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Recombination-Based Telomere Maintenance Is Dependent on Tel1-MRN and Rap1 and Inhibited by Telomerase, Taz1, and Ku in Fission Yeast▽†

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Fission yeast cells survive loss of the telomerase catalytic subunit Trt1 (TERT) through recombination-based telomere maintenance or through chromosome circularization. Although *trt1*Δ survivors with linear chromosomes can be obtained, they often spontaneously circularize their chromosomes. Therefore, it was difficult to establish genetic requirements for telomerase-independent telomere maintenance. In contrast, when the telomere-binding protein Taz1 is also deleted, *taz1*Δ *trt1*Δ cells are able to stably maintain telomeres. Thus, *taz1*Δ *trt1*Δ cells can serve as a valuable tool in understanding the regulation of telomerase-independent telomere maintenance. In this study, we show that the checkpoint kinase Tel1 (ATM) and the DNA repair complex Rad32-Rad50-Nbs1 (MRN) are required for telomere maintenance in *taz1*Δ *trt1*Δ cells. Surprisingly, Rap1 is also essential for telomere maintenance in *taz1*Δ *trt1*Δ cells, even though recruitment of Rap1 to telomeres depends on Taz1. Expression of catalytically inactive Trt1 can efficiently inhibit recombination-based telomere maintenance, but the inhibition requires both Est1 and Ku70. While Est1 is essential for recruitment of Trt1 to telomeres, Ku70 is dispensable. Thus, we conclude that Taz1, TERT-Est1, and Ku70-Ku80 prevent telomere recombination, whereas MRN-Tel1 and Rap1 promote recombination-based telomere maintenance. Evolutionarily conserved proteins in higher eukaryotic cells might similarly contribute to telomere recombination.

Telomeres are specialized DNA-protein complexes at the ends of linear chromosomes and function as protective caps to prevent normal chromosomal ends from end-to-end fusions and recombinational events (13). In most eukaryotic organisms, telomeric DNA is composed of short GT-rich repeat sequences. This DNA is synthesized by telomerase, a reverse transcriptase (RT) tightly associated with its own template RNA. The very end of telomeric DNA is a 3′ single-stranded GT-rich region, known as the G-tail. When cells replicate linear chromosomes without telomerase, telomeric DNA is gradually lost due to the inability of conventional DNA polymerases to fully replicate the ends of DNA molecules. Since GT-rich telomeric repeats provide binding sites for telomere-specific proteins that are important in creating protective caps at telomeres (14), shortening or loss of telomeric repeats leads to DNA damage checkpoint activation and "repair" (fusion and recombination) of telomeric DNA ends.

Given the telomere's ability to suppress DNA repair and DNA damage checkpoint responses, one might expect that functional telomeres would have the ability to prevent detection by DNA repair and DNA damage checkpoint proteins. However, DNA double-strand break (DSB) repair protein complexes, such as Ku70-Ku80 and Mre11-Rad50-Nbs1 (MRN), are bound to telomeres and necessary for normal telomere maintenance (63). Likewise, the checkpoint sensor complexes Rad1-Rad9-Hus1, Rad17-Rfc2-5, ATM, and ATR-ATRIP associate with telomeres and contribute to telomere maintenance (46, 56, 64).

The catalytic subunit of telomerase is known as telomerase RT (TERT) (49). In the fission yeast *Schizosaccharomyces pombe*, TERT is encoded by *trt1*$^+$ gene (45), and *trt1*Δ cells progressively lose their telomeric DNA. The viability of *trt1*Δ cells drops to the lowest level around 120 divisions after deletion of *trt1*$^+$, but cells eventually recover in growth and establish survivors. Most cells survive by circularizing all chromosomes to bypass the need for telomerase, while some cells survive by maintaining their telomeres, presumably through recombination (44). The telomerase regulatory subunit Est1 is also essential for telomerase-dependent telomere maintenance (4).

Taz1 is a telomeric double-stranded DNA-binding protein in *S. pombe* (12) thought to be the counterpart of two mammalian telomere proteins TRF1 and TRF2 (53). Taz1 is important for recruitment of Rap1 and Rif1 to telomeres (10, 30), and deletion of the *taz1*$^+$ gene leads to massive telomere elongation by telomerase (12, 44). Taz1 also promotes replication of telomeric GT-rich repeats by DNA polymerases (39), and telomeres in *taz1*Δ cells are less protected against fusions by nonhomologous end-joining (NHEJ) mediated by Ku70-Ku80 and ligase IV (19). Interestingly, simultaneous deletion of *taz1* and *trt1* genes allows cells to robustly maintain telomeres rather than circularizing chromosomes (44). However, it remained unclear how telomeres are maintained in *taz1*Δ *trt1*Δ cells.

Studies in other organisms have established that telomerase-negative cells can survive loss of telomeric repeats by mecha-

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nisms that involve homologous recombination (HR) among telomeres (16, 47, 58). In *Saccharomyces cerevisiae*, these recombination-based survival mechanisms have been divided into two major types: type I survivors are characterized by amplification of subtelomeric Y′ elements but have relatively short telomeric GT-rich repeats, whereas type II survivors are characterized by long telomeric GT-repeat tracts. The HR protein Rad52 is required for generation of both types of survivors (58), whereas Rad51, Rad54, and Rad57 are essential for generation of type I survivors. The Mre11-Rad50-Xrs2 (MRX) complex and Sgs1 DNA helicase are important for generation of type II survivors (27, 57). Furthermore, Tel1 (ATM) and Mec1 (ATR) kinases play redundant roles in generating type II survivors (60).

In the present study, we identify Rad22 (an ortholog of *S. cerevisiae* Rad52), MRN, Tel1 (ATM), and Rap1 as contributing positively to recombination-based telomere maintenance in *taz1*Δ *trt1*Δ cells. Unexpectedly, efficient Taz1-dependent binding of Rap1 to telomeres is not required to promote recombination-based telomere maintenance. We also show that TERT-Est1, Taz1, and Ku70-Ku80 represent three redundant but interdependent mechanisms that operate at telomeres in preventing telomere recombination. Surprisingly, the ability of TERT to negatively regulate telomere recombination is not dependent on its RT activity and requires only the N-terminal half of the protein.

MATERIALS AND METHODS

Yeast strains. The fission yeast strains used in the present study were constructed by standard techniques (1) and are listed in Table S1 in the supplemental material. Original sources for most mutations and epitope-tagged genes utilized in the present study were previously described (46), except for *est1*Δ (4), *rap1*Δ (30), and *rap1-HA* (30). For liquid growth curve experiments, appropriate heterozygous diploid cells were sporulated on ME plates, and the resulting tetrads were dissected on a YES plate (1). Genotypes of haploid strains were then determined based on growth on PMG minimal medium lacking selective amino acids. Triple mutant strains (*taz1*Δ *trt1*Δ plus additional deletion) used in Fig. 2 were generated by deleting the indicated gene from *taz1*Δ *trt1*Δ survivor cells by transformation of a deletion construct DNA or by genetic cross of a *taz1*Δ *trt1*Δ survivor strain with a strain carrying the desired deletion mutation. Triple mutant cells generated by transformation of *taz1*Δ *trt1*Δ survivor cells may not be epigenetically identical to triple mutant cells generated by genetic cross. However, for *taz1*Δ *trt1*Δ *rad32*Δ cells, strains produced by transformation and strains produced by genetic cross all generated survivor cells carrying circular chromosomes after repeated restreaking on agar plates. The remaining triple mutant strains (*taz1*Δ *trt1*Δ *rad22*Δ, *taz1*Δ *trt1*Δ *rad50*Δ, *taz1*Δ *trt1*Δ *nbs1*Δ, *taz1*Δ *trt1*Δ *tel1*Δ, and *taz1*Δ *trt1*Δ *rad3*Δ) were generated by genetic crosses, whereas *taz1*Δ *trt1*Δ *pku70*Δ and *taz1*Δ *trt1*Δ *est1*Δ strains were generated by transformation. *taz1*Δ *trt1*Δ *pku70*Δ *tel1*Δ strains were generated by transforming *taz1*Δ *trt1*Δ *pku70*Δ cells with a *tel1*Δ construct. *taz1*Δ *est1*Δ strains were generated by transforming *taz1*Δ cells with an *est1*Δ construct. *taz1*Δ *pku70*Δ *est1*Δ strains were generated by transforming *taz1*Δ *pku70*Δ cells with an *est1*Δ construct. *rap1*Δ *trt1*Δ cells were generated either by crossing *trt1*Δ cells carrying pWH5-trt1$^+$ plasmid with *rap1*Δ cells or by transforming *rap1*Δ cells with a *trt1*Δ construct. *taz1*Δ *trt1*Δ *rap1*Δ strains were generated by transforming *taz1*Δ *trt1*Δ cells with a *rap1*Δ construct. Except for liquid growth curve experiments, cells were extensively restreaked on plates to ensure that cells reached their terminal phenotype before preparation of the chromosomal DNA plugs or genomic DNA.

Plasmids. Plasmid pREP81x-taz1$^+$ carries *taz1*$^+$ gene behind the weakest version of *nmt1* promoter, *S. cerevisiae LEU2* gene, and *S. pombe* autonomous replication sequence (*ars1*$^+$). Plasmid pKAN-trt1$^+$ contains the ~5.5-kb *S. pombe* genomic KpnI fragment bearing the *trt1*$^+$ gene, *kanMX4* marker, and *S. pombe ars1*$^+$ (23). Plasmids pKAN-trt1-D590A, pKAN-trt1-D743A, pKAN-trt1-ΔNsi, pKAN-trt1-ΔPac, and pKAN-trt1-Δ[Nde-Xho] are essentially the same as pKAN-trt1$^+$, except that they carry the indicated mutant alleles. Plasmids pKAN-trt1:Cmyc9, pKAN-trt1-D590A:Cmyc9, and pKAN-trt1-D743A:Cmyc9 express the indicated Trt1 alleles with the myc9 tag at the C terminus. Plasmid pWH5-trt1$^+$ contains the ~11-kb *S. pombe* genomic HindIII fragment bearing the *trt1*$^+$ gene, *S. cerevisiae LEU2* gene, and *S. cerevisiae* 2μ replication origin.

Cell growth analysis. Liquid culture growth curve experiments were performed essentially as previously described (46). After cell cultures were used to inoculate 4×10^4 cells/ml in fresh YES liquid medium, the remaining cells were collected by centrifugation, washed once in SP1 buffer (1), and frozen at −80°C for later preparation of agarose plugs.

Pulsed-field gel electrophoresis (PFGE). Chromosomal DNA samples were prepared in agarose plugs, and NotI-digested DNA samples were fractionated with the CHEF-DR III system (Bio-Rad) as previously described (46). The telomeric repeat probe and C, I, L, and M probes were prepared as previously described (44).

ChIP analysis. Exponentially growing cells were processed for chromatin immunoprecipitation (ChIP) analysis as previously described (46, 48). Protein G-Sepharose beads (GE Amersham) were added to whole-cell extracts preincubated with monoclonal antibody 9B11 (anti-myc), 12CA5 (anti-HA), or no antibody (mock). After extensive washes, bead-bound DNA was recovered by using Chelex-100 resin (Bio-Rad) (48). Recovered DNA from ChIP was analyzed by SYBR green-based real-time PCR (Bio-Rad) using BAM136 and BAM137 primers (TAS1 region [46]). Fold enrichment values were calculated based on difference in Ct values between ChIP (antibody) and mock (no antibody) samples. For dot blot analysis, ChIP and input DNA samples were denatured by boiling at 100°C for 10 min in 0.4 M NaOH and 10 mM EDTA, snap chilled on ice, and blotted onto a Hybond XL membrane (GE Amersham Biosciences). Dot blots were then hybridized with a telomeric probe, and hybridization signals were quantified by using ImageQuant software. Percent precipitated DNA for ChIP samples were calculated as 100 × (ChIP – mock)/input.

RESULTS

***taz1*Δ *trt1*Δ cells show accelerated telomere fusions compared to *trt1*Δ cells.** Haploid *taz1*Δ *trt1*Δ cells generated by sporulation of heterozygous diploid *taz1*Δ/*taz1*$^+$ *trt1*Δ/*trt1*$^+$ cells reproducibly went through an initial low-viability phase soon after germination (44) (Fig. 1D; see also Fig. S1D in the supplemental material), although there was no discernible difference in the initial size of colonies or spore viability (see Fig. S1A and B in the supplemental material). In contrast, *trt1*Δ cells initially grew well, reached their slowest growth phase around day 10, and then recovered (Fig. 1B and C; see also Fig. S1C in the supplemental material). In both cases, reduction in cell growth rate was accompanied by an increase in appearance of highly elongated cells that can no longer divide and frequently contain fragmented DNA stuck between two dividing nuclei (44; data not shown).

To better understand the cause of the low viability exhibited by *taz1*Δ *trt1*Δ and *trt1*Δ cells, we analyzed changes in telomere stability by PFGE of NotI-digested chromosomal DNA during the course of liquid cell growth experiments (Fig. 1). When chromosomal DNA of *trt1*Δ cells was analyzed by Southern blotting with probes corresponding to telomeric fragments (C, I, L, and M probes, Fig. 1A), a heterogeneous distribution of slower-migrating bands gradually appeared as cells divided and became sick (Fig. 1B and C, right panels). Based on sequential hybridization with individual probes, we confirmed that some of these altered mobility bands represent inter- and intrachromosomal fusions (data not shown). These fusion bands no longer contain telomeric GT-rich repeats (Fig. 1B and C, middle panels), indicating that fusion events might occur after the total loss of telomeric repeats. Alternatively, the fusion events could be responsible for the loss of telomeric repeats. In any case, interchromosomal fusions, which create dicentric chromosomes, are detrimental to cell viability. Therefore, nondisjunction of fused chromosomes and/or fusion-breakage cycles, caused by uncapped chromosomal ends, are likely to be re-

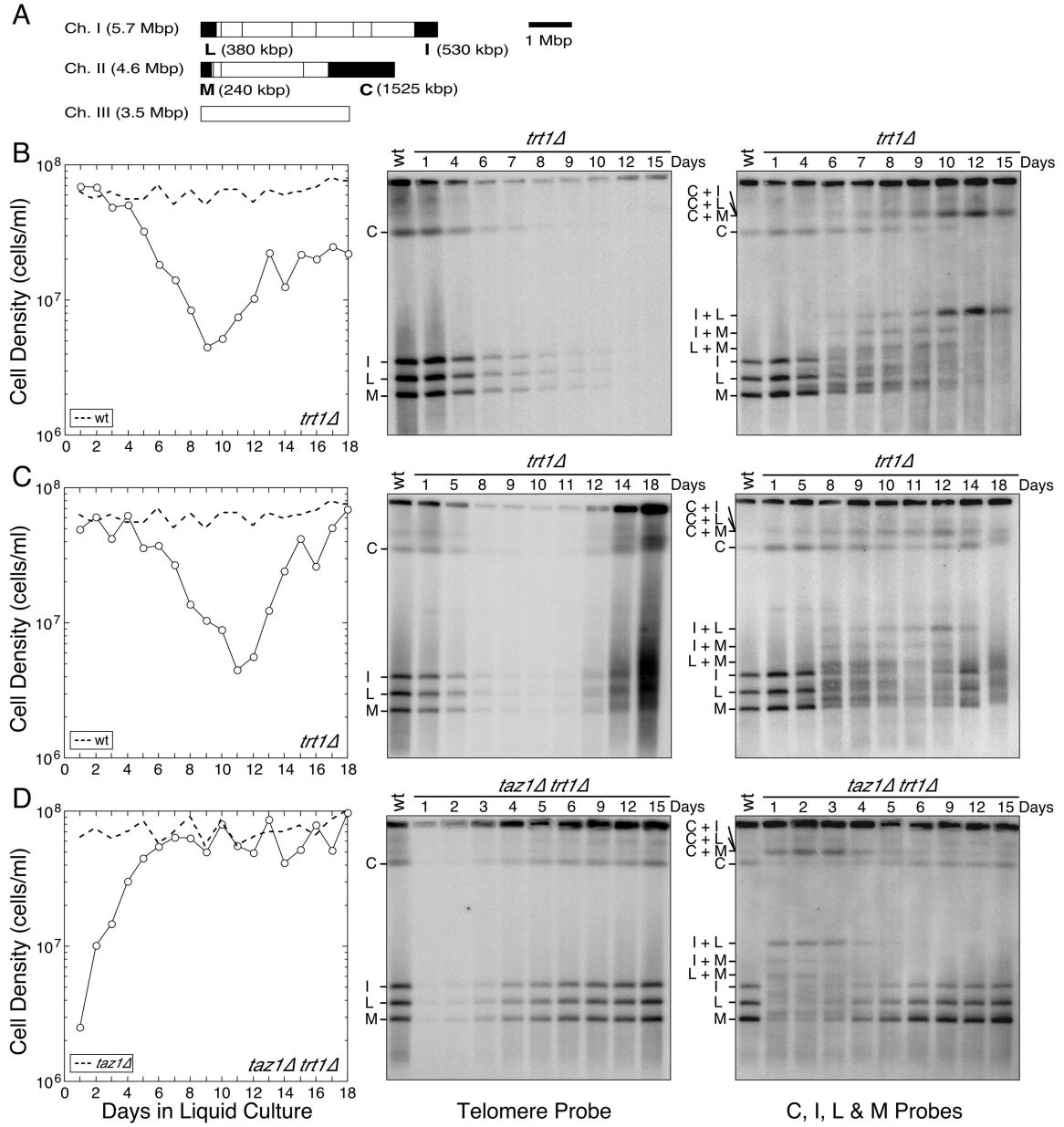

FIG. 1. PFGE analysis for *trt1*Δ and *taz1*Δ *trt1*Δ cells. (A) NotI restriction map of *S. pombe* chromosomes. The telomeric fragments C, I, L, and M are filled in black. (B to D) *trt1*Δ/*trt1*$^+$ or *taz1*Δ/*taz1*$^+$ *trt1*Δ/*trt1*$^+$ diploid strains were sporulated, and *trt1*Δ (B and C) or *taz1*Δ *trt1*Δ (D) cells were selected. For *trt1*Δ cells, two independent cultures, which produced survivors with an intermediate growth rate (B) or a wild-type-like growth rate (C), are shown. The left panels show the growth characteristics of cells after germination in liquid culture. For reference, results from growth curve experiments for wild-type (*trt1*$^+$) or *taz1*Δ cells are also plotted (dotted lines). The middle panels show PFGE results for NotI-digested *S. pombe* chromosomal DNA hybridized with telomere GT-rich repeat specific probe. The right panels show PFGE results for NotI-digested *S. pombe* chromosomal DNA hybridized with probes for the C, I, L, and M bands.

sponsible for a loss of cell viability in *trt1*Δ cells. We also found that when our blots are hybridized to C, I, L, and M probes, additional diffused bands are detected just above the I, L, and M bands when the cells are in the low-viability phase (Fig. 1B and C). We are uncertain of the exact identities of these bands, but they do not seem to contain much telomeric GT-rich repeat sequences since they are not detected by a telomere probe. These diffused bands might be created by fusion-breakage cycles, or they might represent DNA repair intermediates, which could run abnormally on the pulsed-field gel due to their structures.

