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A novel allele of fission yeast *rad11* that causes defects in DNA repair and telomere length regulation

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ABSTRACT

Replication protein A (RPA) is a heterotrimeric single-stranded DNA-binding protein involved in DNA replication, recombination and repair. In *Saccharomyces cerevisiae*, several mutants in the *RFA1* gene encoding the large subunit of RPA have been isolated and one of the mutants with a missense allele, *rfa1-D228Y*, shows a synergistic reduction in telomere length when combined with a *yku70* mutation. So far, only one mutant allele of the *rad11*⁺ gene encoding the large subunit of RPA has been reported in *Schizosaccharomyces pombe*. To study the role of *S.pombe* RPA in DNA repair and possibly in telomere maintenance, we constructed a *rad11-D223Y* mutant, which corresponds to the *S.cerevisiae rfa1-D228Y* mutant. *rad11-D223Y* cells were methylmethane sulfonate, hydroxyurea, UV and γ-ray sensitive, suggesting that *rad11-D223Y* cells have a defect in DNA repair activity. Unlike the *S.cerevisiae rfa1-D228Y* mutation, the *rad11-D223Y* mutation itself caused telomere shortening. Moreover, Rad11-Myc bound to telomere in a ChIP assay. These results strongly suggest that RPA is directly involved in telomere maintenance.

INTRODUCTION

Replication protein A [RPA, also known as human single-stranded DNA-binding protein (SSB) or replication factor A (RFA)] is a heterotrimeric single-stranded DNA-binding protein consisting of three subunits: RPA1 (70 kDa), RPA2 (36 kDa) and RPA3 (14 kDa) (1). RPA was originally identified as an essential factor for SV40 DNA replication *in vitro* (2–4). RPA is also required for nucleotide excision repair and mismatch repair *in vitro* (5–7). Moreover, RPA stimulates the activities of eukaryotic homologous pairing proteins *in vitro* (8–13). These biochemical studies have suggested that RPA is involved in DNA replication, recombination and repair *in vivo*.

Functions of RPA *in vivo* have been well studied in *Saccharomyces cerevisiae* (14–17). The *RFA1* gene encoding the large subunit (70 kDa) of RPA is essential for cell viability. So far, several *rfa1* mutants have been isolated. One of the mutants, *rfa1-44*, is sensitive to UV and X-ray irradiation and is also defective in HO-endonuclease-induced plasmid-to-chromosome gene conversion (16). Another mutant, *rfa1-D228Y*, was isolated in a screen for suppressors of the defect in direct repeat recombination in *rad1 rad52* double mutants (15). *rfa1-D228Y* mutants display increased levels of direct repeat recombination, decreased levels of heteroallelic recombination and UV sensitivity. Although the *rfa1-D228Y* mutation itself does not affect telomere length, synergistic reduction in telomere length is observed in the *yku70 rfa1-D228Y* double mutant, suggesting a role for RPA in telomere maintenance in the absence of Ku heterodimer (18).

Telomeres, the specialized structures at the ends of eukaryotic chromosomes, ensure chromosome stability by protecting chromosome ends from degradation and fusion (19). In *Schizosaccharomyces pombe*, proteins that bind to telomere ends have been shown to positively or negatively influence telomere length (20–25). Telomere length is also controlled by DNA repair or DNA damage checkpoint proteins including Ku heterodimer, Rad32/Rad50/Nbs1 complex, and Rad3/Rad26 complex (26–32). Ku heterodimer binds to double-strand break (DSB) ends and is required for non-homologous end-joining repair (33), while Rad32/Rad50/Nbs1 complex is required for homologous recombination (HR) repair (34). Rad3/Rad26 complex is required for replication and the DNA damage checkpoint (35).

Telomere ends acquire G-rich single-stranded overhangs in S phase in both *S.cerevisiae* and *S.pombe* (36,37). The G-rich overhang is required for telomere elongation, because it is used for binding of the RNA component in the telomerase complex (38). *Schizosaccharomyces pombe* Pot1 is thought to bind to the G-rich overhang *in vivo* (25,39). As RPA is a single-stranded DNA-binding protein, it might function on the G-rich single-stranded overhang. However, there is no direct evidence suggesting that RPA functions at telomere ends in wild-type cells.

In *S.pombe*, the genes encoding the three subunits of RPA, *ssb1*⁺ (p68 subunit gene), *ssb2*⁺ (p30 subunit gene) and *ssb3*⁺

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(p12 subunit gene), have been cloned (40). The reconstituted SpSSB/RPA complex expressed in *Escherichia coli* is active in single-stranded DNA binding and the T-antigen-dependent unwinding of SV40 ori DNA (40). However, the *in vivo* function of *S.pombe* RPA is poorly understood. So far, only one *rad11/ssb1* mutant allele has been reported (41). This *rad11* mutant is UV and γ-ray sensitive, but the DNA damage checkpoint is intact.

To understand the function of *S.pombe* RPA in DNA repair and possibly in telomere maintenance, we created a *rad11-D223Y* mutant in which the asparagine at position 223 is mutated to tyrosine, which corresponds to the *S.cerevisiae rfa1-D228Y* mutant. In this work, we examined the DNA damage sensitivity and telomere length of the *rad11-D223Y* mutant. We provide here the first evidence suggesting that RPA is involved in telomere maintenance.

