Roles of heterochromatin and telomere proteins in regulation of fission yeast telomere recombination and telomerase recruitment

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When the telomerase catalytic subunit (Trt1/TERT) is deleted, a majority of fission yeast cells survives by circulating chromosomes. Alternatively, a small minority survives by maintaining telomeric repeats through recombination among telomeres. The recombination-based telomere maintenance in trt1Δ cells is inhibited by the telomere protein Taz1. In addition, catalytically inactive full length Trt1 (Trt1-CI) and truncated Trt1 lacking the T-motif and reverse transcriptase (RT)-domain (Trt1-ΔT/RT) can strongly inhibit recombination-based survival. Here, we investigated the effects of deleting the heterochromatin proteins Swi6 (HP1 ortholog) and Clr4 (Suv39 family of histone methyltransferases), and the telomere capping complex subunits Poz1 and Ccq1, on Taz1- and Trt1-dependent telomere recombination inhibition. The ability of Taz1 to inhibit telomere recombination did not require Swi6, Clr4, Poz1 or Ccq1. While Swi6, Clr4 and Poz1 were dispensable for the inhibition of telomere recombination by Trt1-CI, Ccq1 was required for efficient telomere recruitment of Trt1 and Trt1-CI-dependent inhibition of telomere recombination. We also found that Swi6, Clr4, Ccq1, the checkpoint kinase Rad3 (ATR ortholog) and the telomerase regulatory subunit Est1 are all required for Trt1-ΔT/RT to inhibit telomere recombination. However, since loss of Swi6, Clr4, Rad3, Ccq1 or Est1 did not significantly alter the recruitment efficiency of Trt1-ΔT/RT to telomeres, these factors are likely to enhance the ability of Trt1-ΔT/RT to inhibit recombination-based survival by contributing to the negative regulation of telomere recombination.

Telomeres, the ends of eukaryotic chromosomes, must fulfill two essential functions to achieve stable inheritance of intact chromosomes. First, telomeres must protect chromosome ends from uncontrolled degradation, end-to-end fusion, and recombination. Second, telomeres must allow complete replication of linear chromosome ends, which cannot be fully replicated by replicative DNA polymerases (1).

In most eukaryotic organisms, telomeric DNA is composed of short GT-rich repeat sequences and extended by telomerase, which utilizes its tightly bound RNA subunit as a template for de novo telomeric repeat DNA addition (2). On the other hand, recombination-based telomerase-independent mechanisms can also extend telomeric GT-rich repeats in various model organisms when telomerase is inactivated and in ~10% of human tumors (3).

In multi-cellular organisms including humans, expression levels of telomerase subunits, overall telomere repeat length, as well as composition and modification status of various telomere bound proteins, are carefully regulated based on tissue types and developmental stages (4). In fact, studies have uncovered connections between dysfunctional telomeres and various human age-related diseases and cancer in recent years (4,5).

While most of the telomeric GT-rich repeats are composed of double-stranded DNA (dsDNA), telomeric DNA terminates with a 3’ GT-rich single-stranded DNA (ssDNA), commonly referred to as G-tail. Since telomerase cannot act on blunt ends (6), the G-tail is essential for telomere extension by telomerase. Both the G-tail and the dsDNA portion of the GT-rich telomere repeats are coated by various sequence specific telomere-binding proteins (7), which are critical to
prevent telomere bound DNA repair and DNA damage checkpoint proteins from causing telomere fusions and permanent cell cycle arrest (8,9).

Interestingly, various DNA repair and checkpoint factors, such as Ku70-Ku80, Mre11-Rad50-Nbs1 (MRN), ATM and ATR-ATRIP, play critical roles in telomere maintenance (10-12). In addition, the formation of heterochromatin structure at telomeres has been observed in many organisms, and the regulation of heterochromatin formation has been suggested to contribute to the proper protection of telomeres (13). However, it is still not fully understood how heterochromatin structures might affect the ability of telomere specific factors and DNA damage response proteins to regulate telomere functions.

Therefore, we decided to investigate how recombination-based telomere maintenance and recruitment of telomerase are affected by loss of various telomere-associated proteins or proteins involved in the formation of telomere heterochromatin in fission yeast Schizosaccharomyces pombe. Fission yeast cells utilize telomere proteins that are highly conserved with mammalian telomere proteins (7). Moreover, the mechanism of heterochromatin formation is very well conserved between fission yeast and mammalian cells (14).

The catalytic subunit of telomerase, known as TERT (telomerase reverse transcriptase) (2), is encoded by the trt1+ gene in fission yeast (15). The Trt1 subunit forms a stable complex with its regulatory subunit Est1 and a telomerase RNA TER1 (16-18). All three subunits are essential for telomere extension by telomerase in fission yeast. In addition, Ccq1, a subunit of the Pot1 telomere-capping complex (composed of Pot1-Tpz1-Poz1-Ccq1) is critical for the recruitment of telomerase to telomeres and the inhibition of recombination at telomeres (19,20) (Figure 1 and supplemental Table S1).

When Trt1 is deleted, fission yeast cells progressively lose their telomeric DNA and viability. However, trt1Δ cells can eventually generate survivors either by circularizing chromosomes or by maintaining telomeric repeats through recombination among telomeres (21). Chromosome circularization is a much more frequently used mode of survival in fission yeast (21); however, rare survivors, which utilize a homologous recombination (HR)-based mechanism to maintain linear chromosomes, can be selected in serially diluted trt1Δ liquid cultures, since “linear” trt1Δ survivors have a selective advantage in competitive growth conditions, given that they grow faster than circular survivors (21).

The “linear” mode of telomerase-independent survival in fission yeast requires the HR protein Rad22Rad52, Tel1ATM, the MRN complex, Rqh1RecQ-like DNA helicase, and the telomere protein Rap1 (22,23). Thus, linear survivors appear to resemble budding yeast Type II recombination survivors or the mammalian alternative lengthening of telomeres (ALT) mode of telomere maintenance (23,24). Moreover, we have previously shown that the telomeric GT-rich repeat specific dsDNA binding protein Taz1 plays a critical role in inhibiting recombination-based telomere maintenance in trt1Δ cells (23), and that taz1Δ trt1Δ cells sustain robust growth with stable linear chromosomes (21,23).

