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# Long telomeres are preferentially extended during recombination-mediated telomere maintenance

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**Most human somatic cells do not express telomerase. Consequently, with each cell division their telomeres progressively shorten until replicative senescence is induced. Around 15% of human cancers maintain their telomeres using telomerase-independent, recombination-based mechanisms that are collectively termed 'alternative lengthening of telomeres' (ALT). In the yeast *Saccharomyces cerevisiae*, ALT cells are referred to as 'survivors'. One type of survivor (type II) resembles human ALT cells in that both are defined by the amplification of telomeric repeats. We analyzed recombination-mediated telomere extension events at individual telomeres in telomerase-negative yeast during the formation of type II survivors and found that long telomeres were preferentially extended. Furthermore, senescent cells with long telomeres were more efficient at bypassing senescence by the type II pathway. We speculate that telomere length may be important in determining whether cancer cells use telomerase or ALT to bypass replicative senescence.**



Telomeres are regions of guanine-rich, repetitive DNA at the ends of chromosomes bound by proteins that protect chromosome ends from being recognized as DNA double-stranded breaks[1]. The telomeres of most human somatic cells undergo progressive shortening owing to incomplete DNA replication and nucleolytic degradation. This process ultimately leads to replicative senescence and is thought to be a barrier to tumorigenesis[2]. Most cancer cells bypass this senescence by activating telomerase[2,3], a specialized reverse transcriptase that elongates telomeres[4]. However, 15% of human cancers bypass senescence by using the telomerase-independent length maintenance mechanisms that are collectively referred to as ALT, which are thought to involve DNA recombination[3,5].

Telomerase-independent telomere maintenance was first discovered in the budding yeast *S. cerevisiae*[6]. Yeast cells that lack telomerase senesce after 60–80 generations, but a small subset of cells can bypass this senescence to become survivors[6]. There are two main classes of survivors (type I and type II), both of which require Rad52-dependent recombination and Pol32-dependent break-induced replication[6–10]. The formation of type I survivors involves the amplification of subtelomeric Y′ sequences and depends on Rad51, Rad52, Rad54, Rad55 and Rad57 (refs. 6–8). Type II survivors show amplification of the $TG_{1-3}$ telomeric repeats and requires Rad52, the MRX complex (consisting of Mre11, Rad50 and Xrs2), Sgs1 and Rad59 (refs. 7,9,11–14).

Type I survivors occur more frequently but grow very poorly, whereas growth of type II survivors is comparable to that of telomerase-positive cells[6,8]. Live cell imaging has shown that senescent cells are arrested at the G2-M transition, with telomeres moving back and forth between the mother and the bud[15]. The telomeres of type I survivors show the same oscillating motion, but telomeres of type II survivors behave like telomeres in telomerase-positive cells[15]. This observation is consistent with the slow growth of type I survivors and wild-type growth of type II survivors, and suggests that the telomeres of type I survivors do not return to a properly capped state.

Type II survivors resemble human ALT cells in that both are defined by the presence of long, heterogeneous telomeric repeats[8,16,17]. Both type II survivors and human ALT cells are thought to amplify their telomeric DNA through recombination-dependent DNA replication[18], probably involving extrachromosomal circular DNA containing telomeric repeat sequences[19,20]. The similarities between type II survivors and human ALT cells make yeast an attractive model in which to study recombination-based telomere maintenance.

We analyzed recombination-mediated telomere extension in *S. cerevisiae* type II survivors immediately after their emergence from a senescent culture. Long telomeres were preferentially extended during type II survivor formation, in contrast to telomerase-mediated telomere maintenance, in which short telomeres are preferentially elongated[21]. Furthermore, increasing the length of telomeres at the point of senescence also increased the efficiency with which cells bypassed senescence by type II recombination. Our results suggest that telomere length at senescence may be an important factor in determining whether neoplastic cells reactivate telomerase or use ALT to maintain their telomeres.