MATERIALS AND METHODS

Schizosaccharomyces pombe strains, media and genetic methods

The *S.pombe* strains used in this work are listed in Table 1. Cells were grown in YPAD medium (1% yeast extract, 2% polypeptone, 2% glucose, 0.04% adenine), YE medium (0.5% yeast extract, 3% glucose) or Edinburgh minimal medium (EMM) with required supplements. Standard procedures were used for propagation and genetic manipulation (42). Sensitivity to γ-rays and UV light was examined as described previously (43). Briefly, exponentially growing cells were irradiated with γ-rays from a ^{60}Co source at a dose rate of 100–200 Gy/h or with UV light from a germicidal lamp (UVP UV-CROSSLINKER, CL-1000) at a dose rate of 50–100 J/m^2/min. Duplicate samples of irradiated cells and unirradiated cells were plated on YPAD plates and incubated at 30°C for 4 days, and the colonies were counted (44). For semi-quantitative analysis of DNA repair activity, the spot assay was employed as described previously (43). Briefly, 3 μl of serial 10-fold dilutions of log-phase cells (0.5×10^7 cells/ml) were spotted onto a YPAD plate or a YPAD plate containing the indicated concentration of methylmethane sulfonate (MMS) or hydroxyurea (HU). All experiments were repeated at least twice and gave similar results.

Construction of plasmid pT7-rad11-D223Y-ura4, which contains the *rad11-D223Y* mutation

Site-directed mutagenesis was carried out by using a Mutan-Super Express Km Kit (TaKaRa) according to the manufacturer's instructions. Briefly, a DNA fragment containing a partial *rad11*$^+$ gene, which was amplified by PCR with a sense primer (5′-AAATAGTGTATCGTCAGGCTA-3′) and an antisense primer, (5′-GATAAAATTGGTAATCCCG-3′) using the *S.pombe* genomic DNA as template, was subcloned into pT7Blue T-Vector, giving the plasmid pT7-rad11. Next the EcoRI–XbaI fragment from pT7-rad11 was inserted into the EcoRI–XbaI site in pKF18, giving the plasmid pKF18-rad11. To introduce the mutation, a DNA fragment was amplified by PCR with a mutation primer (5′-TCCCCACTTTCATAGAGTAAAT-3′) and a selection primer (provided in the Kit) using pKF18-rad11 as template, giving the plasmid pKF18-rad11-D223Y. The NheI–ClaI fragment from pKF18-rad11-D223Y was inserted into the NheI–ClaI site in pT7-rad11, giving the plasmid pT7-rad11-D223Y. Then the *ura4*$^+$ cassette was inserted into the SmaI site in pT7-rad11-D223Y, giving the plasmid pT7-rad11-D223Y-ura4.

Construction of *rad11-Myc* cells and *rad11-D223Y-Myc* cells

To tag Rad11 and Rad11-D223Y with the Myc epitope at the C-terminus, a DNA fragment containing the partial *rad11*$^+$ gene, which was amplified by PCR with a sense primer (5′-GGATCCGTGTTACGCTTTGGGGA-3′) and an antisense primer (5′-TTAATTAATTGAGCAGACTCAATGAAAT-3′) using the *S.pombe* genomic DNA as template, was cloned into the BamHI–PacI site in pFA6a-13Myc-kanMX6, giving the plasmid pFA6a-13Myc-kanMX6-rad11. pFA6a-13Myc-kanMX6 plasmid, which contains 13 copies of the Myc epitope and a kanMX6 marker, was provided by John R. Pringle (University of North Carolina) (45). The resulting plasmid pFA6a-13Myc-kanMX6-rad11 was linearized with NspV and used for transformation of JY746 and YO001.

Determination of recombination rates

The recombination rate was measured between the *ade6B::ura4*$^+$*::ade6X* direct repeats located on chromosome III. *ade6B* and *ade6X* mutations were constructed by destroying the BamHI^{208} and XhoI^{1651} restriction sites, respectively,

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

Strains	Genotype	Source
JY746	*h*$^+$ *leu1-32 ura4-D18 ade6-M210*	M. Yamamoto
JY741	*h*$^-$ *leu1-32 ura4-D18 ade6-M216*	M. Yamamoto
YO001	*h*$^+$ *leu1-32 ura4-D18 ade6-M210 rad11D223Y*	This work
YO002	*h*$^+$ *leu1-32 ura4-D18 ade6-M210 rad11D223Y pku70:LEU2*$^+$	This work
YO003	*h*$^+$ *leu1-32 ura4-D18 ade6-M210 rad11D223Y rad50:ura4*$^+$	This work
YO004	*h*$^+$ *leu1-32 ura4-D18 ade6-M210 rad11:Myc:kanMX6*	This work
YO005	*h*$^+$ *leu1-32 ura4-D18 ade6-M210 rad11D223Y:Myc:kanMX6*	This work
KT120	*h*$^+$ *leu1-32 ura4-D18 ade6-M210 rad50::LEU2*$^+$	(31)
PKu70L	*h*$^-$ *leu1-32 ura4-D18 ade6-M216 pku70::LEU2*$^+$	(44)
TNF79	*h*$^-$ *ade6D ade6B::ura4*$^+$*::ade6X*	This work
YO006	*h*$^-$ *ade6D ade6B::ura4*$^+$*::ade6X rad11D223Y*	This work

using Klenow fragment, resulting in a frame-shift (T. Nakagawa, N. Nitani and H. Masukata, manuscript in preparation). A single colony was inoculated into 2 ml of YE + adenine + uracil medium and incubated for 2 days, and the cells were plated onto EMM + adenine + uracil, EMM + uracil + guanine and EMM + guanine after appropriate dilutions with distilled water. Each amino acid was added at a final concentration of 225 µg/ml. Guanine was added to EMM at a final concentration of 50 µg/ml to avoid colony formation of *ade*⁻ cells on the minimal medium. The number of colonies was counted after 4 days of incubation at 30°C. A fluctuation test was performed to determine spontaneous recombination rates. