performed for a total of six serial propagations (the equivalent of 140 generations). Plates of each passage were stored at 4°C 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, 7): an isolated colony from each of the individual propagation plates was re-propagated onto a sector of a single YPD plate and incubated at 30°C for four days.
To approximate the frequency of escape from senescence, at least 50 colonies were propagated on YPD plates for four days at 30°C. After three passages, the majority of colonies had ceased proliferating and only RAD52-independent survivors were recovered. Surviving colonies were propagated stably for more than 10 serial passages. To generate type II survivors, freshly dissected est2Δ haploid cells were serially diluted in liquid media to a density of $10^5$ cells/ml every 24 hours for 10 days. RAD52 was subsequently deleted in type II survivor cells by mating with rad52Δ cells, followed by dissection and selection of est2Δ rad52Δ 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 hours, 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 (pre-senescent), cells of opposite mating types were mixed on YPD plate and incubated for six to eight hours at 30°C. 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 XhoI, resolved through a 0.75% w/v agarose gel in 1X 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; Zubko and
LYDALL 2006), labeled and used to probe genomic DNA digested with XhoI to reveal a single hybridization fragment at approximately 3 kbp.

**In-gel hybridization.** The assay was performed as described in (ZUBKO and LYDALL 2006) with minor modifications. Genomic DNA was digested with XhoI for four hours and then incubated at 65°C for 20 minutes. Radiolabeled and purified oligo (5'-CACACCCACACCCACACC-3') (100,000 cpm) was added to the DNA and incubated at 37°C for 15 minutes followed by 30 minutes on ice. Samples were subjected to electrophoresis through 0.75% w/v agarose in 0.5X 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) 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 XhoI and subjected to electrophoresis as described above, 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 re-probed with either the CA-rich or 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 (Chen et al. 2001; Larivée and
Welling er 2006; Le et al. 1999; Maringe le and Lydall 2004; Teng and Zakian 1999; Wen
et al. 2006; Zubko and Lydall 2006). We chose a different strain background, S288C [BY4741
and isogenic strain BY4742, for which genome-wide, individual ORF deletions are available for
all non-essential 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 exolA, that promote survival in the absence of RAD52 and telomerase (E. R.,
rad52Δ or S288C tlc1Δ rad52Δ 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 diplo ids, verified their genotypes, and isolated haplo ids. As expected, est2Δ
or tlc1Δ W303 haplo ids underwent senescence at approximately passage two (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 rad52Δ 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 hours (Figures 1K, L). While wild type and $rad52\Delta$ cells from each strain reached a density greater than $10^6$ cells/ml every 24 hours, $est2\Delta rad52\Delta$ cells from both strains exhibited decreased growth potential early during the experiment and failed to recover. Consistent with the growth on plates, S288C $est2\Delta$ or $tlc1\Delta$ (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 (Figures 1K, 1L).