Although the decline in growth rate for independent *trt1*Δ liquid cultures was very reproducible, the extent of recovery was not always the same (Fig. 1B and C; see Fig. S1C in the supplemental material) (44, 46). For the majority of cultures tested, survivors initially grew at a reduced rate compared to *trt1*$^+$ cells and had circular chromosomes (Fig. 1B). Fewer cultures generated survivors that grew similar to *trt1*$^+$ cells and

maintained linear chromosomes, although telomeric bands became much more heterogeneous in later generations (Fig. 1C). Even when cultures were able to establish fast-growing survivors, the growth rate was not always stable, and these cultures sometimes went through a second phase of low viability and slow growth (44; data not shown). Conversely, cultures containing predominantly circular chromosome survivors were often able to improve to a faster-growing state with very diffused weak telomeric bands after an extended period of selection beyond 2 to 3 weeks (data not shown). Such recovery is possible since extremely rare but faster-growing survivors within a population can eventually take over more common but slower-growing survivors in a competitive liquid culture growth environment (3). When telomere repeat length and telomere-associated sequence (TAS) pattern were analyzed by Southern blot hybridization of conventional agarose gels, serially diluted *trt1*Δ liquid cultures often displayed telomere elongation reminiscent of type II survivors in *S. cerevisiae* (see Fig. S4 and S5 in the supplemental material). However, as we have previously reported, these survivors are not always stable and cultures often went through repeated rounds of telomere attrition and low-viability phases (44; data not shown).

For *taz1*Δ *trt1*Δ cells, a heterogeneous distribution of slower-migrating NotI telomeric bands, characteristic of inter- and intrachromosome fusions, was observed at the very early low-viability phase (Fig. 1D, right panel, days 1 to 3), and telomeric repeats were lost from these fused telomeric fragments (Fig. 1D, middle panel). On the other hand, *taz1*Δ *trt1*Δ survivors that arose later (days 9 to 15) grew very well and were able to stably maintain linear chromosomes with sharp distinct bands for telomeric NotI fragments, which also contained stable telomeric repeats (Fig. 1D; see Fig. S1D in the supplemental material). Thus, the absence of Taz1 in *trt1*Δ cells resulted in a much earlier occurrence of telomere fusions, but *taz1*Δ *trt1*Δ cells were eventually able to reproducibly establish survivors with very stable telomeres. When these *taz1*Δ *trt1*Δ survivor cultures were analyzed for telomere length and the TAS region, they were found to contain heterogeneous telomeres and amplified TAS1 sequences (see Fig. S4 and S5 in the supplemental material). Since these cultures contain elongated telomeres, these survivors could be similar to type II survivors in budding yeast. On the other hand, amplified TAS is reminiscent of type I survivors in budding yeast. In any case, these observations indicated that Taz1 and TERT could have partially redundant roles in protecting telomeres from immediate fusions and in regulating recombination events at telomeres.

DNA repair and checkpoint proteins affect telomere maintenance in *taz1*Δ *trt1*Δ cells. Although *trt1*Δ survivor cells with linear chromosomes can be obtained, they often spontaneously circularize their chromosomes. Therefore, it was difficult to establish genetic requirements for telomerase-independent telomere maintenance in *trt1*Δ cells. In contrast, *taz1*Δ *trt1*Δ survivor cells are able to stably maintain telomeres, and thus they can be utilized as a valuable tool in understanding the regulation of telomerase-independent telomere maintenance. In this setup, mutations that lead to the conversion of chromosome structures from linear to circular could identify genes involved in the promotion of telomerase-independent telomere maintenance and/or in protection of telomeres against telomere fusions. One should keep in mind, however, that the genetic requirement for telomere recombination in *taz1*Δ *trt1*Δ and *trt1*Δ cells may not be completely identical.

When the gene for the HR protein Rad22 (Rad52) was eliminated from *taz1*Δ *trt1*Δ cells, telomeric NotI bands (C, I, L, and M) were converted to C+M and I+L bands indicative of circular chromosomes (Fig. 2A). These fusion bands also lacked telomeric repeat sequences (data not shown). Therefore, Rad22-mediated HR is required for telomerase-independent telomere maintenance, much as in *S. cerevisiae*. In contrast, the NHEJ protein Ku70 was not required to maintain telomeres in *taz1*Δ *trt1*Δ cells since elimination of Ku70 did not lead to chromosome circularization (Fig. 2A). We can rule out the possibility that Ku70 is essential for chromosome end fusion, making it impossible for *taz1*Δ *trt1*Δ *pku70*Δ to circularize their chromosomes, based on observations that circular chromosomes can still be generated in *trt1*Δ *pku70*Δ cells (3), *taz1*Δ *trt1*Δ *pku70*Δ *tel1*Δ cells (Fig. 2A), *taz1*Δ *pku70*Δ *est1*Δ cells (Fig. 2A), or *taz1*Δ *trt1*Δ *pku70*Δ cells carrying a *taz1*$^+$ plasmid (see Fig. 5C). Interestingly, after Ku is eliminated, the average telomere size appeared to increase compared to the parental *taz1*Δ *trt1*Δ cells (see Fig. S6 in the supplemental material). Such a phenotype may be an indication that recombination-based telomere maintenance of *taz1*Δ *trt1*Δ cells becomes even more efficient in the absence of Ku.

Next, we found that elimination of any of the MRN complex components (Rad32, Rad50, and Nbs1) or Tel1 leads to chromosome circularization and the complete loss of telomeric repeats in *taz1*Δ *trt1*Δ cells (Fig. 2A; data not shown). Therefore, the MRN complex and Tel1 contribute positively to telomerase-independent telomere maintenance. They may be important either for telomere-telomere recombination or the protection of telomeres from fusions. It was previously shown that the MRN complex and Tel1 work in the same telomere maintenance pathway in fission yeast (8, 46) and that Nbs1-Tel1 interaction recruits Tel1 to sites of DNA damage (67).

Because *S. cerevisiae* Tel1 has been shown to be important in the prevention of NHEJ-dependent telomere fusions in the absence of telomerase (9), we also examined whether the chromosome circularization phenotype we observed for *taz1*Δ *trt1*Δ *tel1*Δ cells depends on Ku by deleting *tel1*$^+$ gene from *taz1*Δ *trt1*Δ *pku70*Δ survivor cells. Since *taz1*Δ *trt1*Δ *pku70*Δ *tel1*Δ cells still circularized their chromosomes (Fig. 2A), Tel1 is likely to prevent telomere fusions by promoting a mechanism(s) essential for telomere maintenance in *taz1*Δ *trt1*Δ cells rather than inhibiting Ku-dependent NHEJ at telomeres. On the other hand, a previous study has found that telomere-telomere fusions can be generated by a Ku-independent but ligase IV-dependent mechanism (37). Therefore, it is still possible that Tel1 may be involved in the inhibition of ligase IV-dependent fusions in *taz1*Δ *trt1*Δ cells.

In *S. pombe*, the MRN-Tel1 pathway and the Rad3-Rad26 (corresponds to *S. cerevisiae* Mec1-Ddc2 or mammalian ATR-ATRIP) pathway function redundantly to prevent telomere dysfunction and chromosome circularization (8, 42, 46). Accordingly, we tested whether the Rad3 checkpoint kinase might have a similar function as Tel1 in telomerase-independent telomere maintenance in *taz1*Δ *trt1*Δ cells. However, in contrast to Tel1, the elimination of Rad3 (ATR) checkpoint kinase did not lead to chromosome circularization in *taz1*Δ *trt1*Δ cells (Fig. 2A). Therefore, Rad3 does not appear to be

