Fission Yeast Taz1 and RPA Are Synergistically Required to Prevent Rapid Telomere Loss[□] [▽]

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The telomere complex must allow nucleases and helicases to process chromosome ends to make them substrates for telomerase, while preventing these same activities from disrupting chromosome end-protection. Replication protein A (RPA) binds to single-stranded DNA and is required for DNA replication, recombination, repair, and telomere maintenance. In fission yeast, the telomere binding protein Taz1 protects telomeres and negatively regulates telomerase. Here, we show that *taz1-d rad11-D223Y* double mutants lose their telomeric DNA, indicating that RPA (Rad11) and Taz1 are synergistically required to prevent telomere loss. Telomere loss in the *taz1-d rad11-D223Y* double mutants was suppressed by additional mutation of the helicase domain in a RecQ helicase (Rqh1), or by overexpression of Pot1, a single-strand telomere binding protein that is essential for protection of chromosome ends. From our results, we propose that in the absence of Taz1 and functional RPA, Pot1 cannot function properly and the helicase activity of Rqh1 promotes telomere loss. Our results suggest that controlling the activity of Rqh1 at telomeres is critical for the prevention of genomic instability.

INTRODUCTION

The maintenance of telomeres is critical for preventing genomic instability, cancer, and senescence. The human telomeric DNA binding protein TRF2 is required for inhibition of inappropriate DNA repair activities such as homologous recombination (HR) and nonhomologous end joining (NHEJ) at telomeres (Zhu *et al.*, 2003; Wang *et al.*, 2004). TRF2 is linked by TIN2 to TRF1, another human telomeric DNA binding protein, which acts as a negative regulator of telomerase (van Steensel and de Lange, 1997; de Lange, 2005). Taz1 is the fission yeast orthologue of TRF2 and TRF1 (Ferreira *et al.*, 2004). In *Schizosaccharomyces pombe*, Taz1 protects telomeres from both HR and NHEJ and is thought to be a negative regulator of telomerase (Cooper *et al.*, 1997; Ferreira and Cooper, 2001). Indeed, deletion of *taz1*⁺ causes massive telomere elongation (Cooper *et al.*, 1997).

The *S. pombe* Trt1, the catalytic subunit of telomerase (Nakamura *et al.*, 1997), interacts with a telomerase subunit, Est1 (Beernink *et al.*, 2003). In *Saccharomyces cerevisiae*, Est1 seems to recruit or activate telomerase in late S phase (Taggart *et al.*, 2002; Schramke *et al.*, 2004). Both *taz1-d trt1-d* and *taz1-d est1-d* double mutants lose telomeric DNA more quickly than *trt1-d* and *est1-d* single mutants, respectively, suggesting that Taz1 also protects telomeric DNA from degradation in the absence of telomerase activity (Nakamura *et*

al., 1998; Beernink *et al.*, 2003; Miller *et al.*, 2006). Moreover, deletion of *taz1*⁺ causes a significant increase in the length of G-rich overhangs whose generation depends on the Rad50–Rad32–Nbs1 complex and Dna2 (Tomita *et al.*, 2003, 2004).

Pot1 was identified as a distant homologue of the telomere-binding protein α subunit of *Oxytricha nova* (Gray *et al.*, 1991; Baumann and Cech, 2001). The N-terminal domain of Pot1 contains oligonucleotide/oligosaccharide-binding (OB) fold, which binds specifically to the single-stranded G-rich telomeric sequence of fission yeast in vitro (Baumann and Cech, 2001). Deletion of the *pot1*⁺ gene results in rapid loss of telomeric DNA as well as chromosome missegregation and chromosome circularization (Baumann and Cech, 2001).

Replication protein A (RPA, also known as single-stranded DNA-binding protein or replication factor A) is a heterotrimeric single-stranded DNA-binding protein consisting of the subunits RPA1 (70 kDa), RPA2 (36 kDa), and RPA3 (14 kDa) (Wold, 1997). The four single-stranded DNA binding domains of RPA have the characteristic OB fold. RPA is well conserved in eukaryotes and is required for DNA replication, recombination, and repair as well as for signaling pathways elicited by DNA damage (Zou and Elledge, 2003; Zou *et al.*, 2006). In *S. cerevisiae*, RPA has been shown to bind to telomeres in late S phase and to act in the telomerase pathway (Schramke *et al.*, 2004). Moreover, a mutant allele of the large subunit of RPA, *rfa1-D228Y*, confers a defect in telomere maintenance in the absence of the DNA end protection protein, yKu70 (Smith *et al.*, 2000). The aspartic acid residue at position 228 of Rfa1 is highly conserved from yeast to humans, and it lies within the loop region opposite the DNA-contacting surface of the DNA binding domain in the crystal structure (Bochkarev *et al.*, 1997). To study the importance of this conserved aspartic acid in telomere maintenance in *S. pombe*, we previously created a *rad11-D223Y* mutant, in which the analogous aspartic acid at position 223