## RESULTS

### Telomere extension events during survivor formation

The single telomere extension (STEX) assay was developed to analyze telomerase-mediated telomere extension events at individual telomeres at nucleotide resolution[21,22]. Here we modified the assay to allow us to study recombination-mediated telomere extension events as cells

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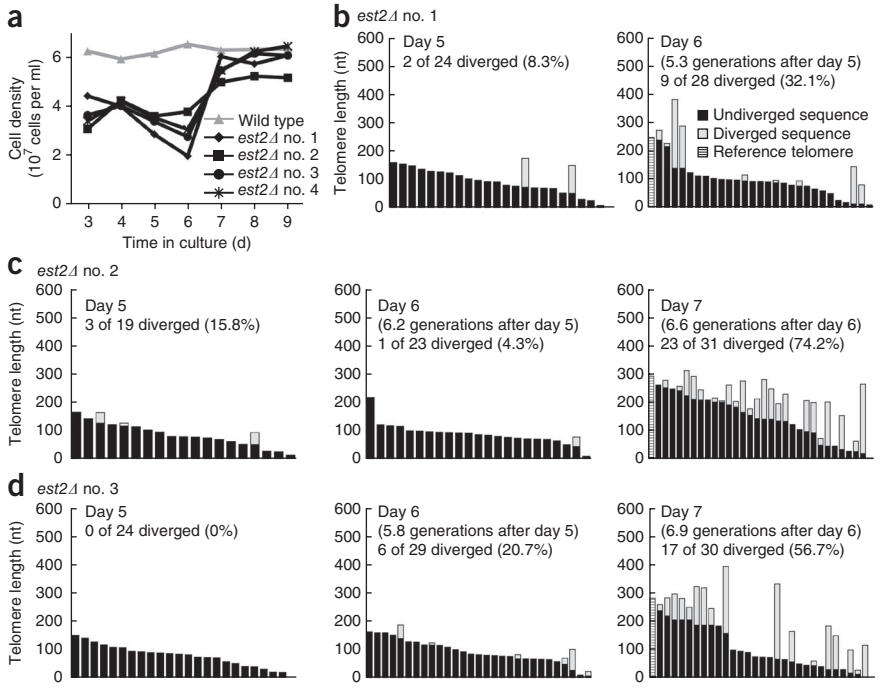


**Figure 1** STEX analysis of type II survivor formation. (**a**) Senescence was monitored in liquid culture by serially passaging four *est2Δ* spore products derived from an *est2Δ*/+ heterozygous diploid. (**b**–**d**) Analysis of sequenced VI-R telomeres from three *est2Δ* strains at days 5, 6 and 7: *est2Δ* no. 1 (**b**), *est2Δ* no. 2 (**c**) and *est2Δ* no. 3 (**d**). Analysis of telomeres from *est2Δ* no. 4 is shown in **Supplementary Figure 1**. Each bar represents an individual VI-R telomere and bars are sorted by the length of the undiverged sequence. The black portion of each bar represents the undiverged or unextended region of the telomere. The light gray portion represents the diverged or extended region of the telomere. For each *est2Δ* strain, the longest telomere without divergent sequence (hashed bar) is used as a reference telomere to which all other telomeres are compared to determine whether divergence has occurred.

senesced and as survivors emerged. For this assay, we deleted one copy of *EST2*, which encodes the catalytic subunit of telomerase[23], in a diploid strain. The resulting *est2Δ*/+ heterozygous diploid was sporulated and the haploid meiotic progeny were isolated on agar plates. After about 25 generations of growth, we transferred wild-type and telomerase-negative *est2Δ* strains to liquid culture. After 24 h, we measured cell density and diluted the cultures to a cell density of $5 \times 10^5$ cells per ml in fresh medium. We repeated the measurements and dilutions seven times at 24-h intervals. Growth in liquid culture allowed us to look specifically at type II survivors because type II survivors have a substantial growth advantage over type I survivors[8]. Although type I survivors arise more frequently, they grow poorly.