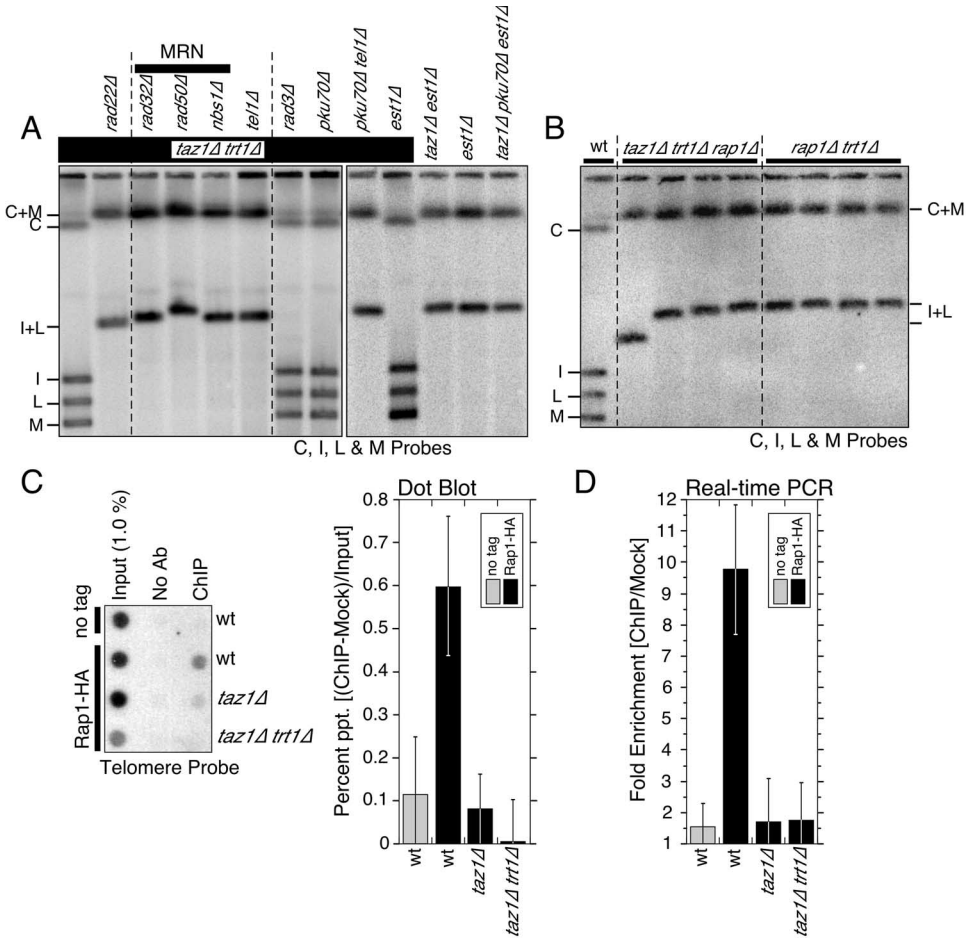

FIG. 2. Determination of genes required for telomere maintenance in *taz1*Δ *trt1*Δ cells. (A) Rad22 and MRN-Tel1 are required for telomere maintenance in *taz1*Δ *trt1*Δ cells, whereas Rad3, Ku70, and Est1 are dispensable. Multiple independently derived clones were analyzed by PFGE after extensive restreaking on YES agar plates, but only one representative clone per genotype is shown. Chromosomal plugs were prepared from cells that were grown at least 150 generations after the strains were generated. (B) Rap1 is essential for telomere maintenance in *taz1*Δ *trt1*Δ or *trt1*Δ cells. Independently derived strains were restreaked extensively on YES agar plates before analysis by PFGE. (C and D) Rap1 association with telomeres requires Taz1 in both wild-type (*trt1*$^+$) and *trt1*Δ cells. Telomere association of hemagglutinin-tagged Rap1 was determined by ChIP assay using dot blot hybridization with a telomeric probe (C) or quantitative PCR with primers against the TAS1 sequence (D). Error bars represent the standard deviation from at least three independent experiments.

essential for telomere maintenance when Taz1 and Trt1 are both missing from cells, although we found that *taz1*Δ *trt1*Δ *rad3*Δ cells showed a reduction in average telomere length compared to *taz1*Δ *trt1*Δ cells (see Fig. S6 in the supplemental material). We can rule out the possibility that Rad3 is essential for generating circular chromosomes since the reintroduction of the *taz1*$^+$ plasmid into *taz1*Δ *trt1*Δ *rad3*Δ cells led to chromosome circularization (see Fig. 5A). A recent study has also found that *taz1*Δ *trt1*Δ *rqh1*Δ cells (Rqh1 is ortholog of *S. cerevisiae* Sgs1) cannot maintain linear chromosomes (32). Taken together, the recombination-based telomere maintenance mechanism observed in *taz1*Δ *trt1*Δ cells has genetic requirements very similar to those of *S. cerevisiae* type II survivors, which require Rad52, the MRX complex, Tel1, Mec1, and Sgs1 (27, 57, 58, 60).

Elimination of Est1 leads to chromosome circularization even in the absence of Taz1. We also examined the effect of eliminating the telomerase regulatory subunit Est1 from *taz1*Δ or *taz1*Δ *trt1*Δ cells. In *S. cerevisiae*, Est1 is essential for telomerase function in vivo; it contributes to efficient telomere recruitment and activation of the telomerase catalytic subunit Est2 (TERT) (5, 18, 55). Similarly, *S. pombe* Est1 coimmunoprecipitates with Trt1, and *est1*Δ cells progressively lose telomeres (4). Therefore, we expected that deletion of *est1*$^+$ would have similar effects on telomere maintenance as the deletion of *trt1*$^+$.

As we have previously shown, when *taz1*Δ *trt1*Δ cells are generated by deleting *trt1*$^+$ from *taz1*Δ cells, survivors with linear chromosomes are generated exclusively, since preexistence of the *taz1*Δ mutation strongly favors cell survival via recombination-based telomere maintenance over cell survival via chromosome circularization (44). Accordingly, we expected to see exclusive occurrences of survivors with linear chromosomes when we deleted *est1*$^+$ from *taz1*Δ cells. Surprisingly, the deletion of *est1*$^+$ from *taz1*Δ cells still led to chromosome circularization (Fig. 2A). In contrast, when we deleted *est1*$^+$ from *taz1*Δ *trt1*Δ survivor cells, we found that all clones were able to stably maintain telomeres (Fig. 2A). In fact, the loss of

Est1 appeared to lead to telomere lengthening in *taz1*Δ *trt1*Δ background (see Fig. S6 in the supplemental material). Thus, the loss of Est1 in the absence of Trt1 might further contribute to more enhanced telomere recombination. On the other hand, it appears that the presence of TERT, even when *est1*$^+$ is deleted in *taz1*Δ cells, can inhibit the efficient use of recombination to maintain telomeres. Such a difference in the ability to generate recombination-based survivors between *taz1*Δ *est1*Δ and *taz1*Δ *trt1*Δ cells might provide an explanation for the previous observation that *est1*Δ/*est1*$^+$ *taz1*Δ/*taz1*Δ diploid cells could not produce *taz1*Δ *est1*Δ haploid cells, whereas *trt1*Δ/*trt1*$^+$ *taz1*Δ/*taz1*Δ diploid cells could efficiently generate *taz1*Δ *trt1*Δ haploid cells (4).

Taz1-independent role of Rap1 in the promotion of recombination-based telomere maintenance. Next, we decided to test the effect of deleting *rap1*$^+$ from *taz1*Δ *trt1*Δ cells on recombination-based telomere maintenance. Since previous studies have shown that recruitment of *S. pombe* Rap1 to telomeres depends on Taz1 (10, 30), we predicted that the resulting *taz1*Δ *trt1*Δ *rap1*Δ cells would be able to stably maintain linear chromosomes, much like *taz1*Δ *trt1*Δ cells.

Surprisingly, *taz1*Δ *trt1*Δ *rap1*Δ cells were unable to maintain telomeres and circularized their chromosomes (Fig. 2B), indicating that Rap1 is required for TERT-independent telomere maintenance. Furthermore, in agreement with a previous study (39), we found that *rap1*Δ *trt1*Δ cells cannot maintain telomeres even when *rap1*Δ *trt1*Δ cells were generated by deleting *trt1*$^+$ from *rap1*Δ cells (Fig. 2B). Thus, Rap1 contributes to TERT-independent telomere maintenance in both *taz1*$^+$ and *taz1*Δ backgrounds. We confirmed that efficient telomere recruitment of Rap1 to telomeres is indeed dependent on Taz1 (Fig. 2C and D). We also considered the possibility that Rap1 can be efficiently recruited to telomeres in the absence of Taz1 once Trt1 is also eliminated. However, we saw no evidence of Rap1 recruitment to telomeres in *taz1*Δ *trt1*Δ cells (Fig. 2C and D). Thus, Rap1 has a Taz1-independent function in promoting recombination-based telomere maintenance, even when the recruitment of Rap1 to telomeres is greatly reduced or abolished.

Catalytically inactive TERT affects the generation of recombination-based survivors. Our data in Fig. 1D suggested that TERT and Taz1 redundantly provide protection against very rapid occurrence of telomere fusions. To test whether the predicted telomere protection function of TERT in the *taz1*Δ background could be separated from its telomere replication function, we monitored changes in cell growth and telomere structure for *taz1*Δ *trt1-D743A* cells, which were derived from *taz1*Δ/*taz1*$^+$ *trt1-D743A*/*trt1*$^+$ diploid cells. The *trt1-D743A* mutation (see Fig. 4A) has been shown to abolish catalytic activity without affecting protein expression level (23). If the catalytically inactive TERT could still function in telomere protection, we would expect to see that *taz1*Δ *trt1-D743A* cells exhibit a delayed loss of viability compared to *taz1*Δ *trt1*Δ cells.

However, the loss of viability and telomere dysfunction in *taz1*Δ *trt1-D743A* cells occurred even earlier than in *taz1*Δ *trt1*Δ cells. (Fig. 3D; see also Fig. S2D in the supplemental material). While *taz1*Δ *trt1-D743A* cells were extremely sick immediately after the germination of spores, by the time cells were collected on day 1 of the liquid growth experiment, they had already started to recover in growth rate. PFGE analysis revealed the presence of mostly fused telomere bands (C+M and I+L) consistent with circular chromosomes on day 1 (Fig. 3F), but continued serial dilutions led to rapid disappearance of distinct telomeric NotI bands by day 3. The failure to detect distinct telomeric NotI fragments was not due to general degradation of chromosomal DNA, since we can detect expected bands for nontelomeric NotI fragments on ethidium bromide-stained agarose gels (see Fig. S3 in the supplemental material). On the other hand, we did reproducibly observe extremely broad diffused hybridization signals when blots for *taz1*Δ *trt1-D743A* samples were hybridized to probes specific to C, I, L, and M bands (Fig. 3F). Southern blot analysis of conventional agarose gels revealed that *taz1*Δ *trt1-D743A* survivor cultures from serial liquid culture dilution experiments often contained highly amplified TAS sequences but little telomere signal (see Fig. S4 and S5 in the supplemental material; also, data not shown). Such organization of subtelomeric regions is reminiscent of type I survivors from budding yeast. Thus, it appears that the presence of catalytically inactive TERT could prevent *taz1*Δ *trt1-D743A* cells from achieving a stable type II-like linear telomere maintenance mode normally observed in *taz1*Δ *trt1*Δ cells. Taken together, our results suggest that catalytically inactive Trt1-D743A protein can affect recombination efficiency at telomeres in a *taz1*Δ background.

Liquid culture growth experiments also revealed that *trt1-D743A* cells reached their lowest viability 5 to 6 days earlier than *trt1*Δ cells (Fig. 3A; see also Fig. S2C in the supplemental material). This earlier loss of viability correlated with earlier telomere erosion and chromosome fusions (Fig. 3B and C). Although the parental heterozygous diploid cells had telomere length comparable to wt cells (data not shown), *trt1-D743A* cells appear to lose telomeres much faster than *trt1*Δ cells. Previously, *trt1*Δ cells that carry a Trt1-D743A expression plasmid were also shown to lose cell viability earlier than *trt1*Δ cells (23). Analysis of telomere length and the TAS region also revealed that *trt1-D743A* liquid culture survivors contain very little telomere repeat sequences and show much more prominent amplification of the TAS1 sequence than *trt1*Δ survivor cultures (see Fig. S4 and S5 in the supplemental material). Thus, the presence of inactive TERT appears to interfere with the generation of type II-like survivor cells in both *taz1*$^+$ and *taz1*Δ cells even when survivor cells were selected in competitive liquid culture selection conditions.

Taz1 and catalytically inactive TERT inhibit recombination-based telomere maintenance. The recombination-based telomere maintenance in *taz1*Δ *trt1*Δ cells is highly efficient, presumably because changes in the telomeric chromatin structure in the absence of Taz1 allow better access for recombination enzymes to telomeres (12). The *taz1*Δ *trt1-D743A* experiments also suggest that catalytically inactive TERT may be able to inhibit telomere recombination. To directly test whether Taz1 and TERT can inhibit recombination at telomeres, we reintroduced Taz1 or catalytically inactive TERT (Trt1-D590A or Trt1-D743A; Fig. 4A) (23) into *taz1*Δ *trt1*Δ survivor cells and then analyzed the telomere structure by PFGE after repeated restreaking on agar plates.

Reintroduction of Taz1, Trt1-D590A, or Trt1-D743A into *taz1*Δ *trt1*Δ cells interfered with recombination-based telomere maintenance, causing chromosome circularization (Fig. 4B). On the other hand, reintroduction of *trt1*$^+$ into *taz1*Δ *trt1*Δ cells

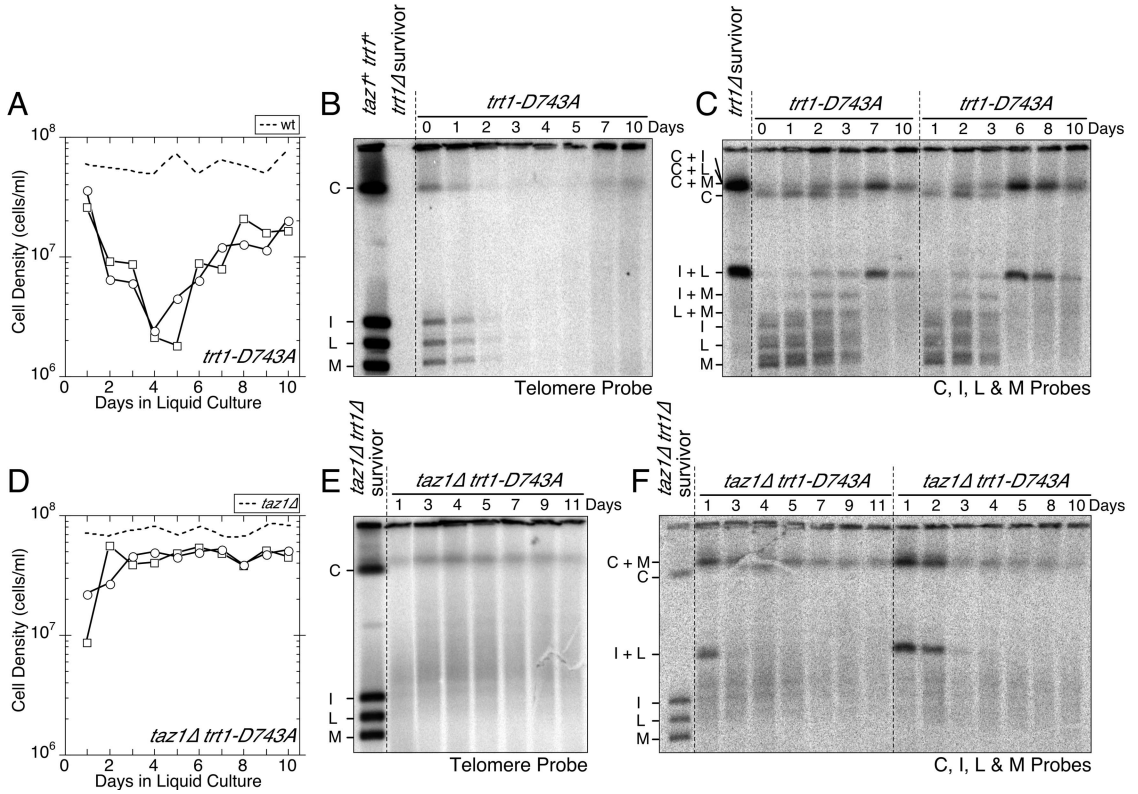

FIG. 3. PFGE analysis for *trt1-D743A* and *taz1*Δ *trt1-D743A* cells. A *taz1*Δ/*taz1*$^+$ *trt1-D743A*/*trt1*$^+$ diploid strain was sporulated, and *trt1-D743A* (A to C) or *taz1*Δ *trt1-D743A* (D to F) cells were selected. (A and D) Liquid growth characteristics of cells after germination. For references, results from growth curve experiments for wild-type and *taz1*Δ cells are also plotted (dotted lines). (B and E) PFGE of NotI-digested *S. pombe* chromosomal DNA hybridized with telomere probe. (C and F) PFGE of NotI-digested *S. pombe* chromosomal DNA hybridized with probes for the C, I, L, and M bands.

resulted in telomere elongation (Fig. 4B and 5D). Wild-type Trt1 is most likely as effective in inhibiting recombination at telomeres as the catalytically inactive Trt1, but since catalytically active Trt1 allows cells to stably maintain telomeric repeats without the help of recombination, chromosomes do not circularize after the reintroduction of wild-type Trt1. Taken together, these results indicate that TERT is as effective as Taz1 in preventing recombination-dependent telomere maintenance, and the inhibitory function of TERT on recombination can be separated from its RT activity.

Since the RT activity of TERT is not necessary to prevent telomere recombination, we next sought to determine whether the RT domain of TERT is necessary to inhibit telomere recombination by constructing a series of truncation mutants (Fig. 4A). The smallest tested TERT fragment that can efficiently inhibit telomere recombination was Trt1-ΔPac (Fig. 4C), which completely lacks the C-terminal RT domain. Thus, the N-terminal half of TERT, which includes the recently crystallized TEN (telomerase essential) domain, the CP motif, and most of the QFP motif (Fig. 4A), represents a functional subdomain that can efficiently inhibit recombination at telomeres even in the absence of Taz1 protein (28, 65).

Est1 and Ku70 are essential for catalytically inactive TERT to inhibit telomere recombination. Next, we searched for mutations that would abrogate the ability of catalytically inactive TERT to inhibit recombination-based telomere maintenance in *taz1*Δ *trt1*Δ cells. Since catalytically inactive TERT is expected to inhibit telomere recombination only if it is recruited to telomeres, factors that are required for the recruitment of TERT to telomeres might be identified from such analyses. Alternatively, factors that are involved in suppressing telomere recombination might also be identified.

