

Fission yeast Rhp51 is required for the maintenance of telomere structure in the absence of the Ku heterodimer

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ABSTRACT

The *Schizosaccharomyces pombe* Ku70–Ku80 heterodimer is required for telomere length regulation. Lack of *pku70*⁺ results in telomere shortening and striking rearrangements of telomere-associated sequences. We found that the rearrangements of telomere-associated sequences in *pku80*⁺ mutants are Rhp51 dependent, but not Rad50 dependent. Rhp51 bound to telomere ends when the Ku heterodimer was not present at telomere ends. We also found that the single-stranded G-rich tails increased in S phase in wild-type strains, while deletion of *pku70*⁺ increased the single-stranded overhang in both G₂ and S phase. Based on these observations, we propose that Rhp51 binds to the G-rich overhang and promotes homologous pairing between two different telomere ends in the absence of Ku heterodimer. Moreover, *pku80 rhp51* double mutants showed a significantly reduced telomere hybridization signal. Our results suggest that, although Ku heterodimer sequesters Rhp51 from telomere ends to inhibit homologous recombination activity, Rhp51 plays important roles for the maintenance of telomere ends in the absence of the Ku heterodimer.

INTRODUCTION

Telomeres are the DNA–protein complexes located at the ends of eukaryotic chromosomes (1–4). Telomere DNA consists of a simple G-rich repeat sequence with a length of 300 bp in yeast. Myb domain-containing telomere-binding proteins such as *Schizosaccharomyces pombe* Taz1 recognize the telomere-repeat sequence (GGTTACA)_n and form distinct DNA–protein structures (5). These structures protect chromosome ends from degradation and aberrant recombination or fusion (6). Telomeres are shortened with each cell division and cells undergo replicative senescence if telomerase is inactive. To prevent telomere shortening, telomere DNA is synthesized by telomerase, which catalyzes the polymerization of telomeric

repeats. Although both *Saccharomyces cerevisiae* and *S.pombe* normally rely on telomerase activity to maintain their telomeres, telomeres can be maintained without this activity. Telomerase-independent telomere maintenance has been well studied in *S.cerevisiae* (7,8). Generation of survivors that lack the gene for a telomerase component requires RAD52-dependent recombination. The majority of survivors have multiple tandem copies of the subtelomeric Y' element and short telomere DNA (type I survivors) (9,10). In contrast, type II survivors (a minor fraction of the survivors) have long, heterogeneous tracts of telomeric repeats (10). In *S.pombe*, deletion of *trt1*⁺, which encodes a catalytic protein subunit of telomerase, leads to progressive loss of telomere DNA and causes cellular senescence (11). There are two modes of survival in those cells. Most survivors have circularized all of their chromosomes, whereas a minor fraction of survivors maintain their telomeres, probably through recombination (12).

Unlike natural chromosome ends (telomere ends), DNA double-strand break (DSB) ends are repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) (13–15). In *S.pombe*, DSBs are mainly repaired by HR. Rad50 and Rad32 (the *S.pombe* homolog of Mre11) are thought to be required for resection of DSB ends to initiate HR (16–19). Rhp51 (the *S.pombe* homolog of Rad51) is thought to bind the single-stranded region of DSB ends to promote homologous pairing and strand exchange (20). Although NHEJ is not a major repair pathway in *S.pombe*, the Ku70–Ku80 heterodimer is required for the repair of linearized plasmids following transformation into cells (21,22). The HR and NHEJ repair pathways are conserved from yeast to mammals. In vertebrates, NHEJ dominates during G₁ to early S phase of the cell cycle and HR is used in late S to G₂ phase (23,24).

Telomere ends are not sensed as DNA DSB ends. However, recent studies have revealed that DNA repair proteins such as the Mre11–Rad50–Xrs2/Nbs1 complex and the Ku70–Ku80 heterodimer bind to telomere ends and play important roles in telomere maintenance (25–29). One might expect that binding of these proteins to telomere ends would result in unnecessary HR or NHEJ. However, Ku heterodimer actually inhibits DNA end joining between telomere DNAs in mice, suggesting that

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Table 1. *Schizosaccharomyces pombe* strains used in this study

| Strain | Genotype | Source |
|--------|---|------------------|
| JY741 | <i>h⁻ leu1-32 ura4-D18 ade6-M216</i> | M. Yamamoto |
| JY746 | <i>h⁺ leu1-32 ura4-D18 ade6-M210</i> | M. Yamamoto |
| LSP11 | <i>h⁺ leu1-32 ura4-D18 cdc25-22</i> | Laboratory stock |
| KT102 | <i>h⁺ leu1-32 ura4-D18 ade6-M216 rad50::ura4⁺</i> | This study |
| KT10b | <i>h⁺ leu1-32 ura4-D18 ade6-M216 rad22::ura4⁺</i> | This study |
| KT10c | <i>h⁺ leu1-32 ura4-D18 ade6-M216 rhp51::ura4⁺</i> | This study |
| HK001 | <i>h leu1-32 ura4-D18 ade6-M216 rad3::aur1 tell1::ura4⁺</i> | This study |
| TK001 | <i>h⁺ leu1-32 ura4-D18 ku70::LEU2 cdc25-22</i> | This study |
| TK002 | <i>h⁻ leu1-32 ura4-D18 ade6-M210</i> | This study |
| TK003 | <i>h⁻ leu1-32 ura4-D18 ade6-M216 pku80::LEU2</i> | This study |
| TK004 | <i>h⁻ leu1-32 ura4-D18 ade6-M216 pku80::LEU2</i> | This study |
| TK005 | <i>h⁻ leu1-32 ura4-D18 ade6-M216 pku80::LEU2</i> | This study |
| TK006 | <i>h⁻ leu1-32 ura4-D18 ade6-H210 pku80::LEU2</i> | This study |
| TK007 | <i>h⁻ leu1-32 ura4-D18 ade6-M210 pku80::LEU2</i> | This study |
| TK009 | <i>h⁺ leu1-32 ura4-D18 ade6-M216 pku80::LEU2 rad50::ura4⁺</i> | This study |
| TK010 | <i>h⁺ leu1-32 ura4-D18 ade6-M216 pku80::LEU2 rad50::ura4⁺</i> | This study |
| TK011 | <i>h⁺ leu1-32 ura4-D18 ade6-M216 pku80::LEU2 rad50::ura4⁺</i> | This study |
| TK012 | <i>h⁺ leu1-32 ura4-D18 ade6-M216 pku80::LEU2 rhp51::ura4⁺</i> | This study |
| TK013 | <i>h⁺ leu1-32 ura4-D18 ade6-M216 pku80::LEU2 rhp51::ura4⁺</i> | This study |
| TK014 | <i>h⁺ leu1-32 ura4-D18 ade6-M216 pku80::LEU2 rhp51::ura4⁺</i> | This study |
| TK015 | <i>h⁻ leu1-32 ura4-D18 ade6-M216 pku80::LEU2 rad22::ura4⁺</i> | This study |
| TK016 | <i>h⁺ leu1-32 ura4-D18 ade6-M216 pku80::LEU2 rad22::ura4⁺</i> | This study |

Ku heterodimer has distinct roles at two types of different DNA end (telomere ends and DSB ends) (27,30–32).