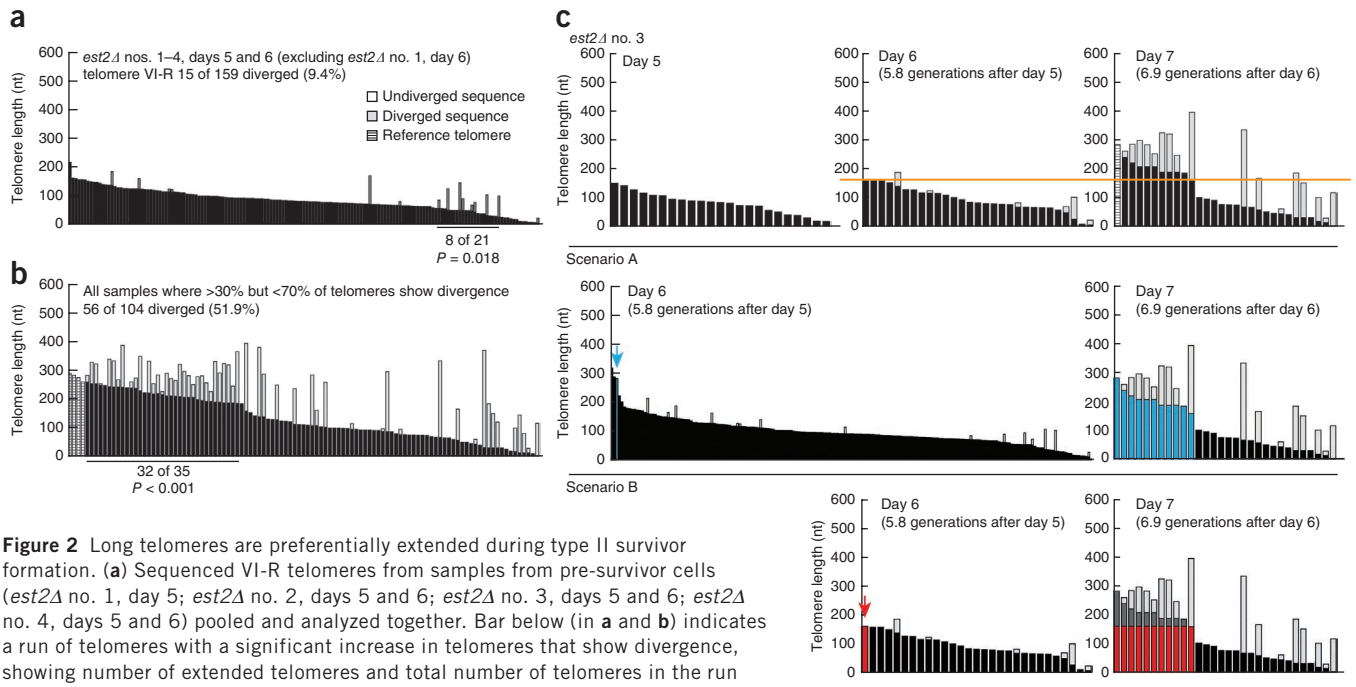


**Figure 2** Long telomeres are preferentially extended during type II survivor formation. (**a**) Sequenced VI-R telomeres from samples from pre-survivor cells (*est2Δ* no. 1, day 5; *est2Δ* no. 2, days 5 and 6; *est2Δ* no. 3, days 5 and 6; *est2Δ* no. 4, days 5 and 6) pooled and analyzed together. Bar below (in **a** and **b**) indicates a run of telomeres with a significant increase in telomeres that show divergence, showing number of extended telomeres and total number of telomeres in the run as well as *P* value (determined by scan statistics; see Online Methods). (**b**) Sequenced telomeres from all samples where >30% but <70% of the telomeres show sequence divergence (*est2Δ* no. 1, day 6, telomere VI-R; *est2Δ* no. 3, day 7, telomere VI-R; *est2Δ* no. 4, day 7, telomeres VI-R and XIV-R) pooled and analyzed together. (**c**) Long unextended telomeres from day 7 samples can be explained by one of two models, both involving the preferential, recombination-mediated extension of long telomeres. Top, *est2Δ* no. 3 data from **Figure 1d**. The orange line highlights the observation that a number of telomeres from the day 7 sample contain undiverged regions that are longer than the longest day 6 telomere. Scenario A: long telomeres from day 7 (blue bars, right) originated from a long telomere from day 6 (blue bar indicated by blue arrow) that was not sequenced because such long telomeres were too rare in the day 6 sample to be detected in our assay. Scenario B: a long telomere from day 6 (red bar indicated by red arrow) was extended between days 6 and 7 and then duplicated several times, giving rise to the long telomeres in day 7 (red bars, right). See text.