Rad3, Est1, and Ku70 are good candidates for proteins that might be involved in the recruitment of TERT to telomeres, since all three proteins have previously been shown to positively contribute to telomere length maintenance in *taz1*$^+$ *trt1*$^+$ cells (3, 4, 46). Moreover, we have already established that these proteins are dispensable for telomere maintenance in *taz1*Δ *trt1*Δ cells (Fig. 2A). Therefore, we reintroduced catalytically inactive TERT into triple-mutant cells (*rad3*Δ, *est1*Δ, or *pku70*Δ combined with *taz1*Δ *trt1*Δ) and examined their telomere structure by PFGE. As a positive control, we also reintroduced Taz1 into these cells. In all cases, reintroduction of Taz1 resulted in circular chromosomes (Fig. 5A to C), indicating that Rad3, Est1, and Ku70 are not necessary for chromosome circularization.

Reintroduction of Trt1-D590A or D743A into *taz1*Δ *trt1*Δ *rad3*Δ cells resulted in efficient chromosome circularization and the complete loss of telomeric repeats (Fig. 5A and data not shown). Conversely, reintroduction of wild-type Trt1 resulted in massive telomere elongation (Fig. 5D), and ChIP analysis confirmed the efficient recruitment of Trt1 to telo-

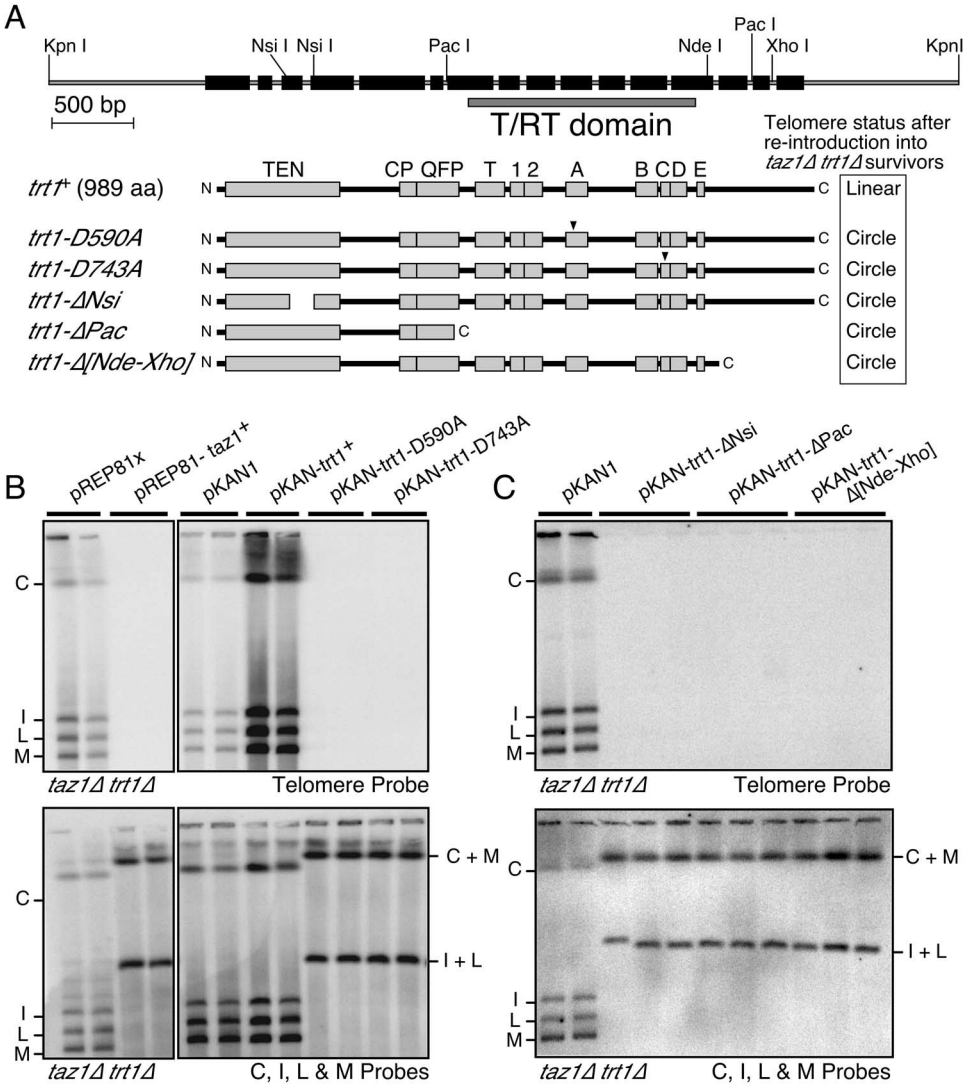

FIG. 4. Taz1 and catalytically inactive TERT inhibit recombination-based telomere maintenance in *taz1*Δ *trt1*Δ cells. (A) The top diagram shows the *S. pombe trt1*$^+$ gene structure. Exons are shown as black boxes, and the relevant restriction sites and the locations of conserved motifs and functional domains are indicated. Below are schematic representations of the various Trt1 constructs tested. (B and C) PFGE analyses of *taz1*Δ *trt1*Δ survivor cells transformed with the indicated plasmids. After transformation with plasmids, the colonies were extensively restreaked on agar plates appropriate for selection of plasmids before analysis by PFGE. The top panel shows hybridization with a telomere probe. The bottom panel shows hybridization with probes for the C, I, L, and M bands.

meres in both *rad3*Δ and *taz1*Δ *rad3*Δ cells (Fig. 5E). Thus, Rad3 is not essential for recruitment of Trt1 to telomeres.

Reintroduction of Trt1-D590A or D743A into *taz1*Δ *trt1*Δ *est1*Δ cells did not lead to chromosome circularization (Fig. 5B). Furthermore, reintroduction of wild-type Trt1 did not result in telomere elongation (Fig. 5D), and Trt1 was no longer detected at telomeres by ChIP analysis in *taz1*Δ *est1*Δ cells (Fig. 5E). Thus, Est1 is essential for recruitment of Trt1 to telomeres. This is the first direct demonstration outside of budding yeast that Est1 is involved in the recruitment of TERT to telomeres (5).

It was surprising that reintroduction of wild-type or catalytically inactive TERT into established *taz1*Δ *trt1*Δ *est1*Δ survivors did not result in chromosome circularization since we earlier showed that *taz1*Δ *est1*Δ cells, generated by deletion of *est1*$^+$ from *taz1*Δ cells, carry circular chromosomes (Fig. 2A). Such a discrepancy might indicate that *taz1*Δ *trt1*Δ survivor cells establish an altered telomeric chromatin environment that no longer allows recruitment of TERT without Est1. In contrast, Est1 might be dispensable for the recruitment of TERT in *taz1*Δ cells, and inactive TERT (due to the lack of Est1) could interfere with the establishment of recombination-based survivors. Alternatively, Est1 might still be required for recruitment of TERT to telomeres even in *taz1*Δ cells, but a gradual decline in Est1 protein level after the deletion of *est1*$^+$ could result in a situation where a limiting amount of Est1 is sufficient for Trt1 recruitment but not RT activation, thus mimicking the situation where catalytically inactive TERT interferes with the generation of recombination survivors. Further careful studies are necessary to distinguish these possibilities.

