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26. Skin biopsies were taken at the time of referral from patients with TEN (at the interface between detached and nondetached skin), MPR (lesional skin), and from healthy controls (non–sun-exposed skin) following informed consent, with one part snap-frozen in liquid nitrogen and the other fixed in 4% paraformaldehyde and routinely processed. Immunohistochemistry was performed on cryosections as described (*20*), using mAb to FasL [A11, Alexis Corp., San Diego, CA (*8*)], mAb to Fas (UB2, Immunotech), and isotype controls.
27. Frozen tissue sections were overlaid with Fas-sensitive Jurkat (human T cell leukemia) cells as described (*21, 22*), with or without preincubation for 30 min with mAb to FasL (NOK1, 2.5 μg/ml, Pharmingen). Jurkat cell apoptosis was determined by flow cytometry using annexin-FITC (Pharmingen) (*23*).
28. Cells were preincubated for 24 hours with IVIG (30 mg/ml; Sando globulin, Novartis, Bern, Switzerland), vehicle [0.9% NaCl and saccharose (51 mg/ml)], or albumin (30 mg/ml in 0.9% NaCl), and thereafter susceptibility to rhsFasL was assessed (*24*).
29. Equimolar amounts of purified fusion proteins Fas-comp (*18*), TNFR1-comp, or albumin were immunoblotted with either IVIG or monoclonal mouse anti-human Fas antibody (ZB4, Immunotech), and then were revealed by using ECL (Amersham). IVIG did not bind to purified comp alone.
30. We thank G. Radlgruber-Steiger for technical assistance; A. Limat and D. Masson for advice; M. Pechère, I. Masouyé, J. Pugin, D. Guggisberg, and P. de Viragh for data and samples from patients; D. Wohlwend for help with fluorescence-activated cell sorting analysis; N. Fusenig for HaCaT cells; and P. Vassalli for discussions. Supported by grants from the Swiss National Science Foundation (L.E.F. and J.T.), the Ernst Schering Research Foundation, the Sir Jules Thorn Charitable Trust, the Ernst and Lucie Schmidheiny Foundation, the Ligue Genevoise contre le Cancer, and the Fondation Medic.

4 August 1998; accepted 16 September 1998

Two Modes of Survival of Fission Yeast Without Telomerase

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Deletion of the telomerase catalytic subunit gene *trt1*$^+$ in *Schizosaccharomyces pombe* results in death for the majority of cells, but a subpopulation survives. Here it is shown that most survivors have circularized all of their chromosomes, whereas a smaller number maintain their telomeres presumably through recombination. When the telomeric DNA-binding gene *taz1*$^+$ is also deleted, *trt1*$^-$ *taz1*$^-$ survivors use the recombinational mode more frequently. Moreover, the massive elongation of telomeres in *taz1*$^-$ cells is absent in the double mutant. Thus, Taz1p appears to regulate telomeric recombination as well as telomerase activity in fission yeast.

In most organisms, the DNA at chromosome ends (telomeres) consists of short GT-rich repeats that are synthesized by a ribonucleoprotein reverse transcriptase called telomerase. In the absence of telomerase, cells continuously lose their telomeric DNA because of incomplete DNA replication and eventually lose the ability to divide. This state is known as senescence.

The catalytic protein subunit of telomerase, TERT (telomerase reverse transcriptase), is phylogenetically conserved (*1*). In the fission yeast *Schizosaccharomyces pombe*, TERT is encoded by the *trt1*$^+$ gene (*2*). As expected for a telomerase mutant, *trt1*$^-$ cells progressively lose their telomeric DNA. They also lose viability, as evidenced by the appearance of irregularly shaped microcolonies, consisting mainly of elongated nondividing cells. Viability of *trt1*$^-$ cells drops to the lowest level around 120 divisions after germination. However, a subpopulation of cells survives: Larger, round colonies containing mostly normal-sized cells eventually reappear upon further restreaking of senescing colonies. Once formed, these survivor strains can continue forming round colonies and divide indefinitely (*2*).