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Table 1. 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
YO001	<i>h⁺ leu1-32 ura4-D18 ade6-M210 rad11-D223Y</i>	Ono <i>et al.</i> (2003)
KT101	<i>h⁺ leu1-32 ura4-D18 ade6-M210 taz1::ura4⁺</i>	This study
YO008	<i>h⁺ leu1-32 ura4-D18 ade6-M210 taz1::ura4⁺ rad11-D223Y</i>	This study
TK034	<i>h⁻ leu1-32 ura4-D18 ade6-M216 taz1::ura4⁺ rad11-D223Y</i>	This study
KT146	<i>h⁻ leu1-32 ura4-D18 ade6-704 rad3::ura4⁺ rad32::ura4⁺</i>	Tomita <i>et al.</i> (2004)
KT007	<i>h⁻ leu1-32 ura4-D18 ade6-M216 trt1::ura4⁺</i>	Tomita <i>et al.</i> (2003)
TK022	<i>h⁹⁰ leu1-32 ura4-D18 ade6-M210 rad11-D223Y</i>	This study
TK023	<i>h⁹⁰ leu1-32 ura4-D18 ade6-M210 rad11-D223Y taz1::ura4⁺</i>	This study
LSP11	<i>h⁺ leu1-32 ura4-D18 cdc25-22</i>	lab stock
YO009	<i>h⁺ leu1-32 ura4-D18 cdc25-22 rad11::Myc:kanMX6</i>	This study
TK024	<i>h⁺ leu1-32 ura4-D18 cdc25-22 rad11-D223Y::Myc:kanMX6</i>	This study
YO004	<i>h⁺ leu1-32 ura4-D18 ade6-M210 rad11::Myc:kanMX6</i>	Ono <i>et al.</i> (2003)
TK019	<i>h⁺ leu1-32 ura4-D18 ade6-M210 rad11::Myc:kanMX6 taz1::ura4⁺</i>	This study
TK020	<i>h⁺ leu1-32 ura4-D18 ade6-M216 rad11::Myc:kanMX6 rad50::LEU2 taz1::ura4⁺</i>	This study
KT021	<i>h⁻ leu1-32 ura4-D18 ade6-M216 taz1::ura4⁺ rad50::LEU2</i>	Tomita <i>et al.</i> (2003)
JK805	<i>h⁹⁰ ade-M210 leu1-32 ura4-D18 rifl12Myc:ura4⁺ rap1::kanMX6</i>	Kanoh and Ishikawa (2001)
KS002	<i>h⁺ leu1-32 ura4-D18 ade6-M210 rap1::kanMX6 rad11-D223Y</i>	This study
YO005	<i>h⁺ leu1-32 ura4-D18 ade6-M210 rad11-D223Y::Myc:kanMX6</i>	Ono <i>et al.</i> (2003)
TK049	<i>h⁻ leu1-32 ura4-D18 ade6-M210 rqh1::LEU2 rad11::Myc:kanMX6</i>	This study
TK050	<i>h⁹⁰ leu1-32 ura4-D18 ade6-M210 rqh1::LEU2 rad11-D223Y::Myc:kanMX6</i>	This study
KT0d1	<i>h⁻ leu1-32 ura4-D18 ade6-M210 rqh1::LEU2 taz1::ura4⁺</i>	This study
TK025	<i>h⁺ leu1-32 ura4-D18 ade6-M210 rqh1::LEU2 taz1::ura4⁺ rad11-D223Y</i>	This study
J876	<i>h⁻ leu1-32 ura4-D18 ade6-704 rqh1-K547A 3HA-top3</i>	Laursen <i>et al.</i> (2003)
J878	<i>h⁻ leu1-32 ura4-D18 ade6-704 rqh1-K547R 3HA-top3</i>	Laursen <i>et al.</i> (2003)
TK069	<i>h⁺ leu1-32 ura4-D18 ade6-704 rad11-D223Y rqh1-K547A</i>	This study
TK074	<i>h⁻ leu1-32 ura4-D18 ade6-M210 rad11-D223Y rqh1-K547R</i>	This study
J564	<i>h⁻ leu1-32 ura4-D18 ade6-704 rqh1::Myc:kanMX6</i>	Laursen <i>et al.</i> (2003)
TK027	<i>h⁻ leu1-32 ura4-D18 ade6-704 rad11-D223Y rqh1::Myc:kanMX6</i>	This study
MGF809	<i>h⁻ leu1-32 ura4-D18 ade6-M216 rad11::GFP:kanMX6</i>	M. G. Ferreira and J. P. Cooper (Cancer Research UK)
TK038	<i>h⁺ leu1-32 ura4-D18 ade6-M210 rad11-D223Y::GFP:kanMX6</i>	This study
TK042	<i>h⁻ leu1-32 ura4-D18 ade6-M210 rad11::GFP:kanMX6 rqh1::Myc:kanMX6</i>	This study
TK043	<i>h⁻ leu1-32 ura4-D18 ade6-M210 rad11-D223Y::GFP:kanMX6 rqh1::Myc:kanMX6</i>	This study
TK037	<i>h⁺ leu1-32 ura4-D18 ade6-M210 rqh1::Myc:kanMX6</i>	This study
TK072	<i>h⁺ leu1-32 ura4-D18 ade6-M210 taz1::LEU2 rad11-D223Y pPC27-pot1-HA:ura4⁺</i>	This study
TK064	<i>h⁺ leu1-32 ura4-D18 ade6-M210 rad11-D223Y pPC27-pot1-HA:ura4⁺</i>	This study

of the large subunit of *S. pombe* RPA (Rad11) is mutated to tyrosine (Ono *et al.*, 2003). Cells harboring the *rad11-D223Y* allele are hypersensitive to methylmethane sulfonate, hydroxyurea, UV, and gamma-radiation, suggesting that *rad11-D223Y* cells have a defect in DNA repair activity. We also found that the *rad11-D223Y* cells have short telomeres and showed that both the wild-type Rad11 protein and the Rad11-D223Y mutant protein bind to telomeric DNA (Ono *et al.*, 2003). These observations strongly suggest that RPA plays important roles at telomeres in both *S. cerevisiae* and *S. pombe*. However, the exact role of RPA in telomere maintenance remains unclear.

In this study, we show that *S. pombe* RPA plays important roles in the maintenance of telomeric DNA. Indeed, *taz1-d rad11-D223Y* double mutant cells lose their telomeric DNA rapidly and completely, indicating that both RPA and Taz1 are critical for the maintenance of telomeric DNA. Our results describe the mechanism by which *taz1-d rad11* double mutant cells lose their telomeric DNA.