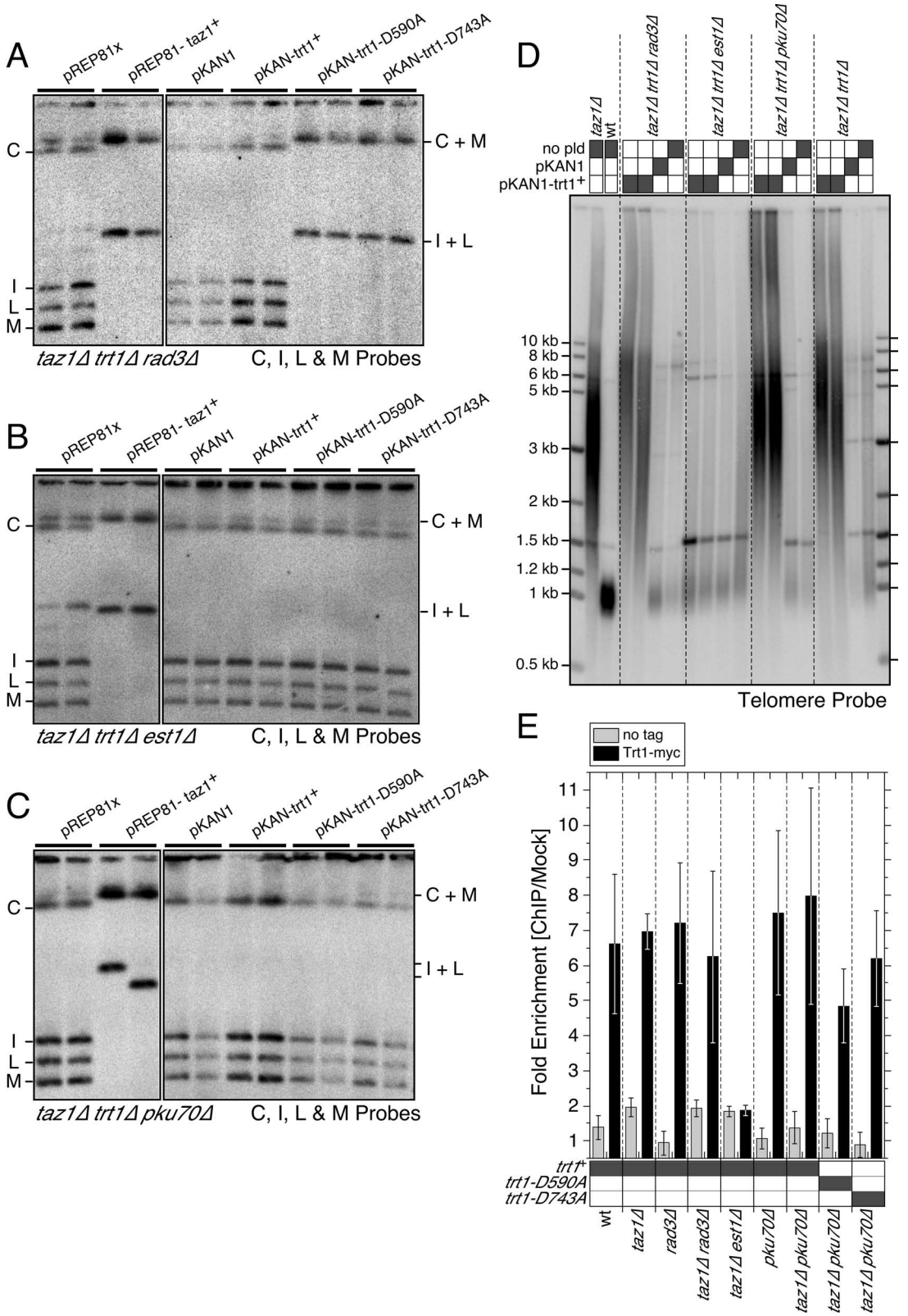

FIG. 5. Est1 and Ku70 are required for catalytically inactive TERT to inhibit recombination-based telomere maintenance in *taz1*Δ *trt1*Δ cells. (A to C) PFGE analysis of *taz1*Δ *trt1*Δ *rad3*Δ, *taz1*Δ *trt1*Δ *est1*Δ, or *taz1*Δ *trt1*Δ *pku70*Δ cells transformed with indicated plasmids after extensive restreaking on appropriate selection plates. (D) Telomere length analysis for the indicated strains. After digestion with EcoRI, genomic DNA was fractionated on a 1% agarose gel and processed for Southern blot analysis with a telomere probe. (E) Trt1 association with telomeres requires Est1, but not Ku70 or Rad3. Telomere association of myc-tagged Trt1 was determined by ChIP assay using quantitative PCR against the TAS1 sequence. For *taz1*Δ *pku70*Δ cells, the recruitment of myc-tagged catalytically dead Trt1 (Trt1-D590A and Trt1-D743A) was also examined. Error bars represent the standard deviation from at least three independent experiments.

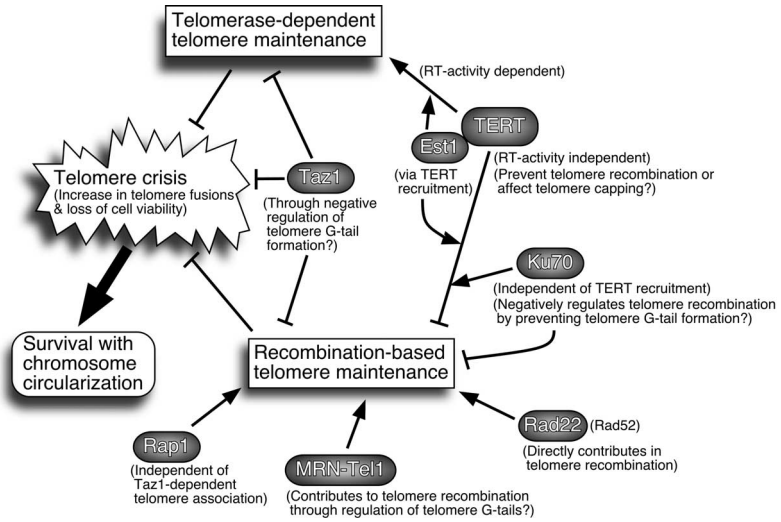

FIG. 6. Summary of genetic interactions found in the current study affecting telomerase-dependent and -independent telomere maintenance and chromosome circularization.

For *taz1*Δ *trt1*Δ *pku70*Δ, reintroduction of Trt1-D590A or D743A did not result in chromosome circularization (Fig. 5C). However, unlike in the case of *taz1*Δ *trt1*Δ *est1*Δ cells, reintroduction of wild-type Trt1 into *taz1*Δ *trt1*Δ *pku70*Δ cells resulted in massive telomere elongation (Fig. 5D). In addition, telomere recruitment for either wild-type or catalytically inactive Trt1 proteins was normal in *taz1*Δ *pku70*Δ cells based on ChIP analysis (Fig. 5E). Trt1 was also recruited efficiently in *pku70*Δ cells (Fig. 5E). Thus, elimination of *pku70*Δ appears to affect telomere recombination independent of TERT recruitment. Indeed, previous studies have shown that *S. pombe* Ku70 has a role in prevention of recombination at telomeres (3, 33). Thus, our results identify Ku70 as a third parallel and redundant factor besides Trt1-Est1 and Taz1 contributing to the prevention of recombination at telomeres.

DISCUSSION

While many proteins contribute to telomere maintenance in eukaryotic cells, it is not well understood how multitudes of proteins work collaboratively or antagonistically in either telomerase-dependent or -independent mechanisms of telomere maintenance. It is also not clear why telomerase-based telomere maintenance is the most prevalent mechanism in eukaryotic cells, since diverse non-telomerase-based mechanisms, such as telomere-telomere recombination and integration of retrotransposons, appear to be almost as effective in maintaining telomeres (7, 41). To understand these issues better, we investigated how the presence or absence of telomere-specific proteins, DNA repair proteins, and DNA damage checkpoint proteins contribute to the prevention or promotion of recombination and fusion events at telomeres in fission yeast.

We first demonstrated that simultaneous loss of Taz1 and TERT results in accelerated telomere fusion compared to the loss of TERT alone (Fig. 1). Therefore, we envision Taz1 and TERT as two major factors that are essential for the prevention of telomere crisis, caused by increased fusion and recombination events at telomeres (Fig. 6). Since the elimination of either Taz1 or TERT alone does not result in immediate telomere crisis, their roles in the protection of telomeres are redundant. Because early telomere crisis cannot be averted by expression of catalytically inactive TERT (Fig. 3; see also Fig. S2 in the supplemental material), we suggest that the rapid loss of telomeric GT-rich repeats likely causes an immediate increase in telomere fusion events. This hypothesis is consistent with a recent study that showed that replication of telomeric repeats in *taz1*Δ cells is critically dependent on TERT (39). GT-rich telomeric repeats also represent the binding site for the telomere capping protein Pot1 (2), so if *taz1*Δ *trt1*Δ cells fail to maintain telomeric repeats, Pot1 might no longer be recruited to protect telomeres and thus lead to catastrophic telomere fusions.

On the other hand, we found that simultaneous elimination of Taz1 and TERT also allows cells to very efficiently maintain telomeres by a Rad22 (Rad52)-dependent recombination mechanism (Fig. 1, 2, and 6). Since reintroduction of Taz1 into *taz1*Δ *trt1*Δ survivors causes chromosome circularization (Fig. 4), Taz1 functions as a potent inhibitor of telomere recombination. Rad22 is in fact essential for preventing NHEJ-dependent fusion of telomeres in *taz1*Δ cells, and *taz1*Δ cells also carry long telomeric G-tails, expected to be a favored substrate for HR (19, 59). However, it is difficult to know how quickly cells can establish long G-tails after the loss of Taz1. When Taz1 and TERT are simultaneously lost, as in the case of *taz1*Δ *trt1*Δ cells derived from *taz1*Δ/*taz1*$^+$ *trt1*Δ/*trt1*$^+$ diploid cells, the inability of *taz1*Δ cells to replicate through telomeres without telomerase (39) might initially dominate telomere dynamics and cause many *taz1*Δ *trt1*Δ cells to quickly lose telomeric repeats and fuse telomeres. Once a nuclease(s) acting at the telomeres (which are normally inhibited by Taz1) degrades the CA-rich strand of telomeres and establishes long G-tails, *taz1*Δ *trt1*Δ cells could then efficiently maintain telomeres through the Rad22-dependent HR mechanism. The fact that *taz1*Δ cells have already established long telomere tracts with extended G-tails can then explain why we observe only linear chromo-

some survivors when *taz1*Δ *trt1*Δ cells are generated by eliminating Trt1 from *taz1*Δ cells (44). In *S. cerevisiae*, combining a temperature-sensitive mutation of the G-tail binding protein Cdc13 (*cdc13-1*) and deletion of telomerase RNA *TLC1* also caused *cdc13-1 tlc1*Δ cells to experience much earlier telomere dysfunction than *tlc1*Δ cells when the cells were grown at a semipermissive temperature (60). Moreover, these cells exclusively generate type II survivors (22, 60). It is worth noting that *cdc13-1* cells grown at semipermissive temperature carry longer G-tails, much like *taz1*Δ cells.