Re-introduction of Taz1 into taz1Δ trt1Δ linear survivor cells strongly induces chromosome circularization due to inhibition of recombination-based telomere maintenance (23). Similarly, re-introduction of catalytically inactive Trt1 (Trt1-CI) or a C-terminally truncated Trt1, which lacks the telomerase-specific T-motif and the reverse-transcriptase domain (Trt1-ΔT/RT), also causes circularization of chromosomes in taz1Δ trt1Δ survivor cells, uncovering a RT-independent role for Trt1 in the inhibition of telomere recombination (23). Moreover, we have established that the non-homologous end-joining (NHEJ) DNA repair protein complex Ku70-Ku80 is essential for inhibition of telomere recombination by Trt1-CI, while Ku70-Ku80 is dispensable for Taz1-dependent inhibition of telomere recombination (23).

Fission yeast Taz1 protein is thought to represent the counterpart of the mammalian telomere proteins TRF1 and TRF2, and binds specifically to the dsDNA portion of telomeric repeats (7,25). In addition to inhibiting telomere recombination in trt1Δ cells, Taz1 is essential for protection of telomeres against NHEJ-dependent telomere fusion in G1-phase (26) and efficient replication of telomeric GT-rich repeats by replicative DNA polymerases (27). Moreover, Taz1 is important for the recruitment of the
telomeric proteins Rap1 and Rif1 to telomeres, and deletion of *taz1*, *rap1* or *rif1* leads to telomerase-dependent expansion of the GT-rich repeat-tract in fission yeast, indicating that they are involved in the negative regulation of telomerase (21,25,28).

Taz1-Rap1-Rif1 are connected to the G-tail binding protein complex Pot1-Tpz1-Poz1-Ccq1 via direct protein-protein interaction between Rap1 and Poz1 (19) (Figure 1). In fact, the fission yeast Taz1-Rap1-Poz1-Tpz1-Pot1-Ccq1 complex has been proposed to represent an evolutionarily conserved telomere protection complex resembling the “shelterin” complex assembled at mammalian telomeres (7,19) (supplemental Table S1).

Studies have further shown that Taz1 contributes to the formation of heterochromatin structures at telomeres (25,29). Taz1, Ccq1 and the RNAi machinery redundantly contribute to the formation of telomeric heterochromatin by promoting the recruitment of the Snf2/Hdac-containing Repressor Complex (SHREC) (30). The assembly of heterochromatin in fission yeast involves the methylation of histone H3 on Lysine 9 (H3K9me) by Clr4, an ortholog of mammalian Suv39 family of histone methyltransferases (31). Moreover, fission yeast heterochromatin is enriched for Swi6, a HP1 ortholog that specifically recognizes and binds H3K9me (32) (Figure 1). Deletion of *clr4* or *swi6* has been suggested to elevate recombination among sub-telomeric regions (33). However, the contribution of heterochromatin in the regulation of recombination-based telomere maintenance has not been investigated in fission yeast.

Here, we tested if Taz1- or Trt1-dependent inhibition of telomere recombination requires the presence of heterochromatin proteins (Swi6 and Clr4) or Pot1 complex components (Ccq1 and Poz1). Our results establish that Taz1 and Trt1-CI can efficiently inhibit telomere recombination in the absence of telomeric heterochromatin or the intact telomere-capping complex. On the other hand, our investigations utilizing Trt1-ΔT/RT implicate a subtle contribution of heterochromatin and the checkpoint kinase Rad3\(^{ATR}\) in repression of telomere recombination in fission yeast.

**EXPERIMENTAL PROCEDURES**

Fission yeast strains—Fission yeast strains used in this study were constructed by standard techniques (34), and are listed in supplemental Table S2. Original sources for deletion alleles for *taz1Δ*, *trt1Δ*, *ccq1Δ*, *est1Δ*, *pku70Δ*, and *rad3Δ* were previously described (10,23,35). For swi6Δ, *clr4Δ* and *pou1Δ*, deletion strains were generated by PCR-based deletion methods (36,37) with primers listed in supplemental Table S3. *taz1Δ trt1Δ clr4Δ* and *taz1Δ trt1Δ ccq1Δ* strains were generated by transforming a *clr4Δ* construct or a *ccq1Δ* construct into *taz1Δ trt1Δ* survivor cells. *taz1Δ trt1Δ swi6Δ* strains were generated by transforming a *trt1Δ* construct into *taz1Δ swi6Δ* cells, and *taz1Δ trt1Δ pou1Δ* strains were generated by crossing *taz1Δ trt1Δ* cells carrying pWH5-trt1\(^+\) plasmid with *pou1Δ* cells. *taz1Δ trt1Δ est1Δ*, *taz1Δ trt1Δ pku70Δ* and *taz1Δ trt1Δ rad3Δ* mutants were previously described (23). All triple mutant strains obtained were restreaked extensively on agar plates to ensure that cells reached their terminal phenotype before preparation of the chromosomal DNA plugs or genomic DNA.

Plasmids—plasmid pKAN-trt1\(^+\) contains the 5.5-kb *S. pombe* genomic KanI fragment bearing the trt1\(^+\) gene, kanMX4 marker, and *S. pombe* ars1\(^+\) (38). Plasmids pKAN-trt1-D590A and pKAN-trt1-D743A and pKAN-trt1-ΔT/RT are essentially the same as pKAN-trt1\(^+\), except that they carry the indicated mutant alleles (23). Plasmid pKAN-trt1-ΔT/RT was previously published as pKAN-trt1-ΔPac (23), and it was created by removing ~1.9 kb Pac1-PacI fragment from trt1\(^+\) gene. The Trt1 constructs carried by these plasmids are illustrated in Figure 3A. Plasmids pKAN-trt1:Cmyc9 and pKAN-trt1-ΔT/RT:Cmyc9 express the indicated Trt1 alleles with the myc9 tag fused at the C-terminus.