**Rare escape from senescence in S288C $est2\Delta rad52\Delta$ or $tlc1\Delta rad52\Delta$ strains:** Consistent with our observations from the genetic screen, rare S288C $est2\Delta$ $rad52\Delta$ and $tlc1\Delta$ $rad52\Delta$ colonies escaped senescence. Of 50 individual S288C $est2\Delta$ $rad52\Delta$ or $tlc1\Delta$ $rad52\Delta$ colonies (derived from heterozygous diploids) propagated on plates every four days, two to five colonies consistently sustained growth after passage six (Figures 2B-D), while the majority of colonies became senescent at approximately passage three. Four to ten percent of colonies survived regardless of which telomerase gene was deleted (i.e. $est2\Delta$ $rad52\Delta$ or $tlc1\Delta$ $rad52\Delta$) 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 (Supplementary Figure 1) and was not observed in W303 $est2\Delta$ $rad52\Delta$ or $tlc1\Delta$ $rad52\Delta$ 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 $est2\Delta$ $rad52\Delta$ or $tlc1\Delta$ $rad52\Delta$ survivors arise after extensive telomere loss:** Once established, $RAD52$-independent survivors could be propagated for many generations (Figures 2B-D). Prior to senescence, telomeres in the $est2\Delta$ $rad52\Delta$ or $tlc1\Delta$ $rad52\Delta$ haploids were shorter in length than telomeres in the preceding heterozygous diploid and were also shorter than in wild
type, est2Δ or rad52Δ haploid colonies (Figure 2E, lanes 1, 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 2E, lane 4 and Figure 2F, lanes 3 and 10). When the population regained growth potential (Figure 2B, passage 4; Figure 2E, lanes 5-11; Figure 2F, lanes 4-7 and 11-14), the telomere pattern was similar to that of RAD52-dependent, type II survivors, i.e. multiple discrete fragments from 1-6 kbp representing telomeres containing a variable number of TG1-3 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 a four base-pair sequence, to which long, non-palindromic TG1-3 tracts would be resistant (WEN et al. 2006). Indeed, genomic DNA from RAD52-independent survivors yielded a telomeric pattern characteristic of long TG1-3 tracts (Figure 2F, lanes 11-14). We extracted genomic DNA after growth of RAD52-independent survivors in liquid media for less than 24 hours, 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 est2Δ rad52Δ or tlc1Δ rad52Δ 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 (Figures 1K, L) to 48 hours (Figures 3A, B). We examined the viability of three individual haploid colonies that originated from the same heterozygous diploid (Figures 3A, B,
and data not shown). Dilution every 48 hours nearly abrogated the transient decrease in population doubling time for \textit{est2A} and \textit{tlc1A} colonies (Figures 3A, B). In this particular experiment, one of three S288C \textit{tlc1A rad52A} colonies regained growth potential at day 14, concomitant with a TRF pattern reminiscent of recombination between telomeric DNA (Figure 3B, open circles and Figure 3C, lanes 17-19). In addition, one of the three S288C \textit{est2A rad52A} 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 \textit{est2A rad52A} colonies failed to escape senescence when diluted every 48 hours (data not shown). Thus, the propagation of S288C \textit{est2A rad52A} or \textit{tlc1A rad52A} (but not W303 \textit{est2A rad52A} or \textit{tlc1A rad52A}) cells every 48 hours in liquid culture allowed the emergence of \textit{RAD52}-independent survivors with a TRF pattern suggestive of telomeric DNA recombination (e.g. type II).

The similar TRF pattern between S288C \textit{RAD52}-independent survivors and type II survivors in other strain backgrounds prompted us to test whether the propagation of S288C \textit{est2A} cells in liquid culture followed by deletion of \textit{RAD52} (see Methods) would affect the incidence of \textit{RAD52}-independent survival. We noted that S288C \textit{est2A rad52A} colonies survived at an increased frequency if S288C \textit{est2A} cells had been propagated in culture to generate type II survivors prior to deletion of \textit{RAD52}. This increase was not statistically significant in one experiment (12/50 colonies survived, p>0.05, Fisher’s exact test), however was significant in another experiment (20/50 colonies survived, p<0.01). This result is in accord with a recent finding by Grandin and Charbonneau that the generation of type II survivors permits survival upon the subsequent deletion of \textit{RAD52} (\textsc{Grandin} and \textsc{Charbonneau} 2009) (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). In order to compare strains with similar characteristics, (i.e. transformed with pVL1107 and nearly equivalent average telomere length), we selected S288C passage two (Figure 4A, lane 1) and W303 passage 15 cells (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 hours 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\textsuperscript{EL} est2\textDelta rad52\textDelta\textsuperscript{pVL1107} and S288C\textsuperscript{EL} est2\textDelta rad52\textDelta\textsuperscript{pVL1107}) were serially propagated on plates every four days. Telomere elongation delayed the onset of senescence in both strains (Figures 4C-4J). In S288C\textsuperscript{EL} est2\textDelta rad52\textDelta cells, a higher percentage of colonies sustained growth than prior to telomere elongation (Figure 4C-J and Supplementary Figure 2). Notably, in contrast to W303 without elongated telomeres, telomere elongation in W303 est2\textDelta rad52\textDelta now allowed some cells to sustain growth for more than 15 passages (Figures 4G-J and Supplementary Figure 2). In addition, the TRF pattern in both strains showed evidence of TG\textsubscript{1-3} signal amplification and resistance to restriction endonuclease digestion (Figures 4K, L, M and Supplementary Figure 2). The ability of W303 est2\textDelta rad52\textDelta 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-labelled 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-labelled GT-rich oligonucleotide (e.g. to detect C-rich ssDNA; data not shown). As controls, S288C est2Δ type I survivors (exhibiting Y' amplification) and ku70Δ 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 ku70Δ cells, whose ssDNA signal is sensitive to *E. coli* Exonuclease I (ExoI) and thus specific to the telomere 3’ terminus (WELLINGER et al. 1993), the ssDNA signal observed in late passage tlc1Δ rad52Δ or est2Δ rad52Δ cells was ExoI-resistant (Supplementary Figure 3). 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 Supplementary Fig. 3; note that panels 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 ku70Δ cells.