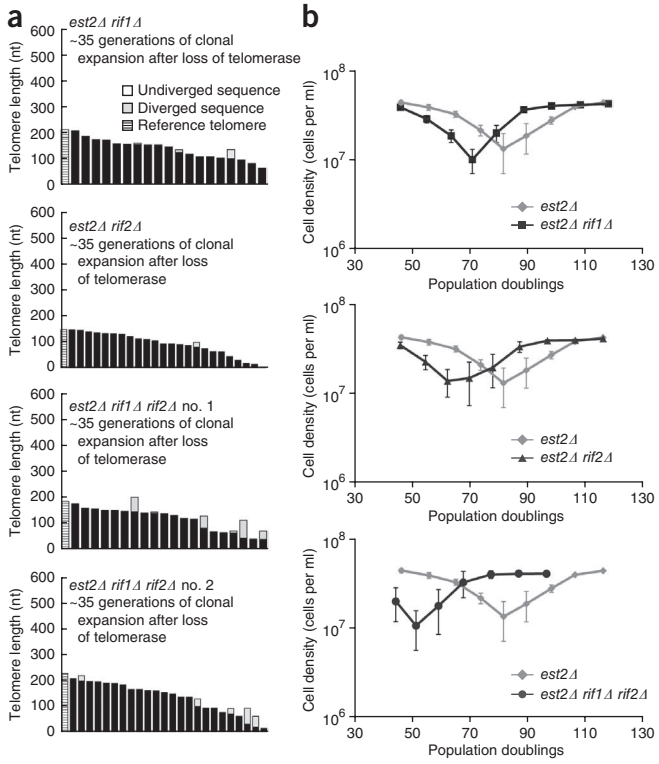


**Figure 3** Telomerase-negative *rifΔ* strains show accelerated senescence. (**a**) Telomere VI-R was sequenced from *est2Δ rif1Δ*, *est2Δ rif2Δ* and two different isolates of *est2Δ rif1Δ rif2Δ* after ~35 generations of clonal expansion following sporulation of an *est2Δ*/+ *rif1Δ*/+ *rif2Δ*/+ diploid. Sequenced telomeres were analyzed as in **Figure 1**. (**b**) Senescence rates were measured in liquid culture by serial passaging of *est2Δ*, *est2Δ rif1Δ*, *est2Δ rif2Δ* and *est2Δ rif1Δ rif2Δ* strains derived from the diploid used in **a**. Mean ± s.e. for at least four independent spore isolates for each genotype are shown.

In liquid culture, the rarer type II survivors quickly outgrow the type I survivors and dominate the culture[8]. As expected, the wild-type strain grew consistently throughout the experiment, whereas growth of the *est2Δ* strains progressively declined (**Fig. 1a**). Senescence occurred around days 5 and 6 for four independent *est2Δ* isolates. By day 7, all four isolates had bypassed senescence.

We isolated DNA from the *est2Δ* isolates on days 5, 6 and 7. We amplified the right telomere of chromosome VI (VI-R) by telomere PCR[24,25]. We then cloned and sequenced the amplified telomeres. Divergence of telomeric sequences is due to the recombination of the imperfect 5′-$(TG)_{0-6}TGGGTGTG(G)_{0-1}$-3′ repeats of *S. cerevisiae* telomeres[21]. Before the emergence of survivors on day 7, telomeric recombination events were rare (**Fig. 1b–d**, **Supplementary Fig. 1** and data not shown), consistent with previous observations[21]. By contrast, most telomeres from day 7 samples (fewer than 7 population doublings after day 6) showed sequence divergence that indicated telomere extension events (**Fig. 1c**,**d** and **Supplementary Fig. 1**). We also analyzed telomeres I-L and XIV-R in samples taken on days 6 and 7 from *est2Δ* isolate no. 4 (**Supplementary Fig. 1** and data not shown). The data from these telomeres were similar to those from telomere VI-R, which suggests that our results are applicable to all telomeres.

## Preferred extension of long telomeres in type II survivors

When we pooled the sequenced telomeres from pre-survivor samples (**Fig. 1b–d** and **Supplementary Fig. 1**: *est2Δ* no. 1, day 5; *est2Δ* no. 2, days 5 and 6; *est2Δ* no. 3, days 5 and 6; *est2Δ* no. 4, days 5 and 6), we found that there was a modest preference for the extension of short telomeres (**Fig. 2a**), which is consistent with previous observations[21,22,26,27].