Our analyses have also uncovered a surprising RT-independent role of TERT in the prevention of recombination at telomeres (Fig. 6). Reintroduction of catalytically inactive versions of TERT was as effective as reintroduction of Taz1 in causing chromosome circularization. The N-terminal 396-amino-acid portion of TERT (Trt1-ΔPac), which includes the recently crystallized TEN domain and the region implicated in telomerase RNA binding, was sufficient for the inhibition (28, 31). Although such results are also consistent with the interpretation that catalytically inactive TERT can disrupt the protective function of telomeres against fusions, we favor the idea that circularization is caused by inhibition of recombination-based survival in *taz1*Δ *trt1*Δ cells since Est1-dependent recruitment of TERT is required to cause circularization and removal of the Ku complex, which may further compromise telomere capping, can reverse the circularization phenotype (Fig. 5).

Our analysis also indicated that the Ku complex is essential for the TERT-dependent protection of telomeres from recombination in the absence of Taz1 (Fig. 5C and 6). While studies in *S. cerevisiae* have clearly demonstrated that the Ku complex plays a very important role in the recruitment of Est2 (TERT) to telomeres (20), we found no evidence that Ku is involved in the recruitment of TERT to telomeres in fission yeast (Fig. 5E). However, the *S. cerevisiae* Ku complex plays the most important role in the recruitment of Est2 in the G_1 phase of the cell cycle (20). Given that exponentially growing *S. pombe* cell cultures contain very few cells in G_1 phase, we cannot rule out the possibility that the recruitment of *S. pombe* TERT to telomeres in G_1 phase might be affected by the loss of the Ku complex. On the other hand, previous studies have found that the Ku70-Ku80 heterodimer is required to prevent recruitment of the HR repair protein Rhp51 (Rad51) to telomeres in fission yeast (33) and is involved in the inhibition of telomere recombination in budding yeast and mammalian cells (6, 51). Therefore, we favor a model in which the Ku complex represents a third independent component involved in repressing recombination at telomeres, in addition to Taz1 and TERT-Est1 (Fig. 6).

Although previous studies have suggested that TERT could have telomere protection roles independent of its extension function (36, 54, 69), these putative "protective" functions of TERT generally still required TERT to be catalytically active. Therefore, even when the overall telomere length was not altered by the expression of TERT, it was difficult to rule out the possibility that TERT might selectively act on extremely short telomeres to improve chromosome stability or cell viability. In fact, the expression of catalytically inactive hTERT variants causes cell death or senescence, probably due to the increase in the loss of telomeric DNA (24, 25, 68). However, these studies also found that cells expressing catalytically inactive hTERT were not able to activate the recombination-based ALT (alternative lengthening of telomeres) survival mechanism to escape cell death, and thus the observations in these studies are consistent with the notion that catalytically inactive TERT can function as an effective protector against telomeric recombination. It was recently reported that catalytically inactive TERT (Est2) and telomerase RNA together could protect telomeres from excessive G-tail formation in *Candida albicans* (26). As mentioned earlier, long G-tails should serve as a good substrate for recombination, and thus the prevention of long G-tail formation may explain why catalytically inactive TERT can function as an inhibitor of telomeric recombination. It is worth noting that a human hTERTα splice variant, which lacks highly conserved critical amino acid residues within the RT domain required for telomerase activity, is expressed in development- and tissue-specific manners and has been shown to function as a dominant-negative inhibitor of telomerase activity (11, 66). A recent study has also shown that *Arabidopsis* POT1A can interact with the N-terminal splicing variant of TERT (52). Therefore, we suggest that the inhibition of telomeric recombination by the naturally occurring hTERTα splice variant could also have an important role in the regulation of telomere maintenance in human cells.

Our genetic analysis has uncovered that recombination-based telomere maintenance in *taz1*Δ *trt1*Δ cells requires the Tel1-MRN (ATM-MRN) complex (Fig. 2A and 6). Previously, type II survivors in *S. cerevisiae* and human ALT cells have been shown to also require the MRX/MRN complex (29, 57). Thus, MRN appears to be universally important for recombination-based telomere maintenance. Interestingly, ATM and MRN are also very important in the protection and/or maintenance of telomeres in *Drosophila* cells, where transpositions of retroelements are utilized in telomere maintenance (7). Moreover, studies in *S. cerevisiae* provide convincing evidence that Tel1-MRX plays a very important role(s) in the recruitment of telomerase components to telomeres (21, 61). Therefore, the ATM-MRN complex contributes positively to telomere maintenance involving telomerase, recombination, and retrotransposons. Since MRN is involved in the generation of G-tails at telomeres (35, 59) and the presence of G-tails is favorable for both efficient recruitment of telomerase and initiation of telomeric recombination (21, 62), ATM-MRN is one of the most important gatekeepers in regulating telomere accessibility to various modes of telomere maintenance. Our current finding that Taz1 and TERT can function as inhibitors of telomere recombination suggests that these telomere-specific factors can in turn regulate the ATM-MRN complex in ensuring that telomeres are protected against recombination and are maintained via telomerase-based extension.

In contrast to the Tel1-MRN complex, our analysis indicated that the Rad3-Rad26 (ATR-ATRIP) complex does not play a significant role in recombination-based telomere maintenance in the absence of Taz1 and TERT (Fig. 2 and 5). On the other hand, in the presence of wild-type Taz1 and Trt1, Rad3-Rad26 appears to play a more important role(s) in telomerase-dependent telomere maintenance than Tel1-MRN, since the deletion of Rad3-Rad26 causes telomeres to become much shorter than cells lacking Tel1 or MRN (8, 46). Our ChIP and chromosome circularization assays suggest that Rad3 is not essential for the recruitment of TERT to telomeres (Fig. 5), although we can-

not rule out the possibility that Tel1-MRN and Rad3-Rad26 are redundantly required for the recruitment of TERT to telomeres, since *tel1*Δ *rad3*Δ cells are incapable of maintaining telomeres even in the presence of wild-type TERT (42). Thus, further studies are required to understand the exact contribution of the Rad3-Rad26 (ATR-ATRIP) complex in the telomerase-dependent telomere maintenance mechanism.

We were surprised by our observation that Rap1 is essential for the maintenance of telomeres in *taz1*Δ *trt1*Δ cells since previous studies established that the recruitment of Rap1 to telomeres largely depends on Taz1, although some residual Rap1 foci at telomeres were occasionally observed by fluorescence microscopy in *taz1*Δ cells undergoing meiosis (10, 30). Although it is still possible that a very small amount of Rap1 (undetectable by ChIP) is recruited to telomeres and contributes to the promotion of recombination or the prevention of fusions at telomeres, our data suggest that Rap1 can positively contribute to linear chromosome maintenance without being efficiently recruited to telomeres (Fig. 6). If there is very weak or transient residual binding of Rap1 undetectable by ChIP, such interaction might involve a Myb domain of Rap1 interacting with telomeric DNA or other telomere proteins. Therefore, it might be interesting to test whether Rap1 lacking a Myb domain might still be able to prevent chromosome circularization after elimination of TERT. A previous genetic study in *S. pombe* has found that the loss of Rap1 exacerbates the cold sensitivity of *taz1*Δ cells, which led the authors to conclude that Rap1 may have Taz1-independent telomere functions (38). However, a *rap1*Δ strain on its own is not cold sensitive, and only Taz1 (but not Rap1) has been found to promote replication of telomeric GT-rich repeats by DNA polymerases (38, 39). Thus, our current observation is the first report wherein *rap1*Δ cells show more severe defects in telomere stability than *taz1*Δ cells. Unlike *S. cerevisiae* cells, in which Rap1 directly binds to the telomeric DNA, recruitment of human Rap1 to the telomere is also dependent on TRF2 (an ortholog of *S. pombe* Taz1) (34). Therefore, it will be interesting to determine whether human Rap1 might also contribute to telomere maintenance independent from its TRF2-dependent recruitment to telomeres. In *S. cerevisiae*, Rap1 plays a well-established role in the transcriptional activation of various genes (40). It is possible that *S. pombe* Rap1 might also contribute to the transcriptional activation of genes responsible for the recombinational mode of telomere maintenance in *taz1*Δ *trt1*Δ cells. However, such a possibility awaits future investigations. It should also be noted that a recent study has provided evidence that Rap1 is necessary to prevent NHEJ in budding yeast cells (50).

Telomerase-based maintenance of telomeres is the most common method of telomere maintenance among eukaryotic cells. This can be partly explained by the fact that telomerase appears to have originated very early in the evolution of eukaryotic cells (43). However, a strong argument can be made that recombination-based or retrotransposon-based telomere maintenance might have even more ancient origins than modern day telomerase-based telomere maintenance (15). So, how could the telomerase-based mechanism be so effective in preventing underlying recombination- or transposon-based modes of telomere maintenance? Certainly, factors that specifically bind to telomeric GT-rich repeats, such as *S. pombe* Taz1, *S. cerevisiae* Rap1, and mammalian TRF1 and TRF2 proteins, play major roles in preventing excessive recombination at telomeres (53). In addition, our observations suggest that TERT is directly involved in preventing alternative telomere maintenance mechanisms. In fact, the N-terminal domain we found to be crucial for the prevention of telomeric recombination is not found in other RT proteins encoded by retrotransposons and viruses (17). It is worth noting that both TRF1/TRF2-type telomere-specific proteins and TERT are absent in *Drosophila* cells and that ALT cells generally lack functional telomerase. Thus, TERT's ability to efficiently compete and protect against other ancient DNA repair and damage response machineries is very important for understanding how telomere maintenance is regulated in eukaryotic cells.

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