We hypothesized that these *trt1*$^-$ cells might survive by a recombinational mode of telomere maintenance, which is mediated by the Rad52 recombination protein in telomerase-negative budding yeast *Saccharomyces cerevisiae* and *Kluveromyces lactis* (*3*). A characteristic feature of this mode of survival is the generation of rearranged and amplified telomeric or subtelomeric regions or both. We therefore isolated *trt1*$^-$ survivors by successively streaking for single colonies on plates and looked for telomere amplification by Southern (DNA) blot hybridization. A telomere-repeat probe and three additional probes that recognize distinct subregions of the telomere-associated sequence (TAS) were used (Fig. 1A) (*4*). Unexpectedly, DNA from *trt1*$^-$ survivors showed no hybridization signals with probes to telomeric repeats, to TAS1, or to TAS2 (Fig. 1B, left), indicating that at least 4 kilobases (kb) of telomeric and subtelomeric DNA was lost. A hybridization signal was observed with a TAS3 probe, which recognizes subtelomeric DNA located at least 5 kb from the chromosome end (Fig. 1B, right). Each independent isolate of survivors showed a uniquely rearranged pattern of subtelomeric DNA restriction fragments that was stably maintained. However, neither telomeric nor subtelomeric regions were amplified in survivors, and thus this mode of survival of *trt1*$^-$ cells differs from the recombination-dependent telomerase-negative survival described

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previously in budding yeast (*3*).

One way the *trt1*$^-$ survivor cells might bypass the need for telomerase would be to circularize their chromosomes. Indeed, when we analyzed DNA from *trt1*$^-$ survivors by pulsed-field gel electrophoresis (PFGE), we found that none of the three chromosomes entered the agarose gel (Fig. 2, A and B), as observed previously for a circular *S. pombe* chromosome II (*5*). This result is not a direct consequence of the *trt1* deletion itself, because DNA from younger generation *trt1*$^-$ cells migrated normally in PFGE. Not I–digested genomic DNA (*6*) was then analyzed by PFGE to look more directly for telomeric fusion events. As expected, the telomeric Not I fragments C, I, L, and M were absent in DNA from *trt1*$^-$ survivor cells, whereas internal restriction fragments were unperturbed (Fig. 2C, left). Probing with telomeric or TAS probes confirmed that the fragments missing in *trt1*$^-$ survivors were precisely the ones that carried telomeric DNA (Fig. 2C, middle). In addition, when the blot was reprobed with specific genes located in telomeric Not I fragments of chromosomes I and II (*7*), these fragments shifted in size exactly as expected for the fusion of the two telomeric fragments from the same chromosome (Fig. 2C, right). Chromosome III has no Not I sites, but similar experiments with Sfi I–digested *S. pombe* DNA showed that it too had fused ends (*7*, *8*). Therefore, we conclude that the *trt1*$^-$ survivor cells had circularized all three of their chromosomes (*9*). The simultaneous deletion of *tel1*$^+$ and *rad3*$^+$ in *S. pombe* has recently also been shown to cause telomeric DNA loss and chromosome circularization (*10*).

When *trt1*$^-$ cells were grown in liquid culture with successive dilution, survivors that maintained linear chromosomes and telomeric repeats were also observed (*11*). Cell growth was followed for *trt1*$^-$ and *trt1*$^+$ haploid strains after sporulation of the *trt1*$^+$/*trt1*$^-$ heterozygous diploid. Whereas the *trt1*$^+$ cells maintained a relatively constant generation time, that of *trt1*$^-$ cells gradually increased from ~2.2 to ~5 hours because of increased cell death (Fig. 3A). As the cells approached the point of lowest viability, the telomeric-repeat sequences shrank almost to the point of complete loss. However, in the later survivor generations, new restriction patterns of weak telomere hybridization were observed (Fig. 3B). For TAS hybridization, the result was more striking because survivor cells had greatly amplified TAS sequences. Survival with maintenance of linear chromosomes, presumably through telomeric recombination, is not as stable as survival by circularization because we observed subsequent loss of telomeric sequence and another round of senescence in some of the liquid cultures (*8*). The poor growth of double mutants in

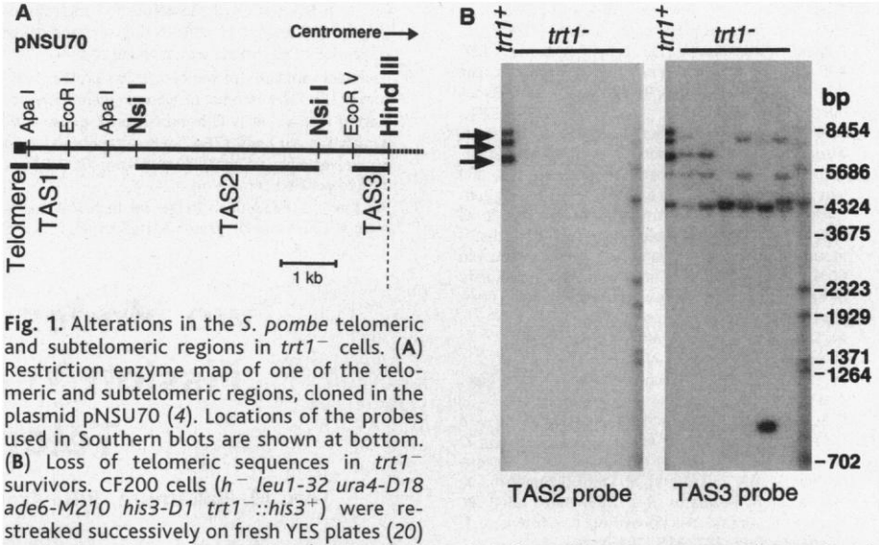

Fig. 1. Alterations in the *S. pombe* telomeric and subtelomeric regions in *trt1*$^-$ cells. (**A**) Restriction enzyme map of one of the telomeric and subtelomeric regions, cloned in the plasmid pNSU70 (*4*). Locations of the probes used in Southern blots are shown at bottom. (**B**) Loss of telomeric sequences in *trt1*$^-$ survivors. CF200 cells (*h*$^-$ *leu1-32 ura4-D18 ade6-M210 his3-D1 trt1*$^-$*::his3*$^+$) were restreaked successively on fresh YES plates (*20*) to obtain *trt1*$^-$ survivors. DNA (~18 μg) from six independent survivors was digested with Hind III, fractionated by electrophoresis in a 0.8% agarose gel, transferred to a nylon membrane, and hybridized to the TAS2 or TAS3 probe. Telomeric Hind III fragments from *trt1*$^+$ cells are indicated by arrows. Telomeric and TAS1 probes gave identical hybridization patterns to that of the TAS2 probe (*8*). Size markers are BstE II fragments of phage λ DNA, the sizes of some of which are indicated. bp, base pair.

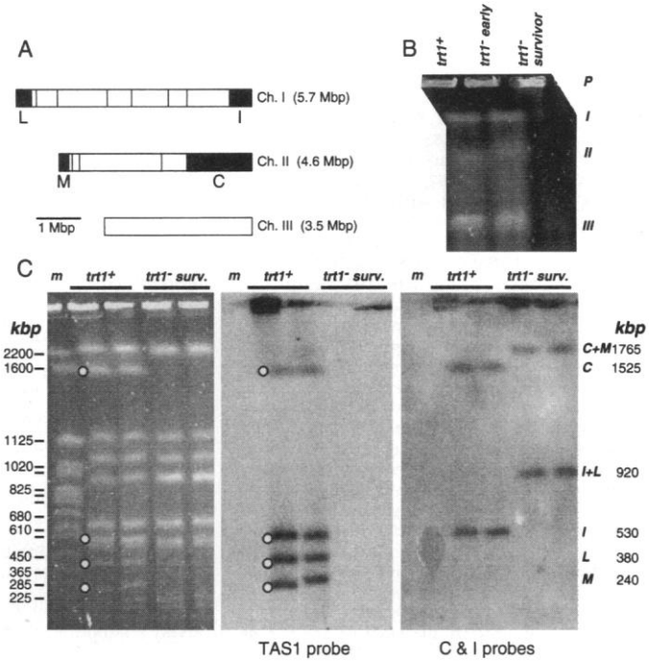

Fig. 2. PFGE fractionation and hybridization analysis of *S. pombe* chromosomal DNAs. (**A**) Not I restriction enzyme map of *S. pombe* chromosomes (Ch.) (*6*). The telomeric fragments C, I, L, and M are shown as black. (**B**) Intact *S. pombe* chromosomal DNA fractionated by PFGE. DNA was prepared from *trt1*$^+$, *trt1*$^-$ presenescent (early), and *trt1*$^-$ survivor cells in agarose plugs (P) (*20*). They were then fractionated in a 0.6% agarose gel with 0.5x TAE [40 mM tris-acetate (pH 8.0) and 1 mM EDTA] buffer at 14°C, with the CHEF-DR II system (Bio-Rad) at 1.5 V/cm (50 V) and a pulse time of 1800 s for 96 hours. DNA from CF199 (*h*$^-$ *leu1-32 ura4-D18 ade6-M210 his3-D1*), CF200, and CF348 (*h*$^-$ *leu1-32 ura4-D18 ade6-M210 his3-D1 trt1*$^-$*::his3*$^+$) was used for lanes designated *trt*$^+$, *trt1*$^-$ early, and *trt1*$^-$ survivor, respectively. (**C**) Not I–digested *S. pombe* chromosomal DNAs were fractionated in a 1% agarose gel with 0.5× TAE buffer at 14°C, with the CHEF-DR II system at 6 V/cm (200 V) and a pulse time of 60 to 120 s for 24 hours. Marker lane (m) contained *S. cerevisiae* chromosomal DNA (Bio-Rad). Not I–digested chromosomal DNA from two independent survivor colonies derived from CF200 was used for *trt1*$^-$ survivor (surv.) lanes, and Not I–digested 972 *h*$^-$ and CF199 DNA was used for *trt1*$^+$ lanes. (Left) Ethidium bromide–stained PFGE agarose gel. White dots indicate restriction fragments C, I, L, and M. (Middle) Hybridization of the same PFGE gel with the *S. pombe* TAS1 probe (Fig. 1A). Hybridization with telomeric probe or TAS2 probe produced an identical pattern (*8*). (Right) Hybridization with probes specific for Not I fragments C and I (*7*). Hybridization with probes against L and M fragments confirmed the identity of the novel fragments as C + M and I + L (*8*). kbp, kilobase pair.

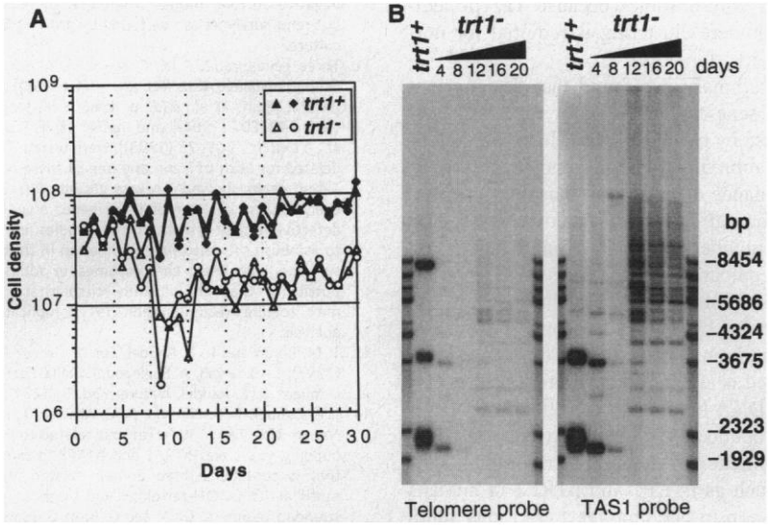

Fig. 3. Growth characteristics and altered telomeric regions of *trt1*$^-$ cells after germination. (**A**) Diploid strain CF248 (*h*$^+$/*h*$^-$ *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216 his3-D1/his3-D1 trt1*$^+$/*trt1*$^-$::*his3*$^+$) was sporulated, and the resulting tetrads were dissected and germinated on YES plates (*20*). The *trt1*$^+$ and *trt1*$^-$ cells were distinguished by growth on his$^-$ plates, and they showed the expected 2:2 segregation pattern. Colonies derived from each spore (two *trt1*$^+$ and two *trt1*$^-$) were grown at 32°C for 3 days and then picked and diluted at 5×10^4 cells/ml in 20 ml of YES. These cultures were grown for 24 hours at 32°C, at which point the cell density was determined by counting in a hemacytometer, and the cells were diluted into 20 ml of fresh YES liquid medium at 5×10^4 cells/ml. The remaining portion of each cell culture was collected by centrifugation, and cell pellets were frozen at −20°C for later preparation of genomic DNA. These procedures were repeated for 30 days. (**B**) Genomic DNA was prepared from *trt1*$^-$ cells collected every 4 days, digested with Nsi I, and fractionated by electrophoresis in a 0.8% agarose gel. Control lanes contain Nsi I–digested DNA from *trt1*$^+$ cells (CF199). DNA was then transferred to a nylon membrane and hybridized to the telomeric or TAS1 probe (Fig. 1A). Size markers are as in Fig. 1B.

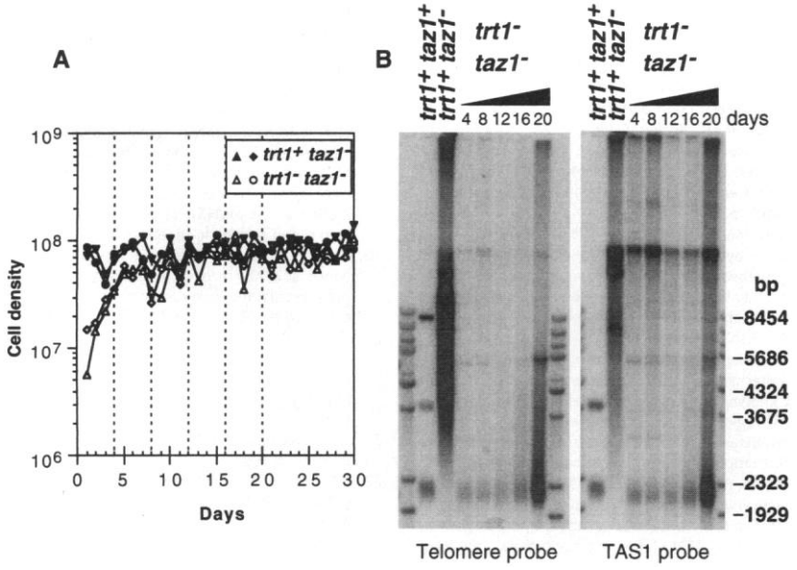

Fig. 4. Growth characteristics and altered telomeric regions of *trt1*$^-$ *taz1*$^-$ cells after germination. (**A**) Diploid strain CF382 (*h*$^+$/*h*$^-$ *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216 his3-D1/his3-D1 trt1*$^+$/*trt1*$^-$::*his3*$^+$ *taz1*$^+$/*taz1*$^-$::*ura4*$^+$) was sporulated, and the resulting tetrads were dissected and germinated on YES plates (*20*). Among the colonies derived from spores, two *trt1*$^+$ *taz1*$^-$ and two *trt1*$^-$ *taz1*$^-$ cells were chosen by restreaking them onto his$^-$ and ura$^-$ plates, and their growth characteristics were followed in YES liquid culture (Fig. 3A). (**B**) Genomic DNA was prepared from *trt1*$^-$ *taz1*$^-$ cells collected every 4 days, digested with Nsi I, and fractionated by electrophoresis in a 0.8% agarose gel. Control lanes contain Nsi I–digested DNAs from *trt1*$^+$ *taz1*$^+$ (CF199) and *trt1*$^+$ *taz1*$^-$ (CF213: *h*$^-$ *leu1-32 ura4-D18 ade6-M210 his3-D1 taz1*$^-$::*ura4*$^+$) cells. DNA was then transferred to a nylon membrane and hybridized to the telomeric or TAS1 probe.

trt1 and homologous recombination-related genes (*12*) has impeded a direct test of the requirements of recombination machinery for this less frequent mode of survival in *trt1*$^-$ cells. Nonetheless, the amplification of subtelomeric sequences is reminiscent of recombination-based survival observed in other yeast (*3*). Some immortalized human cell lines and tumor cells also maintain long telomeres by a telomerase-independent mechanism that is thought to involve recombination (*13*).

We next tested how survival in the absence of telomerase was affected by deletion of the *taz1*$^+$ gene, which encodes a protein (Taz1p) that binds specifically to *S. pombe* telomeric DNA (*14*). Deletion of *taz1* disrupts telomeric chromatin structure, relieves transcriptional repression at telomeres, and causes massive elongation (~10 times increase) of the telomeric DNA tract (*14*). Four different ways of creating *trt1*$^-$ *taz1*$^-$ strains were tested for effects on survival on plates. Deletion of the *taz1* gene from *trt1*$^-$ cells resulted only in cells with circular chromosomes, whereas deletion of the *trt1* gene from *taz1*$^-$ cells resulted only in cells with linear chromosomes (*8*). These two survival outcomes may reflect a predisposition toward either circularization or recombination in cells that started out with shorter or longer telomeres, respectively. In contrast, dissection of spores from a *trt1*$^+$/*trt1*$^-$ *taz1*$^+$/*taz1*$^-$ heterozygous diploid (which had normal telomere length) or spores created by mating *taz1*$^-$ and *trt1*$^-$ cells produced a mixture of cells containing either linear or circular chromosomes upon successive restreaking on agar plates (*15*). Therefore, the balance was shifted toward the presumed recombination mode of survival in the *taz1*$^-$ background because survivors with linear chromosomes could be easily found even on plates. In addition, unlike the *trt1*$^-$ survivors with linear chromosomes that were unstable and still subject to telomere shortening and senescence, the *trt1*$^-$ *taz1*$^-$ cells containing linear chromosomes were stable survivors. The absence of Taz1p creates a more open chromatin structure at telomeres (*14*, *16*), which conceivably gives recombination enzymes better access to telomeric DNA.

We also noticed that sporulation of the heterozygous diploid produced *trt1*$^-$ *taz1*$^-$ cells that appeared to go through an initial low-viability phase after germination (Fig. 4A) (*17*). Thus, it appears as if the loss of *trt1* in a *taz1*$^-$ background accelerates the onset of senescence by at least 100 generations compared with loss of *trt1* in a *taz1*$^+$ background. We suggest that absence of Taz1p from the chromosome ends in a telomerase-negative cell results in an increased rate of chromosome fusion and rearrangement, with concomitant loss of cell viability. Similarly,

the onset of senescence in *trt1*$^-$ cells coincided with the loss of telomeric-repeat sequences, the binding sites for the Taz1p at chromosomal ends. It is interesting that the *taz1* deletion alone does not result in this loss of viability (Fig. 4A). Perhaps the TERT protein itself, in conjunction with Taz1p, is directly involved in maintaining stability at telomeric ends.

The identification of the *trt1*$^+$ gene enabled us to address whether the telomere elongation seen in *taz1*$^-$ cells is caused by telomerase or by recombination (*14*). The much weaker intensity of the telomeric hybridization signals in the *trt1*$^-$ *taz1*$^-$ cells than in the *taz1*$^-$ cells (Fig. 4B) (*18*) indicates that telomerase is largely responsible for the massive elongation of telomere tracts in *taz1*$^-$ cells. On the other hand, telomere tracts of *trt1*$^-$ *taz1*$^-$ cells remained heterogeneous, probably because of increased recombination at telomeric regions (*3*).

Generation times were determined for mitotic growth of stable survivors (*19*). The *taz1*$^-$ and *trt1*$^-$ *taz1*$^-$ cells with linear chromosomes had generation times (116 ± 3 and 119 ± 6 min, respectively) slightly less than that of wild-type cells (127 ± 2 min). In contrast, *trt1*$^-$ and *trt1*$^-$ *taz1*$^-$ cells with circular chromosomes grew ~30% slower (165 ± 5 and 162 ± 4 min, respectively). When DNA was stained with DAPI (4',6-diamidino-2-phenylindole) (*20*), missegregated DNA was commonly seen in strains with circular chromosomes but rarely in cells with linear chromosomes (*8*); missegregation of circular chromosomes may account for slower growth. Previous studies have shown that large circular minichromosomes in fission yeast are not stably maintained, but overexpression of topoisomerase II can rescue this instability (*21*).