We next analyzed the recombination events in samples from emerging survivors. In pre-survivor cultures after 30 generations of clonal expansion, about 7% of telomeres show sequence divergence[21]. Therefore we analyzed samples in which more than 30% of the telomeres showed sequence divergence, to ensure that these samples were from emerging survivors. We did not examine samples in which more than 70% of the telomeres showed sequence divergence because survivor formation had progressed too far in these samples, rendering the data uninformative. In these samples, it was no longer possible to determine how many extension events had occurred on a given telomere and whether extension events showed a preference for telomere length. In samples in which 30–70% of telomeres showed sequence divergence, long telomeres showed preferential extension, whether the samples were analyzed individually (**Fig. 1b–d** and **Supplementary Fig. 1**: *est2Δ* no. 1, day 6, telomere VI-R; *est2Δ* no. 3, day 7, telomere VI-R; *est2Δ* no. 4, day 7, telomeres VI-R and XIV-R) or grouped together (**Fig. 2b**).

The preferential extension of long telomeres can also be seen by comparing day 6 and day 7 telomeres from the same *est2Δ* isolate. The undiverged regions of the day 7 telomeres can be longer than the longest day 6 telomeres (**Fig. 2c**, top, black bars above orange line), which initially seems counterintuitive. However, this observation can be explained in two ways. First, a rare long telomere from day 6 (**Fig. 2c**, scenario A, blue bar indicated by blue arrow), too infrequent in the population to be detected among the 20–30 telomeres sequenced, might have been replicated several times (with daughter telomeres eroding with each round of DNA replication), giving rise

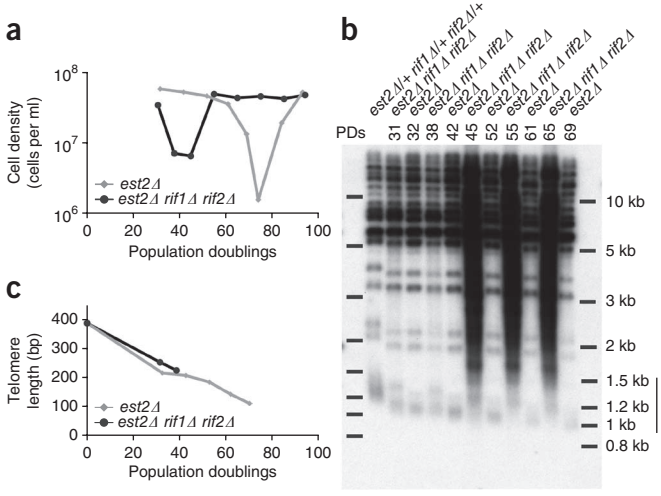


**Figure 4** Telomere shortening rate is unchanged when *RIF1* and *RIF2* are deleted. (**a**) One isolate each of *est2Δ* and *est2Δ rif1Δ rif2Δ* was passaged as in **Figure 3b**. (**b**) At each passage, telomere lengths were determined by denaturing in-gel hybridization (see Online Methods). Vertical bar (right) indicates the position of the terminal restriction fragments of Y′ telomeres, which are present in more than half of yeast telomeres. Larger bands represent non-Y′-containing telomeres. A smear of telomeric signal appears in the *est2Δ rif1Δ rif2Δ* strain after 45 population doublings (PDs), which arise from type II survivors that have undergone telomere repeat amplification. (**c**) The telomere lengths from **b** were quantified and plotted.