Pulsed-Field Gel Electrophoresis (PFGE)—chromosomal DNA samples were prepared in agarose plugs from extensively restreaked strains as previously described (10). NotI-digested DNA samples were fractionated in a 1% agarose gel with 0.5X TAE buffer at 14 °C, using the CHEF-DR III system (Bio-Rad) at 6 V/cm (200 V) and a
pulse time of 60 to 120 seconds for 24 hours. The telomeric repeat C, I, L, and M probes were prepared as previously described (21).

Southern blot analysis–EcoRI-digested DNA was prepared from fission yeast cells and separated in a 1% agarose gel at 100 V for 4 hours. DNA was then transferred to a Hybond XL membrane (GE Amersham Biosciences) for 2 h in transfer buffer (1.5M NaCl, 0.02M NaOH). The membrane was then hybridized with a telomeric repeat DNA probe (10).

Chromatin immunoprecipitation (ChIP) analysis and dot blot–exponentially growing cells were processed for ChIP as previously described (39). For Trt1-myc ChIP, monoclonal anti-myc antibody (9B11, Cell Signaling) was used and ChIP data were quantified using dot blot hybridization. First, 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). Dot blots were then hybridized with a telomeric repeat DNA probe (10), exposed to Phosphorimager cassette (GE), and hybridization signals were quantified by using ImageQuant software. For Rhp51 ChIP, polyclonal anti-Rad51 antibody (A-92, Santa Cruz) was used and quantitative real-time PCR was used to analyze ChIP samples. Percent precipitated DNA values were calculated based on ∆Ct between Input and IP samples after performing several independent triplicate SYBR Green-based real-time PCR (Bio-Rad) using TAS1 primers jk380 and jk381 (39). Two-tailed Student’s t-tests were performed, and P values ≤0.05 were considered as statistically significant differences.

Western blot analysis–whole-cell extracts were prepared from exponentially growing yeast cultures (35) and analyzed by Western blot using monoclonal anti-myc antibody (9B11) or polyclonal anti-Rad51 antibody (A-92). Anti-Cdc2 (y100.4, Abcam) antibody was used as loading control.

RESULTS

Clr4, Swi6, Poz1 and Ccq1 are dispensable for recombination-based maintenance of telomeres in taz1Δ trt1Δ cells–the telomeric repeat binding protein Taz1 is essential for proper maintenance of telomeric heterochromatin in fission yeast (25,29). Taz1 can promote the formation of heterochromatin near telomeric GT-rich repeat sequences by promoting the accumulation of the mammalian HP1 ortholog Swi6 to telomeric repeats, even when telomeric repeats are inserted in the middle of chromosomes (29). Accumulation of Swi6 at telomeric and sub-telomeric regions is dependent on the Suv39 family histone H3 Lysine 9 methyl transferase Clr4 (29).

Unlike taz1Δ cells, which carry massively elongated telomeres, swi6Δ or clr4Δ cells have normal length telomeres (40). Thus, it appears that Taz1’s ability to inhibit telomerase is not dependent on heterochromatin formation at telomeres and sub-telomeres. However, since formation of heterochromatin has been reported to be important for the inhibition of recombination at centromeres, mating type loci, as well as sub-telomeres in fission yeast (33,41), we decided to test if Taz1’s ability to inhibit recombination-based telomere maintenance in trt1Δ cells might require Taz1-dependent heterochromatin formation at telomeres.

Accordingly, we decided to generate taz1Δ trt1Δ swi6Δ and taz1Δ trt1Δ clr4Δ triple mutant strains in order to examine whether the re-introduction of Taz1 would lead to chromosome circularization. Additionally, we tested whether Clr4 or Swi6 is required for the inhibition of recombination-based telomere maintenance by the catalytically inactive Trt1 (Trt1-CI) or the N-terminal Trt1 fragment lacking T-motif and RT domain (Trt1-ΔT/RT) (Figure 3A).

It was possible that Swi6- and Clr4-dependent formation and spreading of heterochromatin at telomeres/sub-telomeres might be essential for telomere maintenance in taz1Δ trt1Δ cells. To test this, we first generated multiple independent triple mutant strains, restrreaked them extensively on YES plates, and tested their telomere status by pulsed-field gel electrophoresis (PFGE). Both taz1Δ trt1Δ swi6Δ and taz1Δ trt1Δ clr4Δ cells were able to stably maintain telomeres (Figure 2B-
Regulation of telomere recombination in fission yeast

Thus, we concluded that neither Swi6 nor Clr4 is required for recombination-based telomere maintenance observed in \textit{taz1Δ trt1Δ} cells.

Previously, it has been proposed that loss of proper telomere capping might allow mammalian tumor cells to survive more efficiently through the recombination-based ALT telomere maintenance mechanism (3). Therefore, in addition to Swi6 and Clr4, we decided to test if loss of the Pot1 telomere-capping complex subunits Poz1 and Ccq1 might affect the ability of Taz1 or Trt1 to inhibit recombination-based telomere maintenance (Figure 1). We did not test the roles of Pot1 or Tpz1, since these proteins are essential for telomere capping and deletion of these genes leads to immediate chromosome circularization (19).

Ccq1 associates with the SHREC heterochromatin effector complex, and it has been proposed that Ccq1 collaborates with Taz1 in promoting sub-telomeric recruitment of the SHREC complex (30). Moreover, Ccq1 has been implicated in the inhibition of recombination at telomeres (19,20). Similar to \textit{taz1Δ} and \textit{ccq1Δ} cells, \textit{poz1Δ} cells are also defective in transcriptional silencing of a marker gene inserted near telomeres, indicative of a failure in proper heterochromatin formation (35). In addition, \textit{poz1Δ} cells carry massively elongated telomeres, suggesting that Poz1 is required for the negative regulation of telomerase (19). We next tested the possibility that the presence of Poz1 or Ccq1 is required for telomere maintenance in \textit{taz1Δ trt1Δ} cells. However, since multiple independently derived \textit{taz1Δ trt1Δ poz1Δ} and \textit{taz1Δ trt1Δ ccq1Δ} strains all stably maintained telomeres after extensive restreaking on YES plates (Figure 2D-E and data not shown), we concluded that the presence of the intact Pot1 telomere capping complex is not essential for recombination-based telomere maintenance in \textit{taz1Δ trt1Δ} cells.