MATERIALS AND METHODS

Strain Constructions and Growth Media

Strains used in this report are listed in Table 1. *cdc25-22 rad11-D223Y* was created by mating *cdc25-22* (LSP11) and *h⁻ rad11-D223Y*. To tag Rad11 protein

or Rad11-D223Y with Myc epitope-tag at the C terminus, the plasmid pFA6a-13Myc-kanMX6-rad11 was linearized with NspV and used for transformation of *cdc25-22* cells, *cdc25-22 rad11-D223Y* cells, *rad11-D223Y* cells, *taz1-d* cells, *rqh1-d* cells, and *rqh1-d rad11-D223Y* cells (Ono *et al.*, 2003). The Myc-tagged Rad11 was fully functional. The *taz1-d rad50-d* double mutant expressing Rad11-Myc protein was created by mating YO004 and KT021. The *taz1-d rad11-D223Y* double mutant used in Figures 4B and 6A was created by transformation of *rad11-D223Y* cells with the *taz1::ura4⁺* disruption fragment (Tomita *et al.*, 2003). The *taz1-d rad11-D223Y* double mutant used in Figure 1C was created by mating YO001 and KT110. Heterozygous diploid strain *taz1⁺/rad11-D223Y taz1⁻/rad11⁺* used in Figure 2 was created by transformation of the diploid created by mating YO001 and JY741 with a *taz1::ura4⁺* disruption fragment (Tomita *et al.*, 2003). Heterozygous diploid strain *trt1⁺/trt1⁻* was created by transformation of the diploid created by mating JY741 and JY746 with the *trt1::ura4⁺* disruption fragment (Tomita *et al.*, 2003). The *rap1-d rad11-D223Y* double mutants were created by transformation of *rad11-D223Y* cells with the *rap1::kanMXn* disruption fragment. *rad11-D223Y rqh1-d* double mutant and *taz1-d rad11-D223Y rqh1-d* triple mutant were created by transformation of the diploid created by mating *rad11-D223Y* (YO001) and *rqh1-d* (KT0d0) with the *taz1::ura4⁺* disruption fragment. *taz1-d rad11-D223Y rqh1-K547A* triple mutant and *taz1-d rad11-D223Y rqh1-K547R* triple mutant were created by transformation of *rad11-D223Y rqh1-K547A* double mutant cells and *rad11-D223Y rqh1-K547R* double mutant cells with the *taz1::ura4⁺* disruption fragment. *rad11-D223Y rqh1-Myc* was created by mating YO001 and J564. *rad11-D223Y-GFP* was created by transformation of *rad11-D223Y* (YO001) with the PCR product of *rad11-GFP-kanMX6*. *h⁺ rqh1-Myc* (TK037) was created by mating JY746 and J564. *rad11-GFP rqh1-Myc* was created by mating MGF809 and TK037. *rad11-D223Y-GFP rqh1-Myc* was created by mating TK038 and TK037. The *taz1-d rad11-D223Y* expressing Pot1 was created by transformation of *rad11-D223Y* cells expressing Pot1 with the *taz1::LEU2* disruption fragment. Cells were grown in YPAD medium (1% yeast extract,

recovered gradually (data not shown). Telomere length of the double mutant was analyzed by Southern hybridization using a telomeric oligonucleotide as a probe (Figure 1B). As shown previously, *rad11-D223Y* single mutant cells had short telomeres, whereas *taz1-d* cells had long telomeres (Figure 1C, lane 2 and 3) (Cooper *et al.*, 1997; Ono *et al.*, 2003). In contrast, we could not detect telomere hybridization signals in the *taz1-d rad11-D223Y* double mutant (Figure 1C, lane 4), suggesting that telomere sequences were lost at chromosome ends. The severe telomere loss seen in *taz1-d rad11-D223Y* cells is particularly curious given that *taz1+* deletion leads to extensive telomere elongation in a wild-type background. Because *S. pombe* cells can survive without telomeres by chromosome circularization (Naito *et al.*, 1998; Nakamura *et al.*, 1998), we analyzed chromosome topology by performing PFGE (Figure 1D). The double mutant between *rad3* (a member of the ATM/ATR family of kinases) and *rad32* (homologues of human and *S. cerevisiae* MRE11) was used as a positive control for chromosome circularization (Nakamura *et al.*, 2002). Although the three chromosomes from wild-type fission yeast enter pulsed field gels, chromosomes from *rad3-d rad32-d* or *taz1-d rad11-D223Y* cells failed to enter the gels (Figure 1E), suggesting that, like *rad3-d rad32-d* chromosomes, the chromosomes of the *taz1-d rad11-D223Y* double mutant are circularized. Chromosome circularization can also be evidenced through an altered *NotI* digest pattern (Naito *et al.*, 1998; Nakamura *et al.*, 1998), because the terminal *NotI* restriction fragments display altered migration patterns when isolated from strains containing circular versus linear chromosomes (Figure 1D). The double mutant did not have *NotI* fragments C, I, L, and M; instead, new bands corresponding to joint fragments between I and L, and C and M, were detected (Figure 1F, lane 2), indicating that the chromosomes of the double mutant are circularized.

We also created the double mutants from short telomere by transforming *rad11-D223Y* cells with a *taz1::ura4+* disruption fragment and from long telomere by removing the plasmid bearing the wild-type *rad11+* gene from the *taz1-d rad11-D223Y* double mutant expressing a wild-type *rad11+* gene, which have long telomere. The resulting double mutants lost their telomeres (data not shown). Thus, the *taz1-d rad11-D223Y* double mutant loses telomere regardless of the length/capping state of the parental telomeres.

Phenotype of the *taz1-d rad11-D223Y* Double Mutant Is Similar to That of *pot1-d* Cells, *taz1-d trt1-d* Double Mutant, and *taz1-d est1-d* Double Mutant

The above-mentioned analyzed double mutants were created from cells that had short or long telomeres. To create the double mutant from cells having normal telomere length, we used a *taz1+/rad11-D223Y taz1+/rad11+* heterozygous diploid, which has normal telomere length (data not shown). We transformed this strain with a *taz1::ura4+* disruption fragment, and dissected spores from the resulting *taz1+/rad11-D223Y taz1-/rad11+* heterozygous diploid. Tetrad dissections revealed that the *taz1-d rad11-D223Y* double mutant progeny formed very small colonies compared with the single mutants (Figure 2A). The *taz1-d rad11-D223Y* double mutant grown in liquid culture for 2 d after sporulation and germination contained a large number of elongated cells (Figure 2B). 4,6-Diamidino-2-phenylindole (DAPI) staining revealed a high frequency of chromosome missegregation (Figure 2B). These chromosome segregation defects are considered to be the reason for the initial growth defect in the double mutant, because chromosome missegregation would cause cell death. Next, the double mutants used in Figure 2A