**The RAD52-independent survivor phenotype shows complex penetrance in diploids:**

Makovets *et al.* recently demonstrated that RAD52-dependent (type I or II) survival in diploids
obtained 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. multi-genic) manner (Makovets et al. 2008). To examine the viability of various diploids created by mating RAD52-independent survivor haploids, we created three different S288C diploid strains: 1) two S288C est2Δ rad52Δ survivors mated together (Survivor X Survivor); 2) an est2Δ rad52Δ survivor mated to a pre-senescent est2Δ rad52Δ colony (freshly isolated from a heterozygous diploid) (Survivor X Pre-senescent); 3) two pre-senescent est2Δ rad52Δ colonies mated together (Pre-senescent X Pre-senescent). 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 X Pre-senescent’ cross would be expected to survive. If the suppressor were recessive, then no ‘Survivor X Pre-senescent’ strains should survive. None of the diploids obtained by mating two pre-senescent est2Δ rad52Δ colonies (by analyzing 50 independent diploids on plates, or by inoculating 30,000 diploid cells in liquid culture) emerged as RAD52-independent survivors (Figures 6B7, B8, and Supplementary Figure 4). However, diploids obtained by mating a RAD52-independent survivor with a pre-senescent est2Δ rad52Δ colony exhibited approximately a 50% incidence of prolonged survival, with some colonies undergoing senescence (Figure 6B, panel 4) and others sustaining growth (Figure 6B, panels 5, 6, and Supplementary Figure 4). Finally, all diploids generated by mating two RAD52-independent haploid survivors continued to grow beyond 11 passages (Figure 6B, panels 1, 2, 3 and Supplementary Figure 4). These RAD52-independent survivors also exhibited a TRF pattern indicative of telomeric tract recombination (Figures 6C, D). Therefore, unlike Makovets et al., who observed dominance of type I or type II survival upon mating to a pre-senescent haploid, the viability of diploids created
by mating a *RAD52*-independent survivor to a pre-senescent haploid was neither dominant nor recessive. The *RAD52*-independent survivor phenotype thus suggests complex multi-genic mechanisms that may share similarities with other survivor phenotypes (Makovets *et al.* 2008; Zubko and Lydall 2006).

**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* (Chen *et al.* 2001; Le *et al.* 1999; Teng and Zakian 1999). 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 (*est2Δ rad52Δ xxxΔ*). Deletion of any of these genes did not affect the incidence of *RAD52*-independent survivors in W303 *est2Δ rad52Δ* or S288C *est2Δ rad52Δ* backgrounds (n=50 for each strain) (Table 1). *RAD5*, which is non-functional 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 *est2Δ rad52Δ* colonies (p>0.05, data not shown). In addition, introduction of wild-type *RAD5* in W303 did not permit the generation of *RAD52*-independent survivors (L.M., unpublished data).

We also examined candidate genes involved in the regulation of chromatin structure or DNA replication (*DNL4, GCN5, HTA1, HTA2, POL32, 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 est2Δ rad52Δ xxxΔ strains (n=50 for each strain).