\textit{Taz1-dependent inhibition of telomere recombination does not require Swi6, Clr4, Poz1 or Ccq1}—having established that Swi6, Clr4, Poz1 and Ccq1 are not essential for telomere maintenance in \textit{taz1Δ trt1Δ} cells, we next tested whether Taz1-dependent inhibition of telomere recombination might require intact telomeric heterochromatin or the telomere capping complex. We found that re-introduction of Taz1 induced chromosome circularization in \textit{taz1Δ trt1Δ swi6Δ, taz1Δ trt1Δ clr4Δ, taz1Δ trt1Δ poz1Δ}, and \textit{taz1Δ trt1Δ ccq1Δ} cells (Figure 3B-E). Thus, we concluded that heterochromatin formation at telomeres or the presence of an intact Pot1 complex is dispensable for Taz1-dependent inhibition of telomere recombination. In addition, since re-introduction of Taz1 was able to cause telomere fusions in all mutant backgrounds, we also concluded that Swi6, Clr4, Poz1 and Ccq1 are not required for fusion of telomeres.

\textit{Inhibition of telomere recombination by Trt1-CI requires Ccq1, while it does not require Swi6, Clr4 or Poz1}—re-introduction of catalytically inactive Trt1 (Trt1-CI) to \textit{taz1Δ trt1Δ} linear chromosome survivor cells induces chromosome circularization due to the inhibition of telomere recombination (23). To test if Swi6, Clr4, Poz1 and Ccq1 are required for the inhibition of telomere recombination by Trt1, we transformed Trt1-CI plasmids (pKAN-trt1-D590A or pKAN-trt1-D743A) into \textit{taz1Δ trt1Δ swi6Δ, taz1Δ trt1Δ clr4Δ, taz1Δ trt1Δ poz1Δ}, and \textit{taz1Δ trt1Δ ccq1Δ} cells (Figure 3A-E).

We found that both Trt1-D590A and Trt1-D743A were able to induce chromosome circularization in \textit{taz1Δ trt1Δ swi6Δ, taz1Δ trt1Δ clr4Δ} and \textit{taz1Δ trt1Δ poz1Δ} cells (Figure 3B-D). Thus, Swi6, Clr4 and Poz1 are dispensable for the inhibition of recombination by Trt1-CI. Re-introduction of wild-type Trt1 led to massive elongation of telomeres in these triple mutant cells, comparable to \textit{taz1Δ trt1Δ} cells complemented with the wild-type Trt1 plasmid (Figure 4). In addition, we observed a comparable precipitation of telomeric repeat DNA by wild-type Trt1 in quantitative ChIP assays among \textit{taz1Δ trt1Δ, taz1Δ trt1Δ swi6Δ, taz1Δ trt1Δ clr4Δ} and \textit{taz1Δ trt1Δ poz1Δ} cells (Figure 5C). Thus, Swi6, Clr4 and Poz1 appear to play no role in telomere recruitment of telomerase or the inhibition of telomerase-dependent telomere elongation in the absence of Taz1.

On the other hand, neither Trt1-D590A nor Trt1-D743A was able to induce chromosome circularization in \textit{taz1Δ trt1Δ ccq1Δ} cells (Figure 3E). Thus, Ccq1 is essential for inhibition of recombination at telomeres by Trt1-CI. Re-introduction of wild-type Trt1 into \textit{taz1Δ trt1Δ...
ccq1Δ cells caused only a very slight increase in telomere length (Figure 4), and cells stably maintained telomeres.

In contrast, a previous study reported rapid telomere loss for taz1Δ ccq1Δ cells when Taz1 was eliminated from ccq1Δ cells to generate taz1Δ ccq1Δ cells (20). In our hands, we observed that taz1Δ ccq1Δ cells generated by genetic cross of single mutant strains initially undergo a very low viability phase, but quickly generate a mixture of survivors that carry either circular chromosomes or stably linear chromosomes (our unpublished observations). The taz1Δ ccq1Δ linear chromosome survivors behaved similarly to taz1Δ trt1Δ ccq1Δ cells carrying either wild-type Trt1 or Trt1-CI plasmids.

In any case, since we found that recruitment of wild-type Trt1 was greatly reduced (but not completely abolished) in taz1Δ ccq1Δ cells compared to taz1Δ cells (Figure 5C), we concluded that efficient recruitment of telomerase to telomeres, promoted by Ccq1, is important for inhibition of telomere recombination by Trt1-CI. On the other hand, since Ccq1 was previously found to be involved in the inhibition of telomere recombination (19) and taz1Δ trt1Δ ccq1Δ cells showed slightly longer telomeres than taz1Δ trt1Δ cells (Figure 4), the ability of Ccq1 to repress telomere recombination might also contribute to Trt1-CI-induced chromosome circularization in taz1Δ trt1Δ cells.

Trt1-ΔT/RT is unable to inhibit recombination-based telomere maintenance in the absence of Swi6, Clr4, and Ccq1—the C-terminally truncated Trt1 (Trt1-ΔT/RT), which lacks both T-motif and reverse transcriptase (RT) domain (Figure 3A), can also efficiently inhibit recombination-based telomere maintenance (23). Thus, we next examined if Trt1-ΔT/RT could still induce chromosome circularization in the absence of Swi6, Clr4, Poz1, or Ccq1.

We found that loss of Swi6 completely abolished Trt1-ΔT/RT-induced chromosome circularization (Figure 3B). On the other hand, we found that chromosomes in ~30% of taz1Δ trt1Δ clr4Δ cells (4 out of 13 examined) became circular after extensive restreaking on YES plates (Figure 3C and data not shown). We are unsure why the telomere status among independent taz1Δ trt1Δ clr4Δ cells is mixed after Trt1-ΔT/RT re-introduction. However, we can conclude that Trt1-ΔT/RT requires the presence of both Swi6 and Clr4 in order to efficiently induce chromosome circularization (Figure 3B-C).