Figure 2. Phenotype of the *taz1-d rad11-D223Y* double mutant is similar to that of *pot1-d* cells, *taz1-d trt1-d* double mutant, and *taz1-d est1-d* double mutant. (A) Colony morphology of *taz1-d rad11-D223Y* double mutant and *trt1-d* cells after tetrad dissection and germination. Heterozygous diploid strains *taz1+/rad11-D223Y taz1-/rad11+* and *trt1+/trt1-* were sporulated and the resulting tetrads were dissected and germinated on YPAD plates. Colonies derived from each spore were grown at 30°C for 3 d. (B) Phase-contrast micrographs (top) and nuclear morphology (bottom) of *rad11-D223Y* single mutant and *taz1-d rad11-D223Y* double mutant stained with DAPI. Colonies used in A were picked and cultured for 2 d in YPAD and then investigated. Approximately 15% of *taz1-d rad11-D223Y* double-mutant cells displayed chromosome missegregation phenotypes. (C) Growth characteristics of *rad11-D223Y* single mutant and *taz1-d rad11-D223Y* double mutant in liquid culture. Colonies used in A were picked and diluted to 0.5×10^4 cells/ml in 20 ml of YPAD. These cultures were grown for 24 h at 30°C, at which point the cell density was determined by counting in a hemacytometer, and the cells were diluted to a cell density of 2.5×10^4 cells/ml in 20 ml of fresh YPAD and incubated at 30°C. These procedures were repeated every 24 h for 14 d. We performed over $n = 3$ independent experiments. (D) Loss of TAS1 sequence of the *taz1-d rad11-D223Y* double-mutant cells used in A was analyzed by Southern hybridization. Genomic DNA was digested with *NsiI*, fractionated by 0.8% agarose gel electrophoresis, transferred to a nylon membrane, and hybridized to the TAS1 probe. Telomeres are indicated by arrows.

were grown in liquid culture with successive dilutions to follow cell growth and telomere structure. When the gene encoding the telomerase catalytic subunit, *trt1+*, is disrupted, the growth rate is initially normal, but thereafter the cells progressively lose viability. At a later stage, their growth rate recovers gradually (Nakamura *et al.*, 1998). In contrast, the growth rate of the spores of the *taz1-d rad11-D223Y* double mutant was very low immediately after dissection (Figure 2C). The growth rate then recovered gradu-

Figure 3. Rad11 protein is enriched at telomeres more in *rad11-D223Y* cells and in *taz1-d* cells than in wild-type cells (A) ChIP assay of Rad11-Myc and Rad11-D223Y-Myc protein. Control *cdc25-22* cells (LSP11), *cdc25-22 rad11-Myc* (YO009) cells and *cdc25-22 rad11-D223Y-Myc* (TK024) cells were incubated at 36°C for 3.5 h to induce arrest at the G2/M boundary. Then, cells were released at the permissive temperature (25°C), and samples were taken every 20 min and subjected to quantitative real-time PCR. Relative enrichment of Rad11-Myc- and Rad11-D223Y-Myc-bound telomeric DNA over background is plotted against time. We performed over $n = 3$ independent experiments. The protein expression level of Rad11-Myc and Rad11-D223Y-Myc was also shown. The positions of Rad11-Myc and Rad11-D223Y-Myc were indicated by arrowheads. For loading control, anti- β -tubulin antibody (Sigma-Aldrich, St. Louis, MO) was used. The relative amounts of Rad11 and Rad11-D223Y signals to β -tubulin signals were used to express the relative amount. The amount of Rad11 protein at time zero point was normalized to 1. Because β -tubulin could not be detected at time 60 min and only slightly detected at time 120 and 140 min (left), the relative protein amounts at these points were not shown. The relative protein amounts at time 60, 120, and 140 min were calculated to be 0.8, 0.8, and 1.4, respectively, from the ratios of Rad11 signals to the signals detected by silver staining (data not shown). (B) ChIP assay of Rad11-Myc protein in control cells (no tag), *taz1-d*, *taz1-d rad50-d* double mutant. Immunoprecipitations were performed with anti-Myc antibody and dot blots were hybridized with a 300-base pair telomere sequence and *ade6*⁺ (see Materials and Methods). (C) Quantification of the data in B, $n = 2$ independent ChIP experiments. (D) The single-strand overhangs in G2 and S phase in *cdc25-22 rad11-Myc* cells and *cdc25-22 rad11-D223Y-Myc* cells were detected by in-gel hybridization. Both *cdc25-22 rad11-Myc* (YO009) cells and *cdc25-22 rad11-D223Y-Myc* (TK024) cells were incubated at 36°C for 3.5 h to induce arrest at the G2/M boundary and were used them 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 *rad11-Myc* cells and

rad11-D223Y-Myc cells were 41 and 30%, respectively), the cells were collected and used as S phase cells (S). Genomic DNA was digested with HindIII and separated by electrophoresis. Then, the gel was dried and hybridized with the ³²P-labeled C-rich (C-probe; top) or G-rich (G-probe; bottom) probe. (E) *rap1-d rad11-D223Y* double mutant do not lose telomeric DNA. The telomere length of the wild-type cells (lane 1, JY746), *rad11-D223Y* cells (lane 2, YO001), *rap1-d* cells (lane 3, JK805), and *rap1-d rad11-D223Y* double-mutant cells (lane 4, KS002) was analyzed by Southern hybridization as shown in Figure 1C.

ally. Southern hybridization analysis of the double mutant at different generations indicated that the double mutant lost the telomeric DNA and the subtelomeric sequence called TAS1 (Nakamura *et al.*, 1998) in the very early generations (Figure 2D). These phenotypes are similar to that of *pot1-d* cells, *taz1-d trf1-d* double mutants, and *taz1-d est1-d* double mutants, which lose telomeres very rapidly (Nakamura *et al.*, 1998; Baumann and Cech, 2001; Beernink *et al.*, 2003).

Rad11-D223Y Mutant Protein Is Enriched at Telomeres More than Rad11 Protein throughout the Cell Cycle

To understand the mechanism of telomere loss in *taz1-d rad11-D223Y* double mutant, we next asked whether the telomere binding of *S. pombe* Rad11 protein is affected by *rad11-D223Y* mutation. We synchronized *S. pombe* cells expressing either Rad11-Myc or Rad11-D223Y-Myc protein by using *cdc25-22* background to block at the G2/M boundary, and Rad11 binding to telomeres was examined by ChIP. The

protein expression level of Rad11-Myc and Rad11-D223Y-Myc were constant throughout the cell cycle, and the *rad11-D223Y* mutation did not affect protein expression level (Figure 3A). Moreover, immunoprecipitation efficiency was not affected by the Rad11-D223Y mutation, indicating that the mutation does not affect the accessibility of the Myc tag to antibody (data not shown). The *S. cerevisiae* RPA is transiently associated with telomeres in late S phase (Schramke *et al.*, 2004; Takata *et al.*, 2004). In contrast, *S. pombe* Rad11 was bound to telomeres most of the cell cycle, and it was not particularly enriched during S phase (Figure 3A). Importantly, Rad11-D223Y-Myc mutant protein was enriched at telomeres more than Rad11-Myc protein throughout the cell cycle. These data are consistent with our previous data from asynchronous cells (Ono *et al.*, 2003). The increase binding could be explained if Rad11-D223Y protein has a higher affinity for telomeric single-strand DNA. If it is the case, *rad11-D223Y* should be a dominant mutation. However, the

telomere length of the diploid cells created from *rad11-D223Y* mutant cells and wild-type cells is normal. Moreover, the *taz1-d rad11-D223Y* double mutant expressing a wild-type *rad11*⁺ gene has elongated telomere as in *taz1-d* cells. These results indicate that *rad11-D223Y* is not a dominant mutation with respect to both the telomere length in *rad11-D223Y* mutant and the complete telomere loss in a *taz1-d* background (data not shown). We therefore hypothesize that the relative enrichment of Rad11-D223Y protein is due to the defect of Rad11-D223Y protein in telomere capping, which could affect telomere structure and/or protein composition at telomere, recruiting more Rad11 to telomere possibly by protein-protein interaction. This defect should be rescued in the presence of wild-type Rad11.

RPA Is Enriched at Telomeres More in *taz1-d* Cells than in Wild-Type or *taz1-d rad50-d* Cells

taz1-d cells have long single-stranded overhangs, whereas *taz1-d rad50-d* double mutant cells have short overhangs. The telomere lengths of these two mutants are almost identical (Tomita *et al.*, 2003). If RPA binds to the single-stranded DNA generated at telomeres in *taz1-d* cells, RPA should be enriched at *taz1-d* telomeres relative to wild-type and *taz1-d rad50-d* telomeres. To test this possibility, we performed a ChIP assay and the percentage of precipitated DNA was calculated as a ratio of input signals and plotted (Figure 3, B and C). Because the histograms in Figure 3C represent the percentage of input telomeric DNA, they are corrected for telomere length changes (Loayza and De Lange, 2003). Indeed, Rad11-Myc was enriched at telomeres more in *taz1-d* cells than in wild-type cells or in *taz1-d rad50-d* double mutant cells. These results suggest that RPA binds to the single-stranded DNA generated at telomeres in *taz1-d* cells.

These results imply that the enrichment of Rad11-D223Y at telomeres might stem from increased levels of single-strand overhang. To test this possibility, we examined the G-rich overhangs in the *rad11-D223Y* mutant in both G2 and S phase. A *cdc25-22* strain background was used to synchronize *rad11-D223Y* mutant. As shown previously, the G-rich overhangs increased in S phase in wild-type cells (Kibe *et al.*, 2003) (Figure 3D). The signal intensity corresponding to G-rich overhangs in *rad11-D223Y* mutant was same as that in wild-type cells in both G2 and S phase (Figure 3D). The signals disappear by addition of *Escherichia coli* Exonuclease I (data not shown), indicating that the signals detected in this assay are present at the terminus of the telomere. These results indicate that the enrichment of Rad11-D223Y mutant protein at telomere is not due to increased single-strand overhangs in *rad11-D223Y* mutant.

rap1-d rad11-D223Y Double Mutant Cells Do Not Lose Telomeric DNA

Deletion of fission yeast *rap1*⁺ leads to telomere elongation, increase in the length of G-rich overhangs, derepression of telomeric silencing and meiotic defects, all reminiscent of the phenotypes seen in *taz1-d* cells (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001; Miller *et al.*, 2005). However, unlike *taz1-d* cells, *rap1-d* cells are not cold sensitive (Miller *et al.*, 2005). Recently, it was reported that *taz1-d* cells, but not *rap1-d* cells, suffer replication fork pausing at the telomere/subtelomere boundary (Miller *et al.*, 2006). Therefore, we considered that telomere loss in the *taz1-d rad11-D223Y* double mutant may be related to the defect in replication fork progression. We created a *rap1-d rad11-D223Y* double mutant. Unlike *taz1-d rad11-D223Y* double mutant, *rap1-d rad11-D223Y* double mutant cells were not elongated and the cell viability of *rap1-d rad11-D223Y* double mutant

was similar to that of wild-type cells (data not shown). Importantly, *rap1-d rad11-D223Y* double mutant showed retention of telomeric DNA (Figure 3E). This result suggests that the abrupt telomere loss in the *taz1-d rad11-D223Y* double mutant may be related to the replication fork pausing at the telomere/subtelomere boundary. Moreover, our results suggest that single-strand overhangs are not the cause of telomere loss in *taz1-d rad11-D223Y* double mutants, because both *rap1-d* cells and *taz1-d* cells have long single-strand overhangs. Interestingly, the telomere in *rap1-d rad11-D223Y* double mutant was shorter than that of *rap1-d* single mutant, indicating that the *rad11-D223Y* mutation suppresses the long-telomere phenotype of *rap1-d* cells. These data suggest that Rad11-D223Y mutation inhibits telomerase activity either directly or indirectly.