The polymerase delta subunit POL32 has been implicated in break-induced replication and telomerase-independent telomere maintenance (LYDEARD et al. 2007), and pol32Δ 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 rad52Δ pol32Δ and est2Δ rad52Δ pol32Δ colonies remained small after microdissection (data not shown). However, when serially passaged on plates, rad52Δ pol32Δ colonies gained growth potential and became viable to a similar extent as other colonies, whereas est2Δ rad52Δ pol32Δ 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 (tlc1Δ rad52Δ, passage 9) with a pol32Δ single mutant haploid were sporulated and tested for viability. The resulting tlc1Δ rad52Δ pol32Δ haploids exhibited heterogeneous colony sizes (data not shown). Serial propagation was possible in tlc1Δ rad52Δ pol32Δ cells arising from a colony with an initial size comparable to a tlc1Δ rad52Δ colony, whereas tlc1Δ rad52Δ pol32Δ cells arising from a microcolony failed to sustain growth (Figure 7C and data not shown). TRF analysis of viable tlc1Δ rad52Δ pol32Δ cells revealed a telomeric DNA pattern reminiscent of telomere tract recombination and “type II” survivors (Figure 7D). We were unable
to obtain viable est2Δ rad52Δ pol32Δ 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 (Chen et al. 2001; Le et al. 1999; Lundblad and Blackburn 1993; Maringele and Lydall 2004; Teng et al. 2000; Teng and Zakian 1999). 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. RAD52-independent survival was inherited as an apparent multi-genic trait, similar to other RAD52-dependent survival phenotypes (Makovets et al. 2008; Zubko and Lydall 2006), however, targeted deletion of genes required for survival in the absence of telomerase and 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.

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 (Chen et al. 2001; Le et al. 1999; 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 \textit{RAD52}-independent and \textit{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 (Suppl. Figure 3). This 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 non-coding 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 \textit{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 \textit{et al.} 2001; LIN \textit{et al.} 2005; MUNTONI \textit{et al.} 2009; MUNTONI and REDDEL 2005; WANG \textit{et al.} 2004; WILLIAMS \textit{et al.} 2005). For example, in \textit{K. lactis} the roll-and-spread mechanism of DNA synthesis in telomerase-deficient and telomerase template-mutated strains does not absolutely require \textit{RAD52} (MCEACHERN and BLACKBURN 1996). It is possible that the G-rich telomeric ssDNA in \textit{RAD52}-independent survivors (Figure 5, Suppl. Figure 3) 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 inter-telomeric BIR to lengthen other telomeres (MCEACHERN and HABER 2006; NATARajan and MCEACHERN 2002).
Similar events might explain the abrupt emergence of RAD52-dependent survivors harbouring 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.* suggest the involvement of Pol32, a non-essential subunit of DNA polymerase δ, in segmental duplication promoting genomic instability through a RAD52-independent mechanism of template switching between microsatellites, or microhomologous sequences (PAYEN *et al.* 2008). 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 non-homologous end joining machineries (PAYEN *et al.* 2008). Although the synthetic lethality of the *est2Δ rad52Δ pol32Δ* and *tlc1Δ rad52Δ pol32Δ* 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*) promote 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 multi-genic 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 pre-senescent est2Δ rad52Δ population argues against a simple recessive or dominant extragenic suppressor mutation. Interestingly, the incidence of escape from senescence in diploids created by mating two est2Δ rad52Δ haploid strains is less frequent than in an est2Δ rad52Δ 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 by-pass 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 there exists a means to survive 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 by-pass the re-acquisition of telomerase activity during tumorigenesis (MUNTONI et al. 2009; MUNTONI and REDDEL 2005).
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NOTE ADDED IN REVISION

During the revision of this manuscript, Grandin and Charbonneau showed that telomerase-deficient cells with long telomeres (e.g. type II survivors) could survive the subsequent deletion of RAD52 (Grandin and Charbonneau 2009). 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 ILT (inter-lengthening of telomeres) 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 (rif2Δ 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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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 one to six (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. J) Wild type S288C telomeres are longer than W303 telomeres. Genomic DNA was digested with XhoI and the membrane was hybridized to a telomeric probe. K-L) W303 est2Δ rad52Δ (K) and S288C tlc1Δ rad52Δ and est2Δ rad52Δ (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 x 10^8 cells/ml). Every 24 hours, cell density was measured using a hemacytometer, and the culture was diluted to 10^5 cells/ml.

Figure 2. Rare S288C est2Δ rad52Δ colonies continue to proliferate and exhibit a TRF (terminal restriction fragment) pattern typical of telomeric tract recombination. A) Schematic of the senescence assays on plates, from passage one to six (see details in Materials and Methods). B-D). Typical S288C est2Δ rad52Δ survivors (or tlc1Δ rad52Δ; 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 one through ten (20 to 200 generations). DNA was digested with XhoI 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 est2Δ rad52Δ at increasing passages. Black arrows at left indicate Y' elements. F) Telomere Southern blot of S288C est2Δ rad52Δ survivors at increasing passages. Genomic DNA was digested with either
**Figure 3.** A subset of S288C est2Δ rad52Δ or tlc1Δ rad52Δ cells escape senescence in liquid culture and exhibit telomeric tract recombination. A-B) Cells were isolated from a fresh dissection plate (isolates 1-3), inoculated into YPD media, grown to saturation (1-2 x 10^8 cells/ml), and diluted to 10^5 cells/ml every 48 hours. 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 two days. Genomic DNA was isolated from cells and digested with *XhoI*. The membrane was hybridized to a telomeric probe. Marker sizes are indicated at left, kbp.

**Figure 4.** Telomere length correlates with the incidence of escape from senescence. A) Telomere elongation in S288C est2Δ rad52Δ and W303 est2Δ rad52Δ 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 two days) on SD-LEU plates. Genomic DNA was digested with *XhoI*. 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: S288C<sup>EL</sup> est2Δ rad52Δ<sup>pVL1107</sup> (C-F); W303<sup>EL</sup> est2Δ rad52Δ<sup>pVL1107</sup> (G-J). Telomere Southern blot on genomic DNA isolated from S288C<sup>EL</sup> est2Δ rad52Δ<sup>pVL1107</sup> (K) or W303<sup>EL</sup> est2Δ rad52Δ<sup>pVL1107</sup> (L) cells. DNA was digested with *XhoI* and the membrane was hybridized to a telomeric probe. K) Lane 1, S288C est2Δ rad52Δ after 2 passages with pVL1107 (passage at which
plasmid was removed); lanes 2-14, S288C<sup>EL</sup> est2Δ rad52Δ at increasing passages after plasmid loss. Lower panel: Blot was stripped and re-hybridized to a CDC15 probe as a loading control. Note the under-representation of CDC15 in lanes 4, 5 due to poor growth at these passages. L) Lanes 11, 12, W303 est2Δ rad52Δ after 1 and 15 (passage at which plasmid was removed) passages with pVL1107, respectively. Lanes 1-10, W303<sup>EL</sup> est2Δ rad52Δ at increasing passages after plasmid loss. Lower panel: Blot was stripped and re-hybridized to a CDC15 probe as a loading control. The telomeric signal intensity in lanes 10 and 12 should be interpreted in light of the under-representation of CDC15. M) Southern blot of genomic DNA digested with a mixture of the restriction endonucleases (AluI, HinfI, HaeIII and MspI). The membrane was hybridized to a telomeric probe. Lanes 1-10, W303<sup>EL</sup> est2Δ rad52Δ at increasing passages after plasmid loss; lanes 11-13, S288C (wt, est2Δ, and est2Δ rad52Δ, each at passage 1); lane 14, S288C est2Δ rad52Δ survivor at passage 10; lanes 15-16, W303 est2Δ rad52Δ<sup>++pVL1107</sup> at passages 1 and 15 (pVL1107 was removed at passage 15). For each panel, marker sizes are indicated at left in kbp.