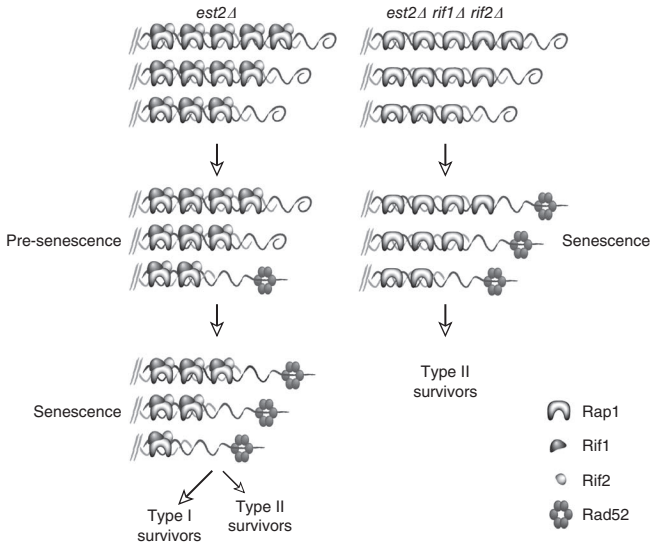


**Figure 5** Model for the senescence of *est2Δ* and *est2Δ rif1Δ rif2Δ* strains. In an *est2Δ* strain (left), telomeres progressively shorten with each cell division. Short telomeres are uncapped and recruit DNA damage factors such as Rad52. The recruitment of DNA damage factors at one or a few shortened telomeres is insufficient to cause senescence until most or all telomeres have been sufficiently eroded. Bypass of senescence occurs by either the type I pathway (involving subtelomeric Y′ elements) or, less frequently, the type II pathway (involving the telomeric repeats). The type II pathway involves the preferential extension of long telomeres by recombination. In an *est2Δ rif1Δ rif2Δ* strain (right), telomeres become uncapped earlier and this leads to accelerated senescence. However, as the telomere shortening rate is unaffected by deletion of the *RIF* genes, telomeres are relatively long at senescence when compared to telomeres in an *est2Δ* strain. The longer telomeres recombine more efficiently, resulting in a marked increase in the fraction of *est2Δ rif1Δ rif2Δ* survivors that are type II. The Rad52 protein complex is depicted as a hexamer for simplicity.

to the long telomeres in day 7 (blue bars), most of which were then extended (light gray portion). Alternatively, the longest detectable telomere from day 6 (**Fig. 2c**, scenario B, red bar indicated by red arrow) could have been extended and, after being replicated several times (with daughter telomeres eroding with each round of DNA replication), given rise to the set of longer telomeres in day 7 (red and dark gray bars; dark gray portion indicating extended region), most of which were extended again (light gray portion of red and dark gray bars). Cells harboring this set of long telomeres in day 7 (blue bars in scenario A, red bars in scenario B) outcompete other senescing cells with shorter (more extensively eroded) telomeres, resulting in the enrichment of these longer telomeres. For both explanations long telomeres are preferentially extended, which is consistent with the statistical analysis (**Fig. 2b**).

### Telomere length affects type II survivor emergence

Given that long telomeres are preferentially extended during the emergence of type II survivors, we reasoned that senescent cells with long telomeres would favor the bypass of senescence by the type II pathway rather than the type I pathway. Therefore, we next examined telomere length in mutant backgrounds in which type II survivors are increased. Rif1 and Rif2, which are recruited to telomeres through the C-terminal domain of Rap1 (refs. 28,29), inhibit exonucleolytic processing at telomeres by the MRX complex[30]. In a telomerase-negative background, the removal of Rif1 or Rif2 (especially Rif2) greatly increases the fraction of type II survivors[11]. All survivors generated from a telomerase-negative *rif1Δ rif2Δ* strain are type II (ref. 11). As it has been proposed that the number of type II survivors increased because Rif1 and Rif2 inhibit type II recombination[11], telomeric recombination should be increased even in *rifΔ* pre-survivor cells. However, when we sequenced telomeres from pre-survivor *est2Δ rif1Δ*, *est2Δ rif2Δ* and *est2Δ rif1Δ rif2Δ* mutants after around 35 generations of clonal expansion following loss of telomerase, we found no marked increase in type II recombination events (**Fig. 3a**). Although there appears to be a slight increase in recombination events in *est2Δ rif1Δ rif2Δ* pre-survivor mutants, the levels are nowhere near as high as seen in survivors. These observations indicate that the greater proportion of type II survivors in *est2Δ rif1Δ rif2Δ* mutant strains is not due to the removal of inhibition of recombination by the Rif proteins. Therefore, we speculated that the increase in type II survivors might be due to telomerase-negative *rifΔ* mutants having longer telomeres at senescence than telomerase-negative *RIF*+ mutants.