In *S.cerevisiae*, deletion of *YKU70* or *YKU80* results in telomere shortening and loss of the silencing of telomere-adjacent genes (33–35). In addition, deletion of *YKU70* or *YKU80* results in the generation of single-strand 3' G-tails at telomere ends in a cell cycle-independent manner, while the single-strand 3' G-tail appears only in S phase in wild-type cells (36–39). In *S.pombe*, deletion of *pku70⁺* or *pku80⁺* results in telomere shortening (22,40). However, unlike in *S.cerevisiae*, telomere silencing in *pku70* or *pku80* mutants remains intact (21,22). In *S.pombe*, the telomeric repeat sequences are internally flanked by repetitive telomere-associated sequence (TAS) (41). Lack of the functional Ku heterodimer caused striking rearrangements of TAS in a *rad22⁺*-dependent manner, indicating a function for Ku heterodimers in inhibiting recombinational activities near chromosome ends (40). However, the mechanism of the recombination at telomere ends in the absence of Ku heterodimers is not fully understood.

Here we have studied the rearrangement of TAS in *pku80 rhp51* double mutants and *pku80 rad50* double mutants. We found that recombination at the subtelomeric region in the absence of Ku80 is Rhp51 dependent. Rhp51 bound to telomere ends when Ku80 was not present there. Moreover, deletion of *pku70⁺* increased the single-stranded overhang in both G₂ and S phase. Based on these observations, we propose that Rhp51 binds to the G-rich overhang and promotes homologous pairing between two different telomere ends in the absence of the Ku heterodimer.

MATERIALS AND METHODS

Schizosaccharomyces pombe strains, medium and genetic methods

The *S.pombe* strains used in this study are listed in Table 1. All strains are derivatives of JY741 *h⁻* or JY746 *h⁺*. Standard

procedures and medium were used for propagation and genetic manipulation (42). YPAD medium consisted of 1% yeast extract, 2% polypeptone, 2% glucose and 20 µg/ml adenine. All experiments were repeated at least twice with similar results. The *rad50⁺* gene was disrupted by replacing the region between the first and second HindIII restriction sites (nucleotide positions 529–1158 relative to the initiation codon) with the *ura4⁺* gene. *pku70-d* and *pku80-d* were constructed by insertion of the *LEU2* cassette into the EcoRV sites of each of these genes. *rad22-d* and *rhp51-d* cells were constructed by insertion of the *ura4⁺* cassette into the AflII site of the *rad22⁺* gene and the NheI site of the *rhp51⁺* gene, respectively. Other double mutants were constructed by genetic crosses.

Chromatin immunoprecipitation (ChIP)

The ChIP assay described by Takahashi *et al.* (43) was adopted with modification. Cells grown in 100 ml of YPAD medium at 30°C were fixed with formaldehyde. For immunoprecipitation, anti-Rhp51 antibody raised in the laboratory of Dr H. Iwasaki (Yokohama City University) and protein G-coated Dynabeads (Dyna) were used. Immunoprecipitated DNA was extracted and suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA). PCR reactions used the following primers to amplify the telomeric DNA (Top, 5'-CGGCTGACGG-GTGGGGCCCAATA-3'; Bottom, 5'-GTGTGGAATTGAG-TATGGTGAA-3'), subtelomeric DNA (Top, 5'-CTACTT-ACTGCACCCTAACG-3'; Bottom, 5'-AAAGTAGGAGA-ATGAAGAAGTAATC-3') and *ade6⁺* DNA (Top, 5'-AGG-TATAACGACAACAAACGTTGC-3'; Bottom 5'-CAAGGC-ATCAGTGTTAATATGCTC-3').

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed as described by Baumann *et al.* (40). Chromosomes were fractionated in a 1% agarose gel with 0.5× TBE (50 mM Tris-HCl, 5 mM boric acid, 1 mM EDTA, pH 8.0) buffer at 14°C, with the CHEF Mapper PFGE system

(Bio-Rad) using the settings suggested by the manufacturer. DNA was visualized by staining with ethidium bromide (1 $\mu\text{g/ml}$) for 30 min.

In-gel hybridization

In-gel hybridization analysis was performed according to the protocol previously published (36) using a G-rich probe (G-probe, 5'-GATCGGGTTACAAGGTTACGTGGTTAC-ACG-3') and a C-rich probe (C-probe, 5'-CGTGTAAACC-ACGTAACCTTGTAACCCGATC-3'). A plasmid containing the telomere-repeat sequence derived from pNSU70 (12) was used as a double-stranded (ds) DNA and single-stranded (ss)DNA control. An aliquot of 1 μg of genomic DNA was digested with HindIII and electrophoresed on a 0.5% agarose gel in 0.5 \times TAE buffer with 0.01 mg/ml ethidium bromide. The gel was vacuum dried at 45°C until it become thin and warm (~45 min). Single-stranded telomeric DNA probe was labeled with [γ - ^{32}P]ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase. An AlkPhos Direct™ kit (Amersham Pharmacia Biotech) was used for hybridization. The gel was prehybridized in hybridization buffer at 37°C for 15 min and then 10 pmol of probe was added and the incubation was continued at 37°C overnight. The gel was washed twice with primary wash buffer at 37°C for 10 min and then washed three times with secondary wash buffer at room temperature for 5 min. The gel was dried on Whatman paper and exposed to X-ray film for ~2 days. To detect the double-stranded telomere DNA, the gel was treated with denaturing solution (0.5 M NaOH, 150 mM NaCl) for 25 min at room temperature and then treated with neutralizing solution (0.5 M Tris-HCl, pH 8.0, 150 mM NaCl) and reprobed with the C-rich probe (C-probe).

Measurement of telomere length

Telomere length was measured by Southern hybridization according to the procedure described previously (5) using an AlkPhos Direct™ kit module (Amersham Pharmacia Biotech). Briefly, chromosomal DNAs, which were digested with EcoRI, ApaI or NsiI and separated by electrophoresis on an agarose gel, were probed with a DNA fragment containing the telomeric-repeat sequences (telomere) or subtelomeric sequences (TAS1 and TAS3) derived from pNSU70 (a gift from Dr N. Sugawara).

RESULTS

Meiosis and/or germination accelerate rearrangement at the subtelomeric region in *ku80-d* cells

In the wild-type *h⁻* strains used in this study, digestion of total DNA with NsiI generates terminal fragments of 2.2, 3 and 4 kb (Fig. 1A and B, lane 1). In the absence of Ku70-Ku80 heterodimer, several low mobility shifted bands are observed and these bands are thought to be generated by rearrangements of TAS (22,40). However, the mechanism of these rearrangements is not fully understood. In previous studies (22,40), heterozygous diploid strains (*pku70^{+/-}* or *pku80^{+/-}*) were used to obtain *pku70-d* or *pku80-d* haploid cells. In such experiments, meiosis and/or germination might affect the rearrangement of TAS. To avoid these concerns, we made *pku80* disruptants by one-step gene replacement using wild-type

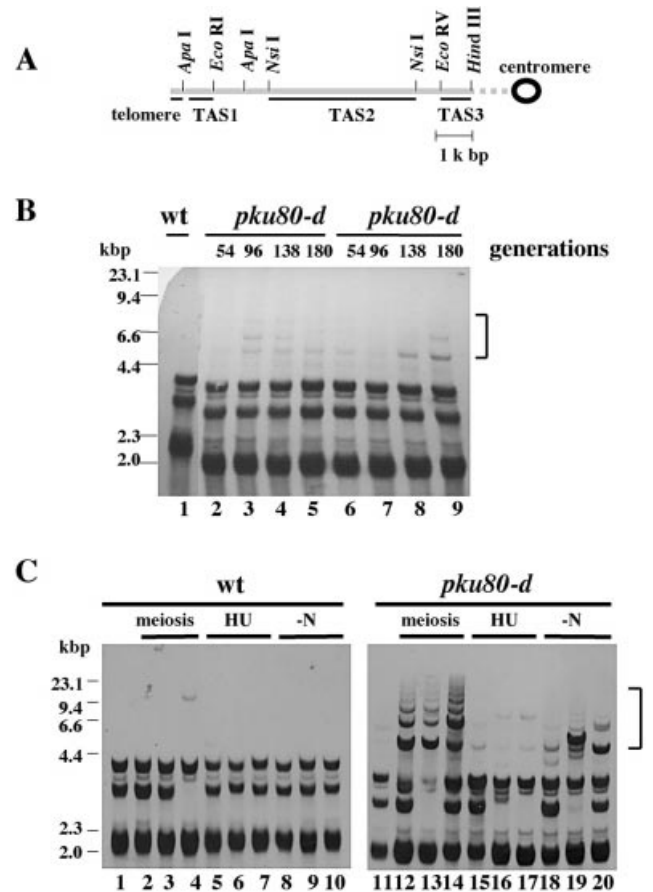


Figure 1. Meiosis and/or germination accelerate the rearrangement of TAS1 in *pku80-d* cells. (A) Restriction enzyme sites in the telomeric and telomere-associated sequences of one chromosome arm cloned in the plasmid pNSU70 (41). The chromosome arm is shown by the long gray bar. The positions of telomere-associated sequences, TAS1, TAS2 and TAS3, used as probes for Southern hybridization assays are underlined. The restriction sites are shown above the long gray bar. (B) Rearrangement of TAS1 in *pku80-d* cells without meiosis. Two independent *pku80-d* cell isolates (lanes 2–5, TK003; lanes 6–9, TK004), which were constructed by one-step gene replacement, were used. The estimated numbers of generations of growth are indicated at the top of the figure. Genomic DNA was digested with NsiI, fractionated on 0.8% agarose gels and analyzed by Southern hybridization. DNAs were hybridized with TAS1 probe. The position of the aberrant band ladder is shown by the bracket. Lane 1, wild-type *h⁻* strain (JY741). (C) Rearrangement of TAS1 in *pku80-d* cells that had undergone meiosis. Three independent *pku80-d* cell isolates (TK005, TK006 and TK007), which were constructed by one-step gene replacement (corresponding to ~42 generations) were crossed with wild-type haploid cells, JY741 (lanes 12–14), treated with 10 mM HU for 12 h (lanes 15–17) or nitrogen starved (-N) for 24 h (lanes 18–20) and grown for ~84 generations on YPAD plates. Genomic DNA was analyzed as described in (B). As a control, freshly prepared *pku80-d* cells, which were constructed by one-step gene replacement, were grown for ~84 generations (lane 11, TK004). Three independent wild-type cell isolates (lanes 2–4) were generated by crossing with wild-type *h⁻* haploid cells (JY741) and wild-type *h⁺* haploid cells (JY746) and grown for ~84 generations on YPAD plates. Three independent wild-type *h⁻* haploid cell isolates (JY741) were treated with 10 mM HU for 12 h (lanes 5–7) or nitrogen starved (-N) for 24 h (lanes 8–10) and grown for ~84 generations on YPAD plates. Lane 1, wild-type *h⁻* strain (JY741).

haploid *h⁻* strains and examined the rearrangement of TAS in two independent *pku80-d* mutants at different generations (Fig. 1B, lanes 2–5 and 6–9). Surprisingly, we detected only a small amount of the shifted bands even after growing the

mutants for ~180 generations. This contrasts sharply with the previous finding that striking rearrangements were observed after growing the disruptants for 160 generations (22). These results suggest that meiosis and/or germination may accelerate the rearrangement of TAS. To test this, we crossed wild-type haploid h^+ strains with h^- *pku80::LEU2* cells, which were constructed by one-step gene replacement using wild-type haploid h^- strains (corresponding to ~42 generations). Then *pku80-d* cells were selected on a –leucine plate and grown for ~84 generations on YPAD plates. In contrast to the findings with *pku80-d* cells that did not undergo meiosis (Fig. 1B), significant amounts of low mobility shifted band ladder were observed in three independent *pku80-d* cell isolates that had undergone meiosis (Fig. 1C, lanes 12–14). These bands were not observed when wild-type h^- strains were crossed with wild-type h^+ strains (Fig. 1C, lanes 2–4). Next, we asked whether nitrogen starvation could induce the rearrangements of TAS. h^- *pku80::LEU2* cells, which were constructed by one-step gene replacement using wild-type haploid h^- strains (corresponding to ~42 generations), were starved of nitrogen for 24 h without mating partners and grown for 84 generations and examined for rearrangement of TAS by Southern hybridization. Although a small amount of rearrangement was observed in three independent *pku80-d* cell isolates that were starved of nitrogen, the band ladder was not observed in these cells (Fig. 1C, lanes 18–20). We also asked whether the treatment of cells with hydroxyurea (HU) could induce the rearrangement of TAS. However, rearrangements were not observed in three independent *pku80-d* cell isolates that were treated with HU for 12 h without undergoing meiosis and grown for 84 generations (Fig. 1C, lanes 15–17). Treatment of wild-type h^- strains with HU or starving them of nitrogen also did not induce the rearrangement of TAS (Fig. 1C, lanes 5–10). These results suggest that, although nitrogen starvation can induce a small amount of rearrangements of TAS, striking rearrangements are induced during meiosis and/or germination in *pku80-d* cells.