In contrast to the relatively small defects in mitotic growth caused by circularized chromosomes, sexual reproduction was severely affected in all three types of stable survivors. In both *trt1*$^-$ and *trt1*$^-$ *taz1*$^-$ cells that carried circular chromosomes, maturation of the spore itself seemed to be affected, because many asci showed only one or two spores and their shapes were aberrant as well (*8*). Meiotic recombination of circular chromosomes is expected to produce dicentric chromosomes that fail to segregate. Meiosis was also defective in the *trt1*$^-$ *taz1*$^-$ cells with linear chromosomes, whereas presenescent *trt1*$^-$ cells that still contained linear chromosomes did not have any visible defects in meiosis (*8*). Therefore, meiotic defects in survivors may also be due to the absence of Taz1p or the absence of Taz1p-binding sites in the case of *trt1*$^-$ cells with circular chromosomes because Taz1p-mediated telomere clustering is required for normal meiosis (*14*, *22*).

In summary, we found that fission yeast escape senescence caused by the loss of telomerase by two distinct mechanisms: (i) circularization of all their chromosomes and (ii) maintenance of linear chromosomes presumably through telomere recombination. The small number of chromosomes in fission yeast compared with *S. cerevisiae* or mammals probably helps favor chromosome circularization by intrachromosomal fusion. The ratio at which these two types of survivors are observed is altered upon deletion of the telomere DNA-binding protein Taz1p. Thus, Taz1p bound at telomeres may suppress their recombination. Other telomere-binding proteins such as hTRF1 and hTRF2 in humans and Rap1p in budding yeast (*14*) may function in a similar manner and thus may also affect survival in the absence of telomerase in these organisms.

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7. Probes specific for telomeric Not I fragments were created by random-primed labeling of gel-purified polymerase chain reaction products with α-^{32}P deoxycytidine triphosphate. Sequences of DNA primers are available at www.sciencemag.org/feature/data/984371.shl
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11. Survival with maintenance of linear chromosomes was not observed when cells were successively restreaked on plates; ~40 independent survivor strains all showed the complete loss of telomeric, TAS1, and TAS2 hydridization indicative of circular chromosomes. Liquid culture selects for cells that grow fastest and allows more cells to explore telomerase-negative survival modes. Therefore, more efficient but rarer survivors can be found by growing in liquid culture.
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15. DNA prepared from the *trt1*$^-$ *taz1*$^-$ cells with circular chromosomes failed to enter the PFGE gels and lost the hybridization signal for telomeric-repeat, TAS1, and TAS2 probes, much like the *trt1*$^-$ cells with circular chromosomes. In contrast, DNA prepared from the *trt1*$^-$ *taz1*$^-$ cells with linear chromosomes ran normally in the PFGE gels and maintained telomere and TAS hybridization signals.
16. J. P. Cooper and T. R. Cech, unpublished observations.
17. Although there were no discernible size differences in the initial colonies formed by *trt1*$^-$ *taz1*$^-$ spores compared with *trt1*$^+$ *taz1*$^+$, *trt1*$^-$ *taz1*$^+$, or *trt1*$^+$ *taz1*$^-$ spores upon germination, restreaking the *trt1*$^-$ *taz1*$^-$ cells from these initial colonies onto agar plates produced colonies with large size variations. Many of the *trt1*$^-$ *taz1*$^-$ cells taken from smaller colonies were elongated, much like the senescing *trt1*$^-$ cells. Normal-sized cells that grew better eventually appeared, much as in the case of the *trt1*$^-$ survivors.
18. When *trt1*$^-$ *taz1*$^-$ cells were created by the deletion of *trt1* from *taz1*$^-$ cells, a gradual decrease in the telomeric hybridization signal was observed. By ~100 cell divisions after the deletion of *trt1*$^+$, the telomeric hybridization signal appeared to reach a new equilibrium, and no further loss of signal was observed. Subsequent reintroduction of the wild-type *trt1*$^+$ gene into these *trt1*$^-$ *taz1*$^-$ cells by transformation of the pWH5-trt1 plasmid produced a strong increase in telomeric-repeat hybridization signal.
19. Growth rates were determined by measuring absorbance at 600 nm of exponentially growing cells in yeast extract medium supplemented (YES) liquid culture (*20*) at 32°C every 30 min.
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23. We thank J. Sperger for discussions and comments on the manuscript, other members of T.R.C.'s lab for discussions, and N. Sugawara for the pNSU70 plasmid. Supported by NIH grant GM28039 (T.R.C.).

5 August 1998; accepted 8 September 1998