**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 XhoI and the membrane was hybridized to a radio-labeled 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 panel A was resolved in the presence of radio-labeled probe). DNA was digested with XhoI and the membrane was hybridized to the radio-labeled C-rich oligonucleotide. Marker sizes are indicated at left in kbp. Lower panel: Blot was stripped and re-hybridized to a CDC15 probe as a loading control. Note that the genomic DNA in lane 7 is slightly under-represented relative to other samples.
**Figure 6.** *RAD52*-independent survival in diploids generated from *RAD52*-independent haploid survivors. Freshly dissected S288C *est2Δ rad52Δ* haploids (‘pre-senescent’) and S288C *est2Δ rad52Δ* haploid survivors (‘survivor’) were mated to generate *est2Δ::est2Δ rad52Δ::rad52Δ* diploid strains. A) Schematic of the senescence assays on plates, from passage one to six (see Materials and Methods). B) Summary senescence assays of representative diploids grown on plates for the indicated number of passages. B1, B2; two representative viable diploids from a Survivor X Survivor cross (passages 1-6), which all survive later passages (e.g. B2 to B3), B4, B5; representative diploids from a Survivor X Pre-senescent cross, some of which do not sustain growth beyond 3 passages (e.g. B4), while others remain viable beyond 12 passages (e.g. B5 to B6), B7, B8; two representatives diploids from Pre-senescent X Pre-senescent crosses (no diploids sustain growth, therefore no data for passages 7-12). See also Supplementary Fig. 3. C) Telomere Southern blot of genomic DNA. Two S288C *est2Δ rad52Δ* haploid survivors at passage 9 (lanes 13, 14) were mated to produce an *est2::NAT/est2::NAT rad52::URA3/rad52::KAN* diploid (lanes 1-10, at indicated passages). Lanes 11-12, S288C *est2Δ* and wild-type haploids. DNA was digested with *XhoI* and the membrane was hybridized to a telomeric probe. D) Telomere Southern blot of genomic DNA from a “Survivor X Pre-senescent” diploid that escaped senescence. S288C *est2Δ rad52Δ* haploid survivor (passage 9; lane 12) was mated to a pre-senescent *est2Δ rad52Δ* haploid (passage 1; lane 13) to produce an *est2::NAT/est2::NAT rad52::URA3/rad52::KAN* diploid (Survivor x Pre-senescent) (lanes 1-11, at indicated passages). Lanes 14-15, S288C haploids at passage 1 (wt, *est2Δ*). DNA was digested with *XhoI* 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 kbp.
Figure 7. Investigating the potential role of POL32 in RAD52-independent survival. A) Schematic of the senescence assays on plates, from passages one to six (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 tlc1Δ rad52Δ pol32Δ haploids obtained from the diploid generated by mating an S288C tlc1Δ rad52Δ survivor (at passage 9) to a haploid pol32Δ colony. Microcolonies could not be propagated for more than three passages (e.g. upper panel), whereas colonies of regular size could be propagated for at least 12 passages (middle and lower panels). D) Telomere Southern blot of DNA of a tlc1Δ rad52Δ pol32Δ viable haploid colony (lanes 6, 8-18); this haploid was obtained from a diploid (lane 3) generated by mating an S288C tlc1Δ rad52Δ survivor (passage 9; lane 1) to a pol32Δ haploid (lane 2). Lanes 4-7; haploids derived from the heterozygous diploid in lane 3. DNA was digested with XhoI and the membrane was hybridized to a telomeric probe. Marker sizes are indicated at left, kbp.

Supplementary Figure 1. 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 NsiI/SalI 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 BglII/PvuII 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 NcoI/AlwNI 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.

**Supplementary Figure 2.** 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.

**Supplementary Figure 3.** 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 est2Δ 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).
Supplementary Figure 4. The RAD52-independent survivor phenotype is inherited 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.
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Table 1. The effect of various gene deletions on escape from senescence in S288C *est2Δ rad52Δ* or W303 *est2Δ rad52Δ* strains. 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 (*est2Δ rad52Δ xxxΔ*). The majority of S288C *est2Δ rad52Δ* and *est2Δ rad52Δ xxxΔ* colonies ceased proliferation after approximately 60 generations. The percentage of *RAD52*-independent survivors recovered from each genotype is indicated. Asterisk (*) indicates the S288C *est2Δ rad52Δ pol32Δ* 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 *est2Δ rad52Δ* or *est2Δ rad52Δ xxxΔ* survivors emerged, and cell populations entered senescence at 40-60 generations.
S288C est2Δ/est2Δ rad52Δ/rad52Δ
haploid parents:

Survivor X Survivor

Survivor X Pre-senescent

Pre-senescent X Pre-senescent

Figure 6
Figure 7

Panel A: Diagram showing sections 1 to 6.

Panel B: Petri dishes with different genotypes:
- **wt** (wild type)
- **est2Δ rad52Δ**
- **est2Δ pol32Δ**
- **rad52Δ pol32Δ**
- **est2Δ rad52Δ pol32Δ**
- **est2Δ**
- **pol32Δ**
- **rad52Δ**

Panel C: Images of microcolony and normal colony for:
- **tlc1Δ rad52Δ pol32Δ**

Panel D: Gel electrophoresis with bands at kbp positions and pass numbers.

Legend:
- **kbp** (kilobase pairs)
- **pass** (passage number)
Suppl. Figure 2
Suppl. Figure 3