Rearrangement at the subtelomeric region is Rhp51 dependent, but not Rad50 dependent

The rearrangements of TAS in *pku70-d* cells are *rad22⁺* dependent, suggesting that HR activity is required for these rearrangements (40). In addition to Rad22, Rhp51 and Rad50 play important roles in homologous recombination and they may affect the frequency of the rearrangement of TAS. To test this, we made *pku80 rhp51* double mutants and *pku80 rad50* double mutants by genetic crosses between h^- *pku80::LEU2* cells (corresponding to ~42 generations) and h^+ *rad50::ura4⁺* cells, h^+ *rhp51::ura4⁺* cells or h^+ *rad22::ura4⁺* cells, respectively. Three independent *pku80 rhp51* double mutants and *pku80 rad50* double mutants and two independent *pku80 rad22* double mutants were selected on –leucine and –uracil plates and examined for the rearrangement of TAS by Southern hybridization. As a control, *pku80-d* cells were crossed with a wild-type h^+ strain. In the h^+ strains used in this study, digestion of total DNA with NsiI generated terminal fragments of 2.2 and 6 kb (Fig. 2, lane 13). Since there is a phenotypic lag of the rearrangement of TAS, each mutant strain was grown for ~168 generations on YPAD plates before it was examined. As reported previously, low mobility bands were observed in three independent *pku80* mutants (Fig. 2,

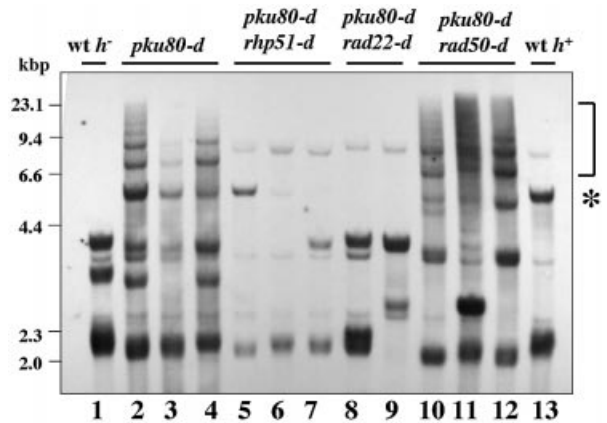


Figure 2. Rearrangement of TAS1 in *pku80-d* cells is Rhp51 dependent but not Rad50 dependent. Freshly prepared *pku80-d* cells, which were constructed by one-step gene replacement (corresponding to ~42 generations), were crossed with wild-type haploid cells (JY741) (lane 2, TK005; lane 3, TK006; lane 4, TK007), crossed with *rhp51-d* cells (KT10c) (lane 5, TK012; lane 6, TK013; lane 7, TK014), crossed with *rad22-d* cells (KT10b) (lane 8, TK015; lane 9, TK016) or crossed with *rad50-d* cells (KT102) (lane 10, TK009; lane 11, TK010; lane 12, TK011) and grown for ~168 generations on YPAD plates. Genomic DNA was analyzed as described in Figure 1B. Lane 1, wild-type h^- strain (JY741); lane 13, wild-type h^+ strain (JY746). The position of the aberrant band ladder is shown by the bracket. The ~6 kb band in lane 5 shown by an asterisk came from h^+ *rhp51-d* cells.

lanes 2–4) (22). However, these bands were not observed in the three independent *pku80 rhp51* double mutants (Fig. 2, lanes 5–7) or the two independent *pku80 rad22* double mutants (Fig. 2, lanes 8 and 9). These results indicate that Rhp51 plays an important role in the rearrangement of TAS. The requirement for both Rhp51 and Rad22 for the rearrangements strongly suggests that these events are due to HR. Next, we examined the NsiI-generated terminal fragments in *pku80 rad50* double mutants. In contrast to the lack of rearrangements in *pku80 rhp51* double mutants, rearrangements of TAS were observed in the three independent *pku80 rad50* double mutants (Fig. 2, lanes 10–12), indicating that the rearrangement of TAS is not Rad50 dependent.

Rhp51 binds to telomere ends in the absence of Ku80

The fact that Rhp51 is required for the rearrangements of TAS suggests that Rhp51 binds to telomeres or subtelomeric regions in the absence of Ku80. To test this, we performed the ChIP assay. Anti-Rhp51 was used for immunoprecipitation and precipitated DNA was amplified by PCR with primers for the telomeric region, subtelomeric region or *ade6⁺* as a control. Telomere DNA was significantly amplified in *ku80-d* cells (Fig. 3B). These results indicate that Rhp51 binds to telomere DNA when the Ku heterodimer is absent from telomere ends. Subtelomeric DNA was not amplified in *ku80-d* cells, suggesting that Rhp51 binds to distal ends of telomere DNA and does not bind to the subtelomeric region. The slight amplification of telomere DNA in wild-type strains might suggest that a small amount of Rhp51 binds to telomere ends in wild-type cells. However, more detailed quantitative analysis is necessary to elucidate whether Rhp51 binds to telomere ends in wild-type strains.

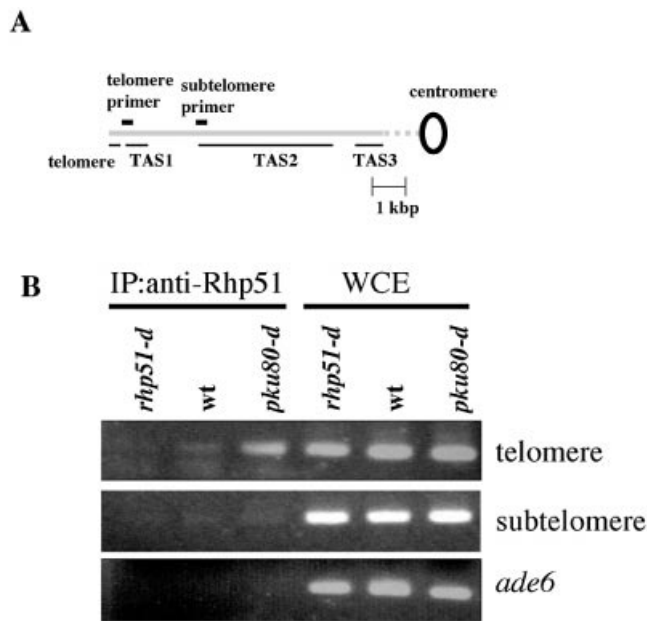


Figure 3. Rhp51 binds to telomere DNA when Ku heterodimer is absent. (A) Schematic presentation of the locations of the telomere and subtelomere primer sets on pNSU70. The chromosome arm is shown by the long gray bar. The positions of telomere-associated sequences, TAS1, TAS2 and TAS3, are underlined. The positions of the telomere and subtelomere primer sets are shown above the long gray bar. (B) ChIP assay of Rhp51. *rhp51-d* cells (KT10c), wild-type cells (JY741) and *pku80-d* cells (TK003) were used. PCR was performed using whole cell extracts (WCE) or chromatin immunoprecipitates (IP, anti-Rhp51) with primers to amplify telomere DNA (telomere), subtelomeric DNA (subtelomere) or DNA from the *ade6*⁺ gene (*ade6*).

Ku heterodimers prevent the generation of the G-rich overhang in both G₂ and S phase

Rad51 from budding yeast and humans binds to ssDNA and promotes homologous pairing and the strand exchange reaction *in vitro* (44,45). Deletion of *YKU70* results in generation of a single-stranded 3' G-tail at telomeres in *S.cerevisiae* (38,39). These facts imply that the deletion of Ku might increase the single-stranded 3' G-tails at telomere ends in *S.pombe*. We therefore investigated the single-stranded 3' G-tails at telomere ends using the in-gel hybridization assay (36) in cells synchronized in G₂ by temperature shift of a *cdc25-22* mutant followed by release into a synchronized cell cycle (46,47). As reported for *S.cerevisiae*, *S.pombe* single-stranded G-rich tails at telomere ends increased in S phase (Fig. 4A, top, and B). Disruption of *pku70*⁺ in the *cdc25-22* mutant background resulted in strong signals corresponding to single-stranded overhangs in both G₂ and S phase (Fig 4A, top, and B). These results indicate that Ku heterodimers prevent the generation of G-rich overhangs in both G₂ and S phase. Our results imply that Rhp51 binds to a single-stranded region at telomere ends.

rhp51 ku80 double mutants have significantly reduced telomere signal

As Rhp51 binds to telomere ends in *pku80-d* cells, the telomere length might be affected in the absence of both Rhp51 and Ku80. To examine this possibility, we analyzed

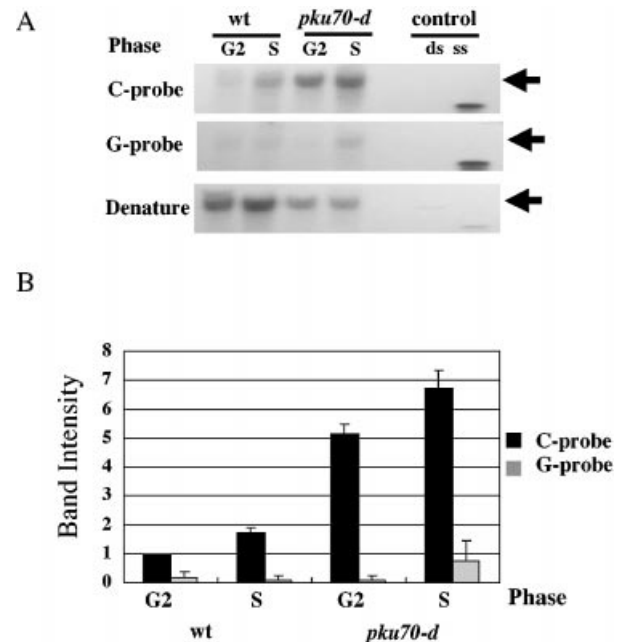


Figure 4. Deletion of *pku70*⁺ increases G-rich overhangs in both G₂ and S phase. (A) The single-stranded overhang in G₂ and S phase in wild-type cells and *pku70-d* cells was detected by in-gel hybridization. A *cdc25-22* strain (LSP11) was used as the wild-type strain. Both wild-type cells (LSP11) and *pku70-d* cells (TK001) were incubated at 36°C for 3.5 h to induce arrest at the G₂/M boundary and were then used as G₂ phase cells (G₂). Then cells were released at the permissive temperature (25°C) to obtain S phase cells. When the septation index became maximal (the septation indices of wild-type cells and *ku70-d* cells were 55 and 78%, respectively), the cells were collected and used as S phase cells (S). Genomic DNA was digested with HindIII and fractionated on 0.5% agarose gels. To detect the G-rich overhang, the semi-dried agarose gels were hybridized with a ³²P-labeled C-rich probe (C-probe, top panel). To detect the C-rich overhang (as a non-specific control), the semi-dried agarose gels were hybridized with ³²P-labeled G-rich probe (G-probe, middle panel) (see Materials and Methods). To detect the double-stranded telomere DNAs, the gel was treated with denaturant and reprobbed with the C-rich probe (Denature, bottom panel). Telomeres are indicated by arrows. (B) Quantitation of the band intensity of the in-gel hybridization assay shown in (A). The band intensity was quantitated using NIH Image 1.62 software. The band intensity obtained from wild-type G₂ cells was taken as 1. The averages of two independent experiments are shown. The intensity corresponding to the signal of the G-rich overhang detected by C-rich probe (C-probe) is shown as a black bar. The intensity corresponding to the non-specific signal detected with the G-rich probe (G-probe) is shown by gray bars. Standard deviations are shown by error bars for two independent experiments.

EcoRI- or ApaI-digested genomic DNA by Southern hybridization to examine the telomere length in the *rhp51 pku80* double mutants used in Figure 2. Although the telomere length of the EcoRI-digested DNA fragment in the *rhp51 pku80* double mutants was almost the same as that in *pku80* single mutants, the telomere hybridization signals were greatly reduced in *rhp51 pku80* double mutants (Fig. 5A, lanes 5–7). We also analyzed the ApaI-digested telomere fragments in the *rhp51 pku80* double mutants used in Figure 2. The telomere hybridization signals were detected in *rhp51* single mutants and *ku80* single mutants (Fig. 5B, lanes 2, 9 and 10). In contrast, we could not detect a telomere hybridization signal in *rhp51 pku80* double mutants (Fig. 5B, lanes 3–5). These results suggest either that undetectable levels of

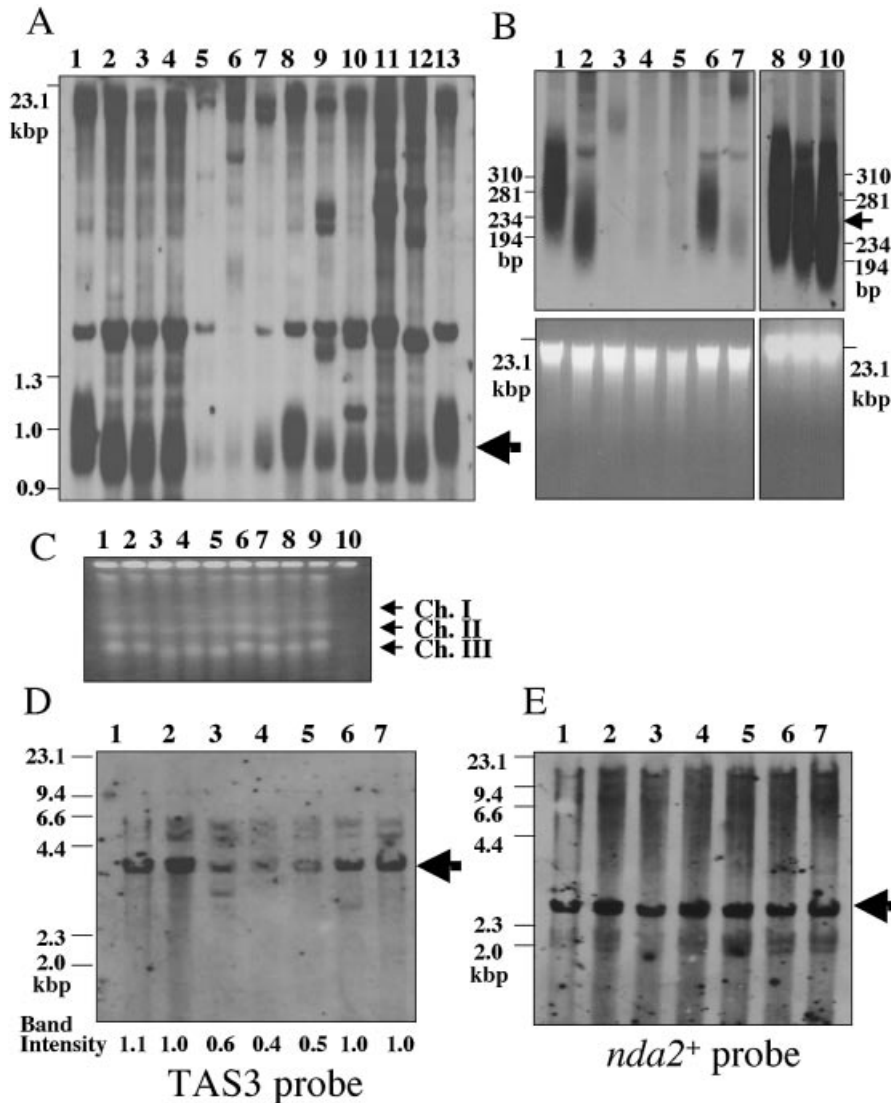


Figure 5. The telomere hybridization signal is very weak in *rhp51 pku80* double mutants. (A) The telomere length of the mutants used in Figure 2 was examined by Southern hybridization. Genomic DNAs of three independent *pku80* mutants (lane 2, TK005; lane 3, TK006; lane 4, TK007), three independent *rhp51 pku80* double mutants (lane 5, TK012; lane 6, TK013; lane 7, TK014), two independent *rad22 pku80* double mutants (lane 8, TK015; lane 9, TK016) and three independent *rad50 pku80* double mutants (lane 10, TK009; lane 11, TK010; lane 12, TK011) were prepared (grown for ~168 generations on YPAD plates). DNAs were then digested with EcoRI, separated on a 1.5% agarose gel and hybridized with a telomere probe. Lane 1, wild-type *h⁻* strain (JY741); lane 13, wild-type *h⁺* strain (JY746). Telomeres are indicated by arrows. (B) The signal intensity of telomere DNA in the mutants used in Figure 2 was examined by Southern hybridization (top panel). Genomic DNAs of *pku80-d* cells (lanes 2 and 10, TK003), three independent *rhp51 pku80* double mutants (lane 3, TK012; lane 4, TK013; lane 5, TK014), two independent *rad22 pku80* double mutants (lane 6, TK015; lane 7, TK016) and *rhp51-d* cells (lane 9, KT10c) were prepared (grown for ~168 generations on YPAD plates). DNAs were then digested with ApaI, separated on a 2% agarose gel and hybridized with a telomere probe. Lane 1, wild-type *h⁻* strain (JY741); lane 8, wild-type *h⁺* strain (JY746). Telomeres are indicated by arrows. To assess the total amount of DNA, DNA in the gel was stained with ethidium bromide before blotting onto the membrane (bottom panel). (C) Intact *S.pombe* chromosomal DNA was fractionated by PFGE. DNA was prepared from wild-type cells (lane 1, TK002), *pku80-d* cells (lane 2, TK003), three independent *rhp51 pku80* double mutants (lane 3, TK012; lane 4, TK013; lane 5, TK014), two independent *rad22 pku80* double mutants (lane 6, TK015; lane 7, TK016), *rhp51-d* cells (lane 8, KT10c), *rad22-d* cells (lane 9, KT10b) and *rad3 tell1* double mutants (lane 10, HK001) in agarose plugs. The DNA was fractionated in a 1% agarose gel. Chromosomes I, II and III (Ch. I, Ch. II and Ch. III) are shown by arrows. (D and E) NsiI-digested DNA fragments were analyzed by Southern blotting using TAS3 probe (D) or *nda2⁺* probe (E). DNA was prepared from wild-type *h⁻* cells (lane 1, JY741), *pku80-d* cells (lane 2, TK003), three independent *rhp51 pku80* double mutants (lane 3, TK012; lane 4, TK013; lane 5, TK014), *rhp51-d* cells (lane 6, KT10c) and wild-type *h⁺* cells (lane 7, JY746). The band intensities quantitated by NIH Image 1.62 software are shown at the bottom of (D). The band intensity obtained from wild-type *h⁺* cells was taken as 1. TAS3 fragment and *nda2⁺* fragment are indicated by arrows. *nda2⁺* probe was prepared by PCR using primers Ch2R-CT (5'-ATGAGAGAAGTAATTTCTGTT-CATGTTGGA-3') and Ch2R-CB (5'-CTCAATGTCAAGATTTCGGCGACAGATATC-3').

telomere DNA are maintained or that the chromosome is circularized because of loss of the telomeric DNA sequence. To distinguish between these two possibilities, we examined the chromosomes in *rhp51 pku80* double mutants by PFGE. Like the chromosomes in *trt1-d* cells, none of the three

chromosomes in *rad3 tell1* double mutants entered the agarose gel (Fig. 5C, lane 10) (12,48). In contrast, three chromosomes were detected in the three independent *rhp51 pku80* double mutants (Fig. 5C, lanes 3–5), indicating that the chromosomes are not circularized in *rhp51 pku80* double mutants.