Deletion of *rqh1*⁺ Suppresses Loss of Telomeric DNA in *taz1-d rad11-D223Y* Double Mutant

To understand the basis for the relative enrichment of Rad11-D223Y versus wild-type Rad11 at telomeres, we sought a mutant that would suppress the enrichment of RPA at telomeres in the *rad11-D223Y* background. The binding of *S. cerevisiae* RPA on stalled replication forks has been shown to be impaired in a *sgs1 mec1-100* cells that harbor mutations in both the *S. cerevisiae* RecQ helicase Sgs1 and the ATR homologue Mec1 (Cobb *et al.*, 2005). Thus, we asked whether deletion of *rqh1*⁺, the fission yeast RecQ helicase, affects telomere binding of Rad11 and Rad11-D223Y. Although deletion of *rqh1*⁺ did not affect telomere binding of the Rad11 protein, it suppressed the relative enrichment of Rad11-D223Y protein at telomeres (Figure 4A). Next, we constructed *taz1-d rad11-D223Y rqh1-d* triple mutants and examined their telomere phenotypes. Strikingly, *taz1-d rad11-D223Y rqh1-d* triple mu-

Figure 4. Deletion of *rqh1*⁺ suppresses loss of telomeric DNA in *taz1-d rad11-D223Y* double mutant (A) ChIP assay of Rad11-Myc protein and Rad11-D223Y-Myc in *rqh1-d* cells. Untagged wild-type control cells (no tag) (JY746), *rad11-Myc* (YO004), *rqh1-d rad11-Myc* (TK049), *rad11-D223Y-Myc* (YO005), and *rqh1-d rad11-D223Y-Myc* (TK050) cells were used. The amount of DNA was calculated by quantitative real-time PCR. For quantification of the data, we performed over *n* = 3 independent ChIP experiments. (B) The telomere length of the wild-type cells (lane 1, JY746), *taz1-d rqh1-d* double mutant (lane 2, KT0d1), *taz1-d rad11-D223Y* double mutant (lane 3, YO008), *taz1-d rad11-D223Y rqh1-d* triple mutant (lane 4, TK025), *taz1-d rad11-D223Y rqh1-K547A* triple mutant (lane 5, TK069), and *taz1-d rad11-D223Y rqh1-K547R* triple mutant (lane 6, TK074) was analyzed by Southern hybridization as shown in Figure 1C.

tant maintained telomeric DNA (Figure 4B, lane 4). PFGE analysis of the genomic DNA indicated that the chromosomes of the *taz1-d rad11-D223Y rqh1-d* triple mutants were not circularized (Supplemental Figure 1A). The suppression of telomere loss in *taz1-d rad11-D223Y* cells by *rqh1* deletion is not due to the loss of long single strand overhang in *taz1-d*, because *taz1-d rqh1-d* double mutant has single strand overhang similar to the *taz1-d* cells (Tomita and Ueno, personal communication). We further asked whether the helicase activity of Rqh1 is required for suppression of telomere loss in the double mutant, using the Rqh1-K547A and Rqh1-K547R mutants, both of which have been shown to have no helicase activity (Laursen *et al.*, 2003). *taz1-d rad11-D223Y rqh1-K547A* and *taz1-d rad11-D223Y rqh1-K547R* triple mutants retained telomere DNA (Figure 4B, lanes 5 and 6). Hence, we conclude that the helicase activity of Rqh1 is directly involved in the suppression of telomere loss in the *taz1-d rad11-D223Y* double mutant.

rqh1-d cells show dramatically increased rates of HR after replication arrest or DNA damage (Stewart *et al.*, 1997; Doe *et al.*, 2000). Therefore, deletion of *rqh1*⁺ may activate a recombination-based telomerase-independent telomere elongation mechanism in the *taz1-d rad11-D223Y* double mutant, thereby explaining the suppression of telomere loss conferred by *rqh1*⁺ deletion. Unfortunately, we were unable to construct a *taz1-d rad11-D223Y rqh1-d trt1-d* quadruple mutant or a *taz1-d rad11-D223Y rqh1-d rad22-d* quadruple mutant to test this hypothesis, suggesting that these mutants are lethal (Laursen *et al.*, 2003). However, we were able to show that a *taz1-d rqh1-d trt1-d* triple mutant failed to maintain telomeric DNA (Supplemental Figure 1B), indicating that telomeric DNA in *taz1-d rqh1-d* double mutant is maintained by telomerase activity. Additional *rad11-D223Y* mutation in *taz1-d rqh1-d* background is unlikely to activate recombination at telomeres as *rad11-D223Y* confers a defect in homologous recombination repair (Ono *et al.*, 2003). Moreover, that a mutation in the helicase domain of Rqh1 has no effect on recombination between sister chromatids (Hope *et al.*, 2006) further suggests that telomeric DNA in *taz1-d rad11-D223Y rqh1-K547A* and *taz1-d rad11-D223Y rqh1-K547R* triple mutants is maintained by telomerase activity. Together, these results suggest that telomeric DNA in the *taz1-d rad11-D223Y rqh1-d* triple mutant is maintained by telomerase activity and that Rqh1 is involved in telomere loss in the *taz1-d rad11-D223Y* double mutant.

Rqh1 Binds to Telomere in *rad11-D223Y* Cells

The involvement of Rqh1 in the telomere loss in *taz1-d rad11-D223Y* cells implies that Rqh1 binds to telomeres in these cells. However, it is difficult to study the telomere binding of Rqh1 in cells that lose telomeric DNA very rapidly. Thus, we studied the telomere binding of Rqh1 in *rad11-D223Y* mutant. ChIP assay shows that Rqh1-Myc protein was enriched at telomere more than twofold in the *rad11-D223Y* mutant compared with the control cells that do not express Rqh1-Myc protein (no tag) (Figure 5A). This result suggests that Rqh1 can bind to telomeres in *rad11-D223Y* mutant and possibly in *taz1-d rad11-D223Y* double mutant. However, Rqh1-Myc protein was not robustly enriched (<2-fold) at telomere in wild-type cells compared with the control cells (Figure 5A). Thus, it remains unclear whether Rqh1 binds to telomere in wild-type cells.

Human and *S. cerevisiae* RPA have been shown to interact with Werner protein (WRN) and Sgs1, respectively (Cobb *et al.*, 2003; Doherty *et al.*, 2005). These findings raise the possibility that *S. pombe* Rqh1 interacts with RPA. Thus, we studied the physical interactions of Rqh1 and RPA by coim-

Figure 5. Rqh1 binds to telomere in *rad11-D223Y* cells and in *taz1-d* cells. (A) ChIP assay of Rqh1-Myc protein in *rad11-D223Y* mutant. Untagged wild-type control (no tag) (JY746), *rqh1-Myc* (J564), and *rad11-D223Y rqh1-Myc* (TK027) cells were used. The amount of DNA was calculated by quantitative real-time PCR. (B) Physical interaction of Rad11 or Rad11-D223Y with Rqh1 in vivo. Cell extracts containing Rqh1-Myc or either Rad11-GFP or Rad11-D223Y-GFP were subjected to immunoprecipitation with anti-Myc and analyzed by Western blotting. *rad11-GFP* (MG809), *rad11-D223Y-GFP* (TK038), *rqh1-Myc* (J564), *rad11-GFP rqh1-Myc* (TK042), and *rad11-D223Y-GFP rqh1-Myc* (TK043) cells were used.

muno-precipitation experiments. Both Rad11-GFP and Rad11-D223Y-GFP were coimmunoprecipitated with Rqh1-Myc (Figure 5B). Thus, both Rad11 and Rad11-D223Y interact with Rqh1. These results suggest that RPA could form a complex with Rqh1 at telomere.

Overexpression of Pot1 Suppresses the Telomere Loss in the *taz1-d rad11-D223Y* Double Mutants

Our results suggest that Rad11-D223Y is enriched at telomeres in *taz1-d rad11-D223Y* cells. Because Pot1 has the ability to bind single-stranded telomere DNA, we envisage the possibility that increased binding of mutant RPA at telomere ends may compete with Pot1 for telomere binding. We therefore asked whether overexpression of Pot1 would suppress telomere loss in the double mutant. We expressed Pot1-HA from its own promoter on a high copy number plasmid (a gift from Peter Baumann, Stowers Institute for Medical Research). The telomere loss phenotype of the double mutant was suppressed by Pot1 overexpression (Figure 6A). This observation suggests that Pot1 cannot function properly in a *taz1-d rad11-D223Y* background or that increased levels of Pot1 compensate for the defects in *taz1-d rad11-D223Y* cells.

DISCUSSION

rad11-D223Y single mutant cells have short telomeres, whereas *taz1-d* cells have long telomeres (Cooper *et al.*, 1997; Ono *et al.*, 2003). Here, we demonstrate that *taz1-d rad11-D223Y* double mutants cannot maintain telomeric DNA. Our result provides the first demonstration that Taz1 and RPA are synergistically required for prevention of rapid telomere loss. *rad11-D223Y* mutant cells have no obvious DNA replication defect, as the timing of replication is unaffected by this mutation (Ono *et al.*, 2003) (data not shown). Therefore, the telomere phenotype observed in *rad11-D223Y* cells is unlikely to be an indirect effect of compromised DNA replication.

Possible Mechanism of Telomere Loss in *taz1-d rad11-D223Y* Double Mutant

The telomere loss phenotype of *taz1-d rad11-D223Y* cells is similar to that of *pot1-d* cells (Figure 2), because telomeres erode rapidly. Moreover, overexpression of Pot1 suppressed

Figure 6. Overexpression of Pot1 suppresses telomere loss in the *taz1-d rad11-D223Y* double mutant. (A) The telomere length of the wild-type cells (lane 1, JY746), *taz1-d* cells (lane 2, KT101), *taz1-d rad11-D223Y* double mutant (lane 3, YO008), and *taz1-d rad11-D223Y* double mutant overexpressing Pot1 (lane 4, TK072) was analyzed by Southern hybridization as shown in Figure 1C. (B) Model of Taz1 and RPA at telomere ends. In wild-type cells, Taz1 and RPA are synergistically required for prevention of rapid telomere loss by controlling activity of Rqh1 helicase and required for Pot1 to function properly at telomere. In *taz1-d rad11-D223Y* double mutant, the mutant RPA (mRPA) containing Rad11-D223Y protein is enriched at telomere compare with the wild-type RPA and that Pot1 cannot function properly in the *taz1-d rad11-D223Y* double mutant. It remains unclear whether Pot1 cannot bind to telomere in the double mutant or Pot1 can bind to telomere but cannot function properly. We assume that Rqh1 helicase extensively unwinds telomere ends and/or DNA break end generated by collapse of stalled replication fork at telomere/subtelomere boundary. Unwound DNA could be degraded by nucleases, which would cause rapid telomere loss.

the telomere loss in *taz1-d rad11-D223Y* double mutant (Figure 6A). These results suggest a link between the mechanism of telomere loss in *pot1-d* cells and the *taz1-d rad11-D223Y* double mutant. One explanation for this observation is that RPA and Taz1 are redundantly required for Pot1 to function properly at telomeres (Figure 6B). Our results suggest that the Rad11-D223Y mutant protein is enriched at telomeres in *taz1-d rad11-D223Y* cells (Figure 3). This enrichment might confer inhibition of Pot1 binding and/or Pot1 function. Indeed, high concentrations of human RPA inhibit telomerase activity in vitro (Cohen *et al.*, 2004). Moreover, *S. cerevisiae*

Rad51-mediated DNA strand exchange is inhibited when single-strand DNA is covered by RPA harboring a *rfal-t11* mutation (Kantake *et al.*, 2003). Our observation that *pot1-d rqh1-d* cells maintain telomeric DNA further suggests a link between the mechanism of telomere loss in *pot1-d* and the *taz1-d rad11-D223Y* cells (Kibe, Imano, and Ueno, unpublished data). A related interpretation is that RPA and Taz1 protect telomeres via two redundant pathways, one or both of which can be bypassed by Pot1 overexpression. Because Rad11-D223Y protein is enriched at telomere compared with the wild-type Rad11 protein, the amount of Pot1 binding to telomere may be decreased in the *rad11-D223Y* cells relative to wild-type cells. To test this possibility, we performed ChIP assay and found that the amount of Pot1 binding to the telomere is not decreased in the *rad11-D223Y* cells relative to wild-type *Rad11* cells (data not shown). This result may suggest that telomere localization of Pot1 is not affected by *rad11* mutation. However, the DNA binding domain located at the N terminal of Pot1 is not required for telomere localization of Pot1 (Bunch *et al.*, 2005). Therefore, it is still possible that the amount of Pot1 binding to the telomere overhang is decreased but Pot1 can bind to telomere through protein-protein interaction.