After Trt1-ΔT/RT re-introduction, taz1Δ trt1Δ ccq1Δ cells were able to stably maintain linear chromosomes (Figure 3E), much like their counterparts expressing Trt1-CI. Thus, Ccq1 is also required for the inhibition of recombination-based telomere maintenance by Trt1-ΔT/RT. On the other hand, Trt1-ΔT/RT was still able to induce chromosome circularization in taz1Δ trt1Δ poz1Δ cells (Figure 3D). Thus, Poz1 appears to play no role in Taz1- or Trt1-dependent inhibition of telomere recombination.

Rad3ATR is required for Trt1-ΔT/RT-dependent inhibition of telomere recombination—we had previously assumed that Trt1-CI and Trt1-ΔT/RT would show identical genetic requirements for the efficient inhibition of telomere recombination. Thus, we had not tested how re-introduction of Trt1-ΔT/RT would affect recombination-based telomere maintenance in taz1Δ trt1Δ est1Δ, taz1Δ trt1Δ pku70Δ, or taz1Δ trt1Δ rad3Δ (23). However, since we now observed that Swi6 and Clr4 are uniquely required for Trt1-ΔT/RT to efficiently inhibit telomere recombination, we next tested how loss of the telomerase regulatory subunit Est1, the NHEJ DNA repair protein Ku70, and the checkpoint kinase Rad3ATR affect the ability of Trt1-ΔT/RT to induce chromosome circularization in taz1Δ trt1Δ survivor cells.

Our previous analyses indicated that Est1 is required for efficient telomere recruitment of Trt1 and Trt1-dependent inhibition of telomere recombination (23). On the other hand, loss of Ku70 abolished Trt1-dependent inhibition of telomere recombination without affecting recruitment of Trt1 to telomeres. Rad3ATR appeared not to contribute to the negative regulation of telomere recombination since Trt1-CI was still able to efficiently induce chromosome circularization in taz1Δ trt1Δ rad3Δ cells (23).

As expected, taz1Δ trt1Δ est1Δ and taz1Δ trt1Δ pku70Δ cells were able to stably maintain linear chromosomes after re-introduction of Trt1-ΔT/RT (Figure 3F). On the other hand, we found that loss of Rad3ATR abolished the ability of Trt1-ΔT/RT to inhibit telomere recombination.
ΔT/RT to induce chromosome circularization in taz1Δ trt1Δ rad3Δ cells (Figure 3F). Thus, Rad3<sup>ATR</sup> is also uniquely required for recombination inhibition by Trt1-ΔT/RT (but not Trt1-CI), much like Swi6 and Clr4.

**Trt1-ΔT/RT shows reduced but significant Ccq1- and Est1-independent telomere association, compared to wild-type Trt1—**quantitative ChIP analysis revealed that Trt1-ΔT/RT was less efficient in precipitating telomeric repeat DNA than wild-type Trt1, indicating that Trt1-ΔT/RT is bound less efficiently to telomeres (Figure 5C-D). Moreover, loss of Swi6, Clr4 or Rad3<sup>ATR</sup> did not significantly reduce telomere association of either wild-type Trt1 or Trt1-ΔT/RT (Figure 5C-D). Thus, we concluded that Swi6, Clr4 and Rad3<sup>ATR</sup> are likely to contribute to the inhibition of telomere recombination, and they are thus needed for the less efficient Trt1-ΔT/RT-induced chromosome circularization. However, they are dispensable for the strong inhibition of telomere recombination imposed by Taz1 or Trt1-CI.

For wild-type Trt1, we observed a significant reduction in precipitation efficiency of telomeric repeat DNA in taz1Δ trt1Δ ccq1Δ and taz1Δ trt1Δ est1Δ cells compared to taz1Δ trt1Δ cells by ChIP assays, consistent with previous studies demonstrating that Ccq1 and Est1 promote the recruitment of telomerase to telomeres (20,23,35). However, we observed a residual Trt1 association significantly above the untagged Trt1 control strain even in the absence of Ccq1 or Est1, suggesting that telomerase can be recruited to telomeres independently of Ccq1 and Est1, at least in a taz1Δ background (Figure 5C).

We have concluded in our previous paper that the loss of Est1 abolishes the recruitment of Trt1 to telomeres in taz1Δ est1Δ cells (23). However, in our earlier experiments, we used quantitative real-time PCR to detect telomerase recruitment to telomeres. The primer pairs used in those PCR are located in the sub-telomeric region immediately adjacent to the telomeric repeat sequences. Thus, while PCR primers are only 250-300 base pairs away from the 3' ends of telomeres in wild-type cells, they are several kilobases away from the 3' ends in taz1Δ cells, due to Trt1-dependent telomere elongation. We had partially corrected for the fact that PCR primers are further away from telomere ends by reducing the number of sonication cycles, but this probably led to an underestimation of telomere-bound Trt1. In the current study, we hybridized a telomeric repeat DNA probe to ChIP samples spotted on a nylon membrane to measure Trt1 recruitment to telomeres, and thus improved the sensitivity of Trt1 detection in taz1Δ cells. Therefore, while both real-time PCR and hybridization methods clearly indicate that Est1 is crucial for the efficient recruitment of Trt1 even in the absence of Taz1, we can now detect Est1-independent recruitment of Trt1 significantly above the untagged background control.

Since the efficiency for telomeric repeat DNA precipitation by wild-type Trt1 was comparable among taz1Δ trt1Δ ccq1Δ, taz1Δ trt1Δ est1Δ and taz1Δ trt1Δ ccq1Δ est1Δ cells (Figure 6C), we could exclude the possibility that Ccq1 and Est1 are redundantly required for recruitment of telomerase to telomeres. In addition, our results implicate the existence of a telomerase recruitment mechanism that is independent of Ccq1 and Est1 in fission yeast.