Why do telomere hybridization signals become very weak in *rhp51 pku80* double mutants? One possible explanation is that some chromosome ends have lost their telomeric DNA altogether, through stochastic telomere rapid deletion events as described in *S.cerevisiae* (49). To see if subtelomeric or more internal sequences are lost or rearranged in *rhp51 pku80* double mutants, NsiI-digested DNA fragments were analyzed by Southern blotting. The band intensity of the DNA fragment hybridized by probe TAS3 became 40–60% in *rhp51 pku80* double mutants (Fig. 5D, lanes 3–5). These reductions were also observed when probe TAS2 was used instead of probe TAS3 (data not shown). In contrast, the band intensity was not reduced when the *nda2+* gene, which is located ~260 kb from telomere ends, was used as a probe (Fig. 5E, lanes 3–5). These results suggest that the subtelomeric region is lost together with telomere DNA in ~50% of the chromosomes in *rhp51 pku80* double mutants. The reduction in band intensity was not observed in *rhp51* and *ku80* single mutants (Fig. 5D, lanes 2 and 6). Our results demonstrate that although the mechanism of rapid deletion is not known, Rhp51 plays important roles in maintaining the telomere and subtelomere in the absence of Ku heterodimer.

We also found that the telomere hybridization signal became weak in one of the *rad22 pku80* double mutants (Fig. 5B, lane 7), but not in the other *rad22 pku80* double mutants (Fig. 5B, lane 6). It has been reported that the telomere hybridization signal could not be detected in *rad22* single mutants (18). However, we detected the telomere hybridization signal in *rad22* single mutants (data not shown). In *S.cerevisiae*, a *rad52* mutation caused an ~125-fold increase in the gross chromosome rearrangement rate (50). Thus the decreased telomere hybridization signal in *rad22 pku80* double mutants might be due to gross chromosome rearrangement. It is possible that Rad22 plays important roles at telomere ends in the absence of Ku heterodimer, because Rad22 forms a complex with Rhp51 and binds to DNA DSB ends (51,52).

DISCUSSION

In the present study we have shown that the rearrangements of TAS in *ku80-d* cells are Rhp51 dependent (Fig. 2). A previous report showed that the rearrangements of TAS in *ku70-d* cells are Rad22 dependent (40). These two results strongly suggest that the rearrangement occurs via HR. We demonstrated, using the ChIP assay, that Rhp51 bound to the distal ends of telomere DNA when Ku heterodimers were not present at telomere ends (Fig. 3). We also showed that a G-rich overhang was significantly increased when Ku heterodimer was not present at telomere ends (Fig. 4). Rhp51 is thought to accumulate at the single-stranded region of DSB ends. Considering all these results, we propose that Rhp51 binds to the single-stranded regions at telomere ends in *ku80-d* cells and the single-stranded ends act as invasive ends for HR.

Consistent with our observations, Ku heterodimers have been suggested to inhibit recombination at telomere ends in *S.cerevisiae* (38,53–56). Moreover, the rearrangement of the subtelomeric regions in *yku70-d* cells is RAD52 dependent (53). The amount of single-stranded overhang at telomere ends is increased in *yku70-d* cells (38,39). These facts suggest

that the mechanism that inhibits unnecessary recombination at telomere ends by Ku heterodimer is conserved between fission and budding yeasts.

We found that the rearrangement of TAS in *pku80-d* cells is not Rad50 dependent (Fig. 2). If the single-stranded overhang is required for the rearrangements of TAS in *pku80-d* cells, one might expect that the rearrangements of TAS would also be Rad50 dependent, because it has been suggested that the Rad50–Rad32 (homolog of Mre11) complex is required for the processing of DSB ends to initiate homologous recombination. We assume that the single-stranded overhang is generated by an unknown nuclease(s) in the absence of the Rad32–Rad50 complex in *pku80-d* cells. Indeed, DSB ends can be processed by an unknown nuclease(s) in the absence of both Mre11 complex and Ku heterodimer in *S.cerevisiae* (57).

How does the rearrangement occur in *pku80-d* cells? In *S.cerevisiae*, telomeres are thought to be maintained by break-induced replication (BIR) in the absence of telomerase (58). We propose that the rearrangement in *pku80-d* cells also occurs by BIR. We found that a 64mer sequence is located repeatedly in the subtelomeric region (Fig. 6A). Notably, TAS1 is surrounded by two of these 64mer sequences (Fig. 6A). We assume that the 64mer sequence adjacent to the telomere ends on one chromosome end invades the 64mer sequence centromere proximal to TAS1 on the other chromosome end by the action of Rhp51 (Fig. 6B). Then the 3' end of the invading strand initiates DNA replication. This would result in amplification of ~1 kb of TAS1 sequence (Fig. 6B). The telomere-repeat sequence (GGTTACA)_n may be cleaved before DNA replication, because there is no telomere repeat sequence in the subtelomeric region. In this scenario, the amplified sequence should not contain the telomere-repeat sequence. This is consistent with previously reported data showing that the amplified band does not contain the telomere repeat sequence (22). In *S.cerevisiae*, a small population of the cells have single-stranded subtelomeric regions in *yku70* mutants (59). Therefore it is possible that a small fraction of *pku80-d* cells possess single-stranded subtelomeric DNA that allows strand invasion. In *S.cerevisiae*, BIR can still proceed in *rad51-d* strains (60). However, when donor sequences are heterochromatic, Rad51 is required for BIR (61). In *S.pombe*, telomere heterochromatin is thought to be maintained in the absence of Ku heterodimer, because telomere silencing is intact in both *pku70-d* and *pku80-d* cells (21,22). These facts explain the importance of the fission yeast Rhp51 for the rearrangement of TAS1 in *pku80-d* cells.

We found that meiosis and/or germination accelerate the rearrangement of TAS in *pku80-d* cells (Fig. 1C). In *S.pombe*, telomeres cluster near the spindle pole body at the onset of meiosis and dramatic nuclear movements, called horsetail movements, occur during meiotic prophase (62–64). The distance between two different chromosome ends would become very close at those stages. This might increase the frequency of the rearrangements between two different chromosome ends in the heterozygous diploid (*pku80^{+/-}*) cells, where the amount of Ku heterodimer is lower than that in the wild-type homozygote. Alternatively, if DSBs are induced near the telomere ends during meiotic recombination, the frequency of the rearrangement would be increased. In this case, the Ku heterodimer might be required for the protection of DSB ends from uncontrolled degradation that might