To test this hypothesis, we sporulated an *est2Δ*/+ *rif1Δ*/+ *rif2Δ*/+ diploid and monitored the growth of the telomerase-negative haploid progeny in liquid culture by serial dilution of cells to $5 \times 10^4$ cells per ml every 24 h (**Fig. 3b**). We found that *est2Δ rifΔ* mutants senesced after fewer population doublings than *est2Δ* strains. We then monitored the telomere lengths of *est2Δ* and *est2Δ rif1Δ rif2Δ* strains as they senesced and found that telomeres shortened at the same rate in both strains (**Fig. 4**). However, as the *est2Δ rif1Δ rif2Δ* strain senesced earlier, its telomeres were more than 100 bp longer than those of the *est2Δ* strain at the point of maximum senescence (**Fig. 4c**). These observations support our proposal that longer telomeres recombine more efficiently, which explains the increase in type II survivors in telomerase-negative *rifΔ* mutants.

Finally, deleting both *RIF1* and *RIF2* greatly accelerated senescence and deletion of *RIF2* had a bigger effect than deletion of *RIF1* (**Fig. 3b**). This finding correlates with the increase in type II survivors in telomerase-negative *rifΔ* mutants; *rif2Δ* mutants show a greater increase in type II survivors than *rif1Δ* mutants[11]. Rif2 has been shown to have a more prominent role than Rif1 in inhibiting telomeric 5′-end resection by the MRX complex[30]. Together, these observations suggest that the function of the Rif proteins, in particular Rif2, during senescence is to prevent premature uncapping of telomeres.

## DISCUSSION

We have analyzed type II recombination-mediated telomere extension events as survivors emerged from a senescent culture. Long telomeres were preferentially extended (**Fig. 2b**,**c**), which was unexpected because previous studies showed that the shortest telomeres triggered senescence in both mouse and yeast[31–33] and that the shortest telomeres were preferentially elongated by telomerase in yeast and human fibroblasts expressing telomerase[21,34]. However, recombination efficiency is directly proportional to the length of the substrate DNA in prokaryotes, yeast and mammalian cells[35–40]. Therefore, we propose that once a senescent cell becomes a survivor, the long telomeres are better substrates for recombination and will recombine first. Any short telomeres will also be extended eventually, otherwise the cell would not be able to escape senescence.

Although Rad52 is important for virtually all recombination events in budding yeast, Rad52-independent recombination can occur if the substrate DNA is sufficiently long[41]. Type II-like survivors, showing amplification of the telomeric repeats, can form in the absence of Rad52 if longer telomeres are present during senescence[42,43]. These results are consistent with our findings that long telomeres recombine

more efficiently and that these long telomeres are preferentially extended during the formation of type II survivors.

Long telomeres were not preferentially extended in pre-survivor cells (**Fig. 2a**). In the pre-survivor samples we analyzed, there was a modest preference for the extension of short telomeres (**Fig. 2a**), consistent with previous observations[21,22,26,27]. Such short telomeres are more likely to be uncapped and to recruit DNA damage checkpoint proteins including Rad52 (ref. 32), which might explain why short telomeres are more likely to be extended by recombination in pre-survivor cells. The recruitment of these proteins at eroded telomeres can start many generations before senescence[32]. In our model, these senescing cells continue to divide until most or all telomeres have been sufficiently eroded and Rad52 is present at all of the telomeres (**Fig. 5**). Consequently, when a senesced cell transforms into a survivor, all of the Rad52-coated telomeres are competent for recombination, with the longer ones being more efficiently extended by recombination.

Alternatively, the modest increase in short telomeres that have been extended in pre-survivor samples (**Fig. 2a**) might be due not to preferential extension of short telomeres but rather to the senescence of cells with short telomeres. Cells that extend such telomeres would be selected to survive, giving the illusion that short telomeres are preferentially extended. This explanation may also account for the slight increase in short telomeres showing divergence even in emerging survivors (**Fig. 2b**).