We were surprised to find that Trt1-ΔT/RT association with telomeres was not significantly reduced by loss of Ccq1 and/or Est1 (Figure 5D, 6D). Since we could not detect significant changes in the recruitment efficiency of Trt1-ΔT/RT to telomeres by deleting Ccq1 or Est1, loss of Trt1-ΔT/RT-induced chromosome circularization could not be solely explained by roles of Ccq1 and Est1 in promoting the efficient recruitment of Trt1 to telomeres. We have observed that taz1Δ trt1Δ est1Δ (23) and taz1Δ trt1Δ ccq1Δ (Figure 4) cells maintain a slightly longer average telomere length than taz1Δ trt1Δ cells. Moreover, Ccq1 has been shown to be important for preventing telomere recombination (19,20). Thus, our results are consistent with the notion that Est1 and Ccq1 also contribute to inhibition of recombination-based survival in fission yeast, independently of Taz1 and Trt1. If this hypothesis is indeed true, one might be able to obtain separation of function mutants for Est1 and Ccq1, which fail to support telomerase recruitment but can still contribute to inhibition of telomere recombination in the future. Such results would then provide independent experimental support for the existence of Trt1-
Rad3ATR is involved in inhibition of Rhp51Rad51 accumulation at telomeres in taz1Δ trt1Δ cells since our results suggest that Swi6, Ctr4 and Rad3ATR are uniquely required for Trt1-ΔT/RT-induced chromosome circularization by contributing to the inhibition of telomere recombination, we next decided to test if loss of Swi6, Ctr4 and Rad3ATR would cause an increase in the recruitment of DNA repair factors involved in telomere recombination. Since we have previously established that the HR protein Rad22 (Rad52 ortholog) is essential for the maintenance of linear chromosomes in taz1Δ trt1Δ cells (23), we initially wanted to test if the recruitment of Rad22Rad52 to telomeres is increased when Swi6, Ctr4 or Rad3ATR are eliminated in taz1Δ trt1Δ cells by ChIP assays. Unfortunately, when we introduced the myc-tagged Rad22Rad52, which we had previously deemed largely functional based on DNA damage sensitivity (39), into taz1Δ trt1Δ cells, we discovered that the resulting cells were unable to maintain stable linear chromosomes (data not shown). Therefore, we turned to another HR repair protein Rhp51 (Rad51 ortholog), since we can utilize an antibody raised against mammalian Rhp51 to monitor the recruitment of Rhp51 to DNA by ChIP (39,42).

We first established that Rhp51Rad51 is essential for linear chromosome maintenance in taz1Δ trt1Δ cells by creating several independently derived taz1Δ trt1Δ rhp51Δ strains, extensively restreaking them on YES plates, and then examining their telomere structure by PFGE (Figure 7A). We also established that the recruitment of Rhp51Rad51 to telomeres is significantly enhanced in taz1Δ trt1Δ cells compared to wild-type cells (Figure 7B).

We then asked if the elimination of Swi6, Ctr4 or Rad3ATR would increase Rhp51Rad51 recruitment to telomeres in taz1Δ trt1Δ cells. Additionally, we examined if the elimination of Ccq1 affects the recruitment of Rhp51Rad51 to telomeres in taz1Δ trt1Δ cells. We did not observe significant differences in Rhp51Rad51 recruitment to telomeres among taz1Δ trt1Δ, taz1Δ trt1Δ swi6Δ, taz1Δ trt1Δ ctr4Δ, and taz1Δ trt1Δ ccq1Δ cells (Figure 7B). In contrast, taz1Δ trt1Δ rad3Δ cells showed a significant increase in the recruitment of Rhp51Rad51 to telomeres over taz1Δ trt1Δ cells. Thus, more investigations are required to establish a molecular mechanism by which loss of Swi6, Ctr4 or Ccq1 suppresses Trt1-ΔT/RT-induced chromosome circularization. However, at least for Rad3ATR, our Rhp51Rad51 ChIP data support the notion that the inability of Trt1-ΔT/RT to induce chromosome circularization may be caused by a reduced protection against telomere recombination in taz1Δ trt1Δ rad3Δ cells.

DISCUSSION

In this study, we took advantage of the unique ability of fission yeast to survive telomere dysfunction by circularizing their chromosomes, in order to understand how telomere recombination and telomerase recruitment is regulated. Since taz1Δ trt1Δ cells are healthy and maintain stable telomeres by utilizing a recombination-based mechanism, mutations that lead to chromosome circularization in taz1Δ trt1Δ cells might identify positive regulators of telomere recombination. Conversely, since we had previously established that Taz1, Trt1-CI and Trt1-ΔT/RT strongly inhibit telomere recombination, mutations that suppress chromosome circularization upon re-introduction of Taz1, Trt1-CI or Trt1-ΔT/RT into taz1Δ trt1Δ cells may identify factors that contribute to the inhibition of telomere recombination. In addition, a subset of mutations that can suppress chromosome circularization induced by Trt1-CI or Trt1-ΔT/RT may identify factors involved in the recruitment of telomerase to telomeres.

We examined how loss of proteins involved in the formation of sub-telomeric heterochromatin and loss of two Pot1 telomere-capping complex subunits affect telomere recombination and/or telomerase recruitment to telomeres. We also re-examined roles of the checkpoint kinase Rad3ATR and the telomerase regulatory subunit Est1 in inhibition of telomere recombination. Our results imply roles for Swi6, Ctr4, Rad3ATR, Ccq1 and Est1 in the protection against telomere recombination, which we propose is crucial for the induction of chromosome circularization in taz1Δ trt1Δ cells upon re-introduction of Trt1-ΔT/RT. However, it should be noted that contributions
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made by Swi6, Clr4 and Rad3<sup>ATR</sup> are minor compared to major inhibitors of telomere recombination, such as Taz1, Trt1 and Ku70.

We also established that Ccq1 and Est1 are both important for the efficient recruitment of Trt1 to telomeres, even in the absence of Taz1, the negative regulator of telomerase. However, since we could still detect a reduced but significant recruitment of Trt1 even in the absence of both Ccq1 and Est1, we propose the existence of a telomerase recruitment mechanism that could function in the absence of Taz1, Ccq1 and Est1.

We have reported previously that <i>taz1Δ est1Δ</i> cells cannot maintain stable linear telomeres and circularize chromosomes when they were generated by deleting <i>est1</i> from <i>taz1Δ</i> cells (23). In contrast, when <i>est1</i> was deleted from <i>taz1Δ trt1Δ</i> cells, the resulting triple mutant cells were able to stably maintain telomeres (23). Based on these observations, we speculated that Trt1 might be able to contribute to the inhibition of recombination-based telomere maintenance in the absence of Est1. Since we can now detect residual Trt1 recruitment to telomeres even in <i>taz1Δ est1Δ</i> background, it further supports the notion that Trt1 indeed could have Est1-independent roles at telomeres.