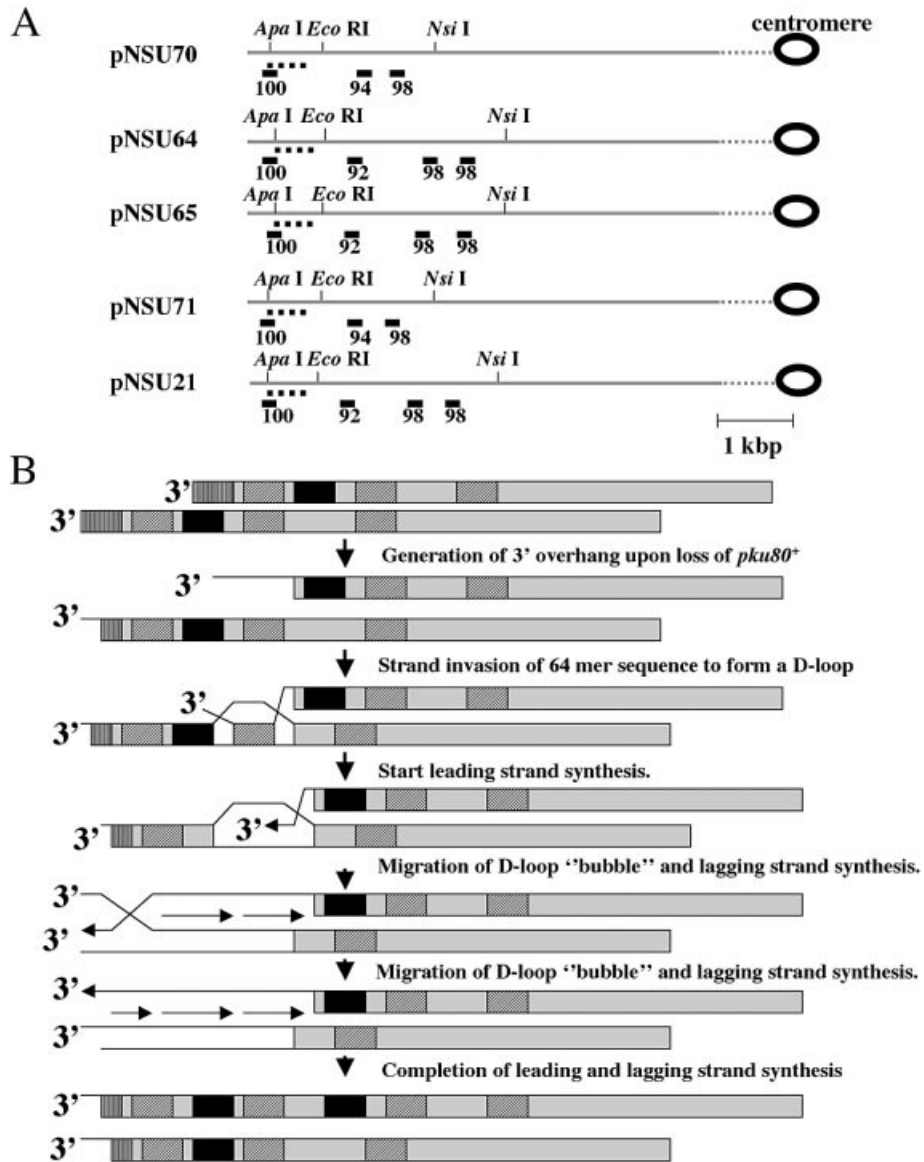


Figure 6. Model. (A) The position of the 64mer sequences in the subtelomeric regions cloned in plasmids pNSU70, pNSU64, pNSU65, pNSU71 and pNSU21 are shown (41). The chromosome arms are shown by long gray bars. The restriction sites are shown above the long gray bars. The 64mer repeat sequence (5'-CTGCCTTACGGCTCGGCTGACGGGTGGGGCCCAATAGTGGGGGCATTGTATTGTGAAAAAAA-3') is underlined. The identity (%) of the 64mer sequence with the other 64mer sequences is shown below the underline. The position of TAS1 is shown by a dotted line. (B) Hypothetical model for the amplification of TAS1 in *pku80-d* cells. The 64mer repeat sequences (diagonal hatched boxes), TAS1 region (closed boxes) and telomere-repeat sequence (vertical hatched boxes) in two different chromosome arms (large gray boxes) are represented at the top. A 3' overhang is generated due to loss of *pku80*⁺. Rhp51 binds to the exposed single-stranded 3' overhang. The ssDNA (64mer sequence) on one chromosome arm invades the homologous region (64mer sequence) on the other chromosome arm and a D-loop is formed. The telomere-repeat sequence (G-rich repeat sequence) is cleaved because there is no such sequence in the subtelomeric region. Then the 3' end of the invading strand initiates DNA replication, leading to a migrating D-loop 'bubble' as described by Formosa and Alberts (82). As a result, TAS1 is amplified. Alternatively, if the D-loop does not migrate to the telomere end, DNA replication will generate a Holliday junction, which will be resolved by an unknown mechanism. In this case, TAS1 is also amplified.

increase the recombination frequency. The other possible explanation for the accelerated rearrangement is that the Ku heterodimer is required for the protection of chromosome ends during germination. It has been reported that in cells with compromised telomere function (*taz1-d*), SpEst1 loss confers a lethal germination phenotype while telomerase loss does not (65). This result demonstrates the importance of protection of the chromosome ends during germination. We also found that nitrogen starvation slightly increases the frequency of the rearrangements of TAS in *ku80-d* cells. When *taz1-d* cells are

subjected to nitrogen starvation, the cells exhibit lethal telomere fusions, which are formed by Ku-dependent NHEJ (66). This fact further suggests the importance of telomere protection during the G₁ state. Nitrogen starvation, meiosis and germination all induce a G₁ state. Therefore a prolonged G₁ state may be the cause of the rearrangement of TAS in *ku80-d* cells. Further investigation of the function of Ku heterodimer at telomere ends will be necessary to fully elucidate the functions of telomeres during meiosis and germination.

Our data reveal that the Ku heterodimer inhibits homologous recombination at telomere ends by preventing Rhp51 from binding to telomere ends. Competition between HR and NHEJ is also observed at DSB ends (67–73). Although the mechanisms governing this competition are not understood, recent studies suggest that DNA damage checkpoint proteins play important roles in controlling the activity of Ku heterodimers in both yeast and mammals (74,75). In *S.cerevisiae* and *S.pombe*, double mutants of Ku components and telomerase components display accelerated senescence (39,40,76). These results suggest that the activity of the Ku heterodimer is important for telomerase-independent telomere maintenance. Similarly, mammalian Ku heterodimers might play important roles in telomere maintenance in telomerase-negative cancer cells. In a subset of human tumor cells, telomeres are maintained without telomerase activity. These cells are called ‘ALT’ cells (77,78) and telomeres of these cells are thought to be maintained by BIR (58,79). Indeed, several proteins involved in HR localize to telomeres in ALT cells (80,81). Given that telomeres are maintained by HR in ALT cells, it is possible that Ku inhibits or regulates this pathway. Therefore investigation of the roles of Ku heterodimers in recombination-dependent telomere maintenance may provide information useful for understanding the development of cancer by the ALT pathway.

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