Point mutations in the helicase domain of Rqh1 suppressed telomere loss in *taz1-d rad11-D223Y* cells as well as deletion of *rqh1*⁺ (Figure 4), suggesting that Rqh1 helicase activity is involved in the telomere loss seen in these cells. Human RPA stimulates the helicase activity of WRN in vitro (Brosh *et al.*, 1999). Therefore, it is conceivable that the elevated levels of telomeric RPA in *taz1-d rad11-D223Y* cells hyperstimulates Rqh1 activity. Up-regulated Rqh1 at telomeres could lead to extensive unwinding of telomeric and subtelomeric DNA, rendering these regions more accessible to nucleases (Figure 6B). This could explain the telomere loss in *taz1-d rad11-D223Y* and possibly *pot1-d* cells. Human Pot1 stimulates WRN helicase activity in vitro (Opresko *et al.*, 2005). In contrast, *S. pombe* Pot1 may inhibit Rqh1 at telomeres in vivo. Our results suggest that controlling the activity of Rqh1 at telomeres is critical for the prevention of genomic instability.

The rapid telomere loss in *taz1-d rad11-D223Y* cells is also similar to that seen in *taz1-d trt1-d* and *taz1-d est1-d* cells (Nakamura *et al.*, 1998; Miller *et al.*, 2006). In *S. cerevisiae*, RPA has been shown to be required for telomerase action (Schramke *et al.*, 2004). Therefore, it could be possible that in the context of the *taz1-d* mutation, the *rad11-D223Y* allele prevents telomerase activation. However, unlike *taz1-d rad11-D223Y rqh1-d* triple mutant cells, the *taz1-d trt1-d rqh1-d* triple mutant failed to maintain telomeric DNA (Supplemental Figure 1B). Moreover, that chromosomes of *pot1-d* cells and *taz1-d rad11-D223Y* cells are circularized, but not to *taz1-d trt1-d* cells, supports the notion that *taz1-d rad11-D223Y* cells deprotect ends from a mechanism distinct from that in *taz1-d trt1-d* cells.

taz1-d cells, but not *rap1-d* cells, accumulate paused replication forks at the telomere/subtelomere boundary, and most of the 1–4-kb telomeres in *taz1-d* are suggested to be synthesized by telomerase, starting from the broken DNA end generated near the telomere/subtelomere boundary (Miller *et al.*, 2006). Unlike *taz1-d rad11-D223Y* cells, *rap1-d rad11-D223Y* cells were able to maintain telomeric DNA (Figure 3E), suggesting that the mechanism of telomere loss in the *taz1-d rad11-D223Y* double mutant may be related to the replication fork pausing induced by Taz1 loss. Therefore, RPA and Taz1 may prevent degradation of the broken telomere ends produced by collapsed replication forks by inhibiting Rqh1 helicase activity (Figure 6B). If the collapsed

replication fork is the reason for the telomere loss in the *taz1-d rad11-D223Y* double mutant, there are several other explanations for the telomere loss. For example, the collapsed replication forks at the telomere/subtelomere boundary are repaired by HR, and normal RPA function is required for this process. However, *taz1-d rad22-d* and *taz1-d rhp51-d* cells maintain telomeres, ruling out a requirement for Rad22- or Rhp51-dependent processes (data not shown). The other possibility is that the collapsed replication forks would require time for HR repair and that compromised checkpoint activation in *rad11-D223Y* cells leads to reduced repair and hence loss of telomeres. However, that *taz1-d rad3-d* and *taz1-d tel1-d* cells maintain telomeres rules out this possibility (Nakamura *et al.*, 2002; Miller and Cooper, 2003). It may also be that *rad11-D223Y* confers a defect in the protection of the stalled replication forks in the absence of Taz1, so that these stalled replication forks are more prone to collapse in the double mutant. In this case, *taz1-d swi1-d* cells might be expected to lose telomeric DNA, because Swi1 is suggested to be required for the protection of collapsed replication forks (Noguchi *et al.*, 2003). However, *taz1-d swi1-d* double mutants did not lose telomeric DNA (data not shown), suggesting that even if *rad11-D223Y* is defective in protection of the stalled replication fork, it is not the reason for telomere loss in the double mutant. Finally, *rad11-D223Y* may compromise de novo telomere addition at the broken DNA ends produce by the collapsed replication forks at the telomere/subtelomere boundary. However, de novo telomere addition is observed in *rad11-D223Y* cells, ruling out this possibility (Cullen and Humphrey, personal communication).

Possible Role of Human RPA at Telomere

RPA is highly conserved from yeast to humans, so the observation that both *S. cerevisiae* and *S. pombe* RPA have important roles in telomere maintenance imply synergism between RPA and TRF1 and/or TRF2 in telomere maintenance in higher eukaryotes. TRF1 cooperates with Pot1 to regulate telomerase access to the 3' telomere overhangs (Loayza and De Lange, 2003). One possible role of human RPA on telomeres could be to regulate telomerase access by cooperating with TRF1 and/or Pot1. T-loops are created through the strand invasion of 3' telomere overhangs into the duplex region of the telomeres, and TRF2 is required for this process (Griffith *et al.*, 1999; de Lange, 2004). Because RPA is required for the strand invasion during DNA double-strand break repair, human RPA might cooperate with TRF2 to form or dissociate T-loops by binding to the single-stranded DNA generated when Pot1 is released during G2 (Verdun *et al.*, 2005). Human WRN and RPA binds to telomeres in S phase (Crabbe *et al.*, 2004; Verdun and Karlseder, 2006). Moreover, RPA interacts with WRN (Doherty *et al.*, 2005). Thus, human RPA might function together with WRN on telomeres in S phase. Further investigation of RPA, Rqh1, Taz1, and Pot1 and their human counterparts will provide clues about mechanisms of telomere maintenance in higher eukaryotes.

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