Based on results presented in the current and previous papers, we can begin to establish a hierarchical order of the numerous proteins involved in telomere maintenance, both for the inhibition of telomere recombination (Figure 8A) and for the telomerase-dependent telomere extension (Figure 8B). We do not imply the existence of linear pathways for the various factors indicated in Figure 8, but their placement is meant to summarize the genetic requirements for either the inhibition or the promotion of telomere recombination and telomerase-dependent telomere elongation.

For inhibition of telomere recombination (Figure 8A), the inhibitory sign directly points from Taz1 to a “telomere recombination” box since we have yet to find mutation(s) that can abolish the Taz1-induced chromosome circularization. On the other hand, for telomerase-dependent telomere extension (Figure 8B), Rap1 and Poz1 are placed below Taz1 since Rap1 and Poz1 are required for the Taz1-dependent inhibition of telomere extension by telomerase (19,28).

Similarly, factors placed below Trt1 or Trt1-ΔT/RT in Figure 8A are those we have shown to be required for chromosome circularization induced by Trt1-C1 or Trt1-ΔT/RT, respectively. For Figure 8B, Est1 and Ccq1 are placed below Trt1 since they are both essential for telomerase-dependent telomere maintenance (16,19,20). Rad3<sup>ATR</sup> and MRN-Tel1 are placed in branched arrows, since they are redundantly required for telomerase-dependent telomere maintenance and recruitment of telomerase to telomeres (10,35,43).

It is particularly intriguing that Taz1, Rap1 and Poz1 show quite different phenotypes with regard to the regulation of recombination at telomeres, despite the fact that deletions of these three factors result in massive telomerase-dependent telomere elongation and loss of transcriptional repression for markers inserted adjacent to the telomeric repeats (28,35,44). In the case of Poz1, we did not find any evidence that this protein plays a role in the repression of telomere recombination. In addition, the fact that <i>taz1Δ trt1Δ poz1Δ</i> cells can stably maintain telomeres indicates that Poz1 is not involved in the promotion of recombination-based telomere maintenance. In contrast, we have previously demonstrated that Rap1 is essential for recombination-based telomere maintenance in <i>taz1Δ trt1Δ</i> cells (23). We were surprised to find that Rap1 can contribute to recombination-based survival in the absence of Taz1, since efficient recruitment of Rap1 to telomeres has been shown to depend on Taz1 in fission yeast (23,28,45) (see also Figure 1). However, the recent finding that Rap1 directly interacts with Poz1 (19) raised the possibility that a Poz1-dependent recruitment of Rap1 to telomeres might be critical for Rap1’s role in the promotion of recombination-based telomere maintenance. However, since <i>taz1Δ trt1Δ poz1Δ</i> cells can stably maintain telomeres, our results indicate that Rap1’s positive contribution in recombination-based telomere maintenance is independent of both Taz1 and Poz1.

By analyzing the spontaneous rearrangement of sub-telomeric regions, the Ku70-Ku80 complex and the formation of sub-telomeric heterochromatin have previously been implicated in the repression of recombination at telomeres (33,42,46). Our chromosome circularization
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assays using Taz1, Trt1-CI and Trt1-ΔT/RT have enabled us to detect the contribution of these factors in the regulation of telomere recombination. In addition, the use of Trt1-ΔT/RT in the chromosome circularization assay has allowed us to identify a previously unknown contribution of Rad3*KTR in the repression of telomere recombination. Factors that affect the recruitment efficiency of telomerase to telomeres, such as Est1 and Ccq1, have also been identified as necessary for the suppression of chromosome circularization by Trt1-CI.

Therefore, the chromosome circularization assay we have developed should be useful in identifying new factors that contribute to the regulation of telomere recombination and telomerase-dependent telomere extension. Since proteins involved in telomere regulation are well conserved between fission yeast and mammalian cells, careful evaluation of factors that affect telomere recombination and telomerase recruitment in fission yeast may provide new insight into the regulation of mammalian telomere recombination and telomerase recruitment.
REFERENCES


FOOTNOTES

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The abbreviations used are: TERT, telomerase reverse transcriptase; MRN, Mre11-Rad50-Nbs1; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ALT, alternative lengthening of telomeres; HR, homologous recombination; NHEJ, non-homologous end-joining; SHREC, Snf2/Hdac-containing Repressor Complex; ChIP, chromatin immunoprecipitation; PFGE, pulsed-field gel electrophoresis.

FIGURE LEGENDS

FIGURE 1. A model of fission yeast telomere proteins discussed in this study. The complex formed by Taz1, Rap1, Poz1, Tpz1, Pot1 and Ccq1 is thought to resemble the mammalian telomere complex shelterin. Taz1, Rap1 and Poz1 are important for the negative regulation of telomerase, while Ccq1, Tpz1 and Pot1 are implicated in the recruitment of telomerase to telomeres. Ccq1 is also associated with the SHREC complex, and it promotes the Clr4-dependent methylation of histone H3 Lysine 9 (H3 K9me) and the accumulation of Swi6 at telomeres.

FIGURE 2. Swi6, Clr4, Poz1 and Ccq1 are dispensable for recombination-based telomere maintenance. A, NotI restriction map of fission yeast chromosomes. Telomeric C, I, L and M (filled black) fragment specific probes were used in Southern blot hybridization shown in Figures 2B-E, 3B-F, 6E and 7A. B-E, telomere status analysis by PFGE for fission yeast strains with the indicated genotypes. Chromosomal DNA was prepared in agarose plugs, digested with NotI, and then used in PFGE. DNA was then transferred to a Nylon membrane and hybridized to C, I, L and M specific probes.

FIGURE 3. Swi6, Clr4, Ccq1, Est1, Ku70, and Rad3 are required for the Trt1-ΔT/RT-dependent inhibition of telomere recombination. A, trt1+ gene structure (top) and schematic diagrams (bottom) for wild-type (trt1+), catalytically inactive Trt1-CI (trt1-D590A, trt1-D743A) and truncated Trt1-ΔT/RT used in this study. B-F, analysis of telomere status by PFGE for the indicated strains transformed with empty, Taz1, Trt1, Trt1-CI or Trt1-ΔT/RT plasmids.