We also found that telomere length in senescent cells determined the probability that those cells would bypass senescence by the type II pathway. Long telomeres recombine more efficiently and therefore favor the utilization of the type II pathway over the type I pathway. This preference for long telomeres is clearly seen in telomerase-negative *rifΔ* mutants (**Fig. 4**). Rif1 and Rif2 inhibit MRX-mediated telomere end-resection[30]. In telomerase-negative *RIF+* strains, as telomeres become progressively shorter with each round of cell division, the amount of telomere-associated Rif1 and Rif2 declines[44]. Eventually, short telomeres lack sufficient Rif1 and Rif2 to inhibit the MRX complex, allowing end-resection, telomere uncapping and induction of senescence. In telomerase-negative strains that lacked Rif1 and Rif2, senescence was accelerated (**Figs. 3b** and **4a**) because MRX-mediated telomere uncapping probably occurs earlier after loss of telomerase in this background. In addition, the rate of telomere shortening was not accelerated in these strains, so telomerase-negative *rifΔ* mutants senesce with longer telomeres than telomerase-negative *RIF+* strains (**Fig. 4c**), resulting in an increase in the fraction of type II survivors (**Fig. 5**).

It is unclear why some cancer cells maintain telomeres by reactivating telomerase and why some cancer cells do so by using ALT. We suggest that the length of the telomeres in neoplastic cells just before activation of a telomere maintenance mechanism may have a key role in determining which mechanism is used. Our work predicts that mutations in genes that cause cells to senesce with long telomeres would favor the use of ALT so it will be of great interest to determine whether these genes are mutated in ALT cells.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

*Note: Supplementary information is available on the Nature Structural & Molecular Biology website.*

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### AUTHOR CONTRIBUTIONS

M.C. designed and carried out the experiments, analyzed the data and wrote the manuscript. J.C.D. did the statistical analysis of the STEX data. R.R. helped to analyze the data and write the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Yeast media and strains.** Standard yeast media and growth conditions were used[45]. Yeast strains used in this study are listed in **Supplementary Table 1**.

**Telomere PCR and sequencing.** Telomeres VI-R and I-L were amplified by PCR as described[21,25]. The telomere I-L PCR also amplified telomere XIV-R, which could be differentiated by analysis of the subtelomeric sequence just upstream of the telomeric repeats. Sequencing was done by GENEWIZ (http://www.genewiz.com). To catch the *est2Δ* cultures immediately after the emergence of survivors, STEX samples where >30% but <70% of the telomeres showed sequence divergence were pooled and analyzed (**Fig. 2b**). These threshold values were determined empirically. Most samples from survivors (as determined by the senescence assays) contain telomeres where >30% show sequence divergence. From our experience, samples where >70% of telomeres show sequence divergence do not yield very meaningful data.

**Scan statistics.** To determine which telomeres were preferentially elongated, a scanning statistic was carried out. The lengths of the undiverged regions of the sequenced telomeres were recorded, as well as whether an extension event occurred. The results were then ordered by telomere length (longest to shortest) and a scanning statistic was carried out using this list on the basis of whether or not an extension event occurred. The scanning statistic was scored by calculating a lambda using a generalized likelihood ratio test (GLRT) with window sizes ranging from five to half the number of telomeres considered. For this GLRT, the null hypothesis was that the probability of having an extension event within any given window of data was the same as an event occurring outside the window. After this calculation, the window with the smallest lambda value was considered to be the most significantly enriched for extension events. To calculate a *P* value for this lambda value, a permutation test was done by randomizing the data 20,000 times, each time calculating the most significant (smallest lambda) value. These random and ordered lambdas were then ranked and the ordered lambda's *P* value was assigned by: $P = \text{rank}/20{,}000$ (refs. 46,47).

**Senescence assays.** Each senescence assay started with *est2Δ*/+ diploids that were propagated for at least 50 generations before sporulation to ensure that telomeres were at a stable equilibrium length. Freshly dissected spores were allowed to form colonies on YPD agar plates after 2 days of growth at 30 °C, or ~25 population doublings. Cells from these colonies were serially passaged in liquid YPD medium at 24-h intervals. For each passage, the cell density in each culture was determined and the cultures were diluted back into fresh YPD medium at a cell density of $5 \times 10^4$ cells per ml. For the STEX experiments (**Fig. 1**), the cultures were diluted to a cell density of $5 \times 10^5$ cells per ml to reduce the number of population doublings between time points and to maximize the chances of catching the cultures soon after survivors had emerged.

**DNA isolation and denaturing in-gel hybridization.** Yeast genomic DNA was isolated using a Yeast DNA Extraction Kit (Thermo Scientific). The DNA was digested with XhoI restriction endonuclease before running on a 0.8% agarose gel. Denaturing in-gel hybridization using a telomeric CA oligonucleotide radiolabeled probe was done as described[48].


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