FIGURE 4. Analysis of telomere length of various triple mutant strains. Southern blot analysis was performed for the indicated strains. The effect of expressing wild-type Trt1 on telomere length was also examined. Genomic DNA was digested with EcoRI, fractionated in a 1% agarose gel, and processed for Southern blot analysis. A probe specific to telomeric repeat DNA was used in hybridization.
FIGURE 5. **Recruitment of Trt1 to telomeres monitored by quantitative ChIP assays.** *A-B*, expression levels of full length Trt1 (*A*) or Trt1-ΔT/RT (*B*) were examined by anti-myc Western blots, and found to be comparable among the various mutant backgrounds. Western blots with anti-Cdc2 antibody were used as loading controls. *C*, recruitment of wild-type Trt1 to telomeres was monitored by quantitative ChIP assays. Percent precipitation of input DNA was determined for each ChIP sample, based on quantification by dot blot hybridization with a telomeric repeat DNA probe. (Representative dot blots are shown below.) Average % precipitation values from at least three independent experiments are plotted, and error bars represent standard deviations. For all strains tested, Trt1-myc showed statistically significant enrichment of telomeric DNA over no tag control (*P* = 0.003 for *taz1Δ trt1Δ ccq1Δ*, *P* = 0.006 for *taz1Δ trt1Δ est1Δ*, and *P* < 0.0002 for other triple mutant strains). Compared to *taz1Δ trt1Δ*, only *taz1Δ trt1Δ ccq1Δ* (*P* = 0.0001) and *taz1Δ trt1Δ est1Δ* (*P* = 0.0007) showed statistically significant reductions in Trt1 recruitment. *D*, recruitment of Trt1-ΔT/RT to telomeres was monitored by quantitative ChIP assays with dot blot hybridization using a telomeric repeat DNA probe. (Representative dot blots are shown below.) Average % precipitation values from at least three independent experiments are plotted, and error bars represent standard deviations. For all strains tested, Trt1-ΔT/RT-myc showed statistically significant enrichment of telomeric DNA over no tag control (*P* < 0.016). Compared to *taz1Δ trt1Δ*, none of the triple mutant strains showed statistically significant changes in % precipitation values (*P* > 0.14).

FIGURE 6. **Wild-type Trt1 and Trt1-ΔT/RT can be recruited to telomeres in the absence of Taz1, Est1 and Ccq1.** *A-B*, expression levels of wild-type Trt1 (*A*) or Trt1-ΔT/RT (*B*) were examined by anti-myc Western blots, and found to be comparable among the various mutant backgrounds. Western blots with anti-Cdc2 antibody were used as loading controls. *C-D*, recruitment of wild-type Trt1 (*C*) or Trt1-ΔT/RT (*D*) to telomeres was monitored by quantitative ChIP assays with dot blot hybridization using a telomeric repeat DNA probe. (Representative dot blots are shown below.) Average % precipitation values from at least four independent experiments are plotted, and error bars represent standard deviations. For all strains tested, Trt1-myc and Trt1ΔT/RT-myc showed statistically significant enrichment of telomeric DNA over no tag control (*P* < 0.006). *E*, telomere status analysis by PFGE indicates that *taz1Δ trt1Δ est1Δ ccq1Δ* cells stably maintain telomeres.

FIGURE 7. **Recruitment of Rad51 to telomeres monitored by quantitative ChIP assays.** *A*, telomere status analysis by PFGE indicates that Rhp51Rad51 is essential for telomere maintenance in *taz1Δ trt1Δ* cells. *B*, recruitment of Rhp51Rad51 to telomeres was monitored by quantitative ChIP assays using real-time PCR. Average % precipitation values from at least four independent experiments are plotted, and error bars represent standard deviations. Comparable levels of Rhp51 were expressed among the different mutant strains, based on anti-Rad51 Western blots. Western blots with anti-Cdc2 antibody were used as loading controls.

FIGURE 8. **A summary of the genetic regulation of telomere recombination (A) and telomerase-dependent telomere extension (B) in fission yeast.** See main text for details.
Khair_Figure2

A

Ch. I (5.7 Mbp)
L (380 kbp)
I (530 kbp)

Ch. II (4.6 Mbp)
M (240 kbp)
C (1525 kbp)

Ch. III (3.5 Mbp)

B

wt  trt1Δ  taz1Δ trt1Δ  taz1Δ trt1Δ swi6Δ  taz1Δ trt1Δ swi6Δ

wt  trt1Δ  taz1Δ trt1Δ  taz1Δ trt1Δ clr4Δ  taz1Δ trt1Δ clr4Δ

C

wt  trt1Δ  taz1Δ trt1Δ  taz1Δ poz1Δ  taz1Δ trt1Δ poz1Δ

wt  trt1Δ  taz1Δ trt1Δ  taz1Δ ccq1Δ  taz1Δ trt1Δ ccq1Δ

D

wt  trt1Δ  taz1Δ trt1Δ  taz1Δ poz1Δ  taz1Δ trt1Δ poz1Δ

wt  trt1Δ  taz1Δ trt1Δ  taz1Δ ccq1Δ  taz1Δ trt1Δ ccq1Δ

E

wt  trt1Δ  taz1Δ trt1Δ  taz1Δ poz1Δ  taz1Δ trt1Δ poz1Δ

wt  trt1Δ  taz1Δ trt1Δ  taz1Δ ccq1Δ  taz1Δ trt1Δ ccq1Δ
A

Taz1 ➔ Trt1 ➔ Trt1-ΔT/RT

Est1  Ccq1  Ku70

Telomere recombination

Rap1  MRN-Tel1  Rad52  Rad51

Likely not involved

Poz1

B

Taz1 ➔ Trt1

Rap1  Poz1

Est1  Ccq1

Swi6  Clr4  Rad3

Telomerase-dependent telomere extension

Rad3  MRN-Tel1

Likely not involved

Ku70  Rad52  Rad51  Swi6  Clr4
Roles of heterochromatin and telomere proteins in regulation of fission yeast telomere recombination and telomerase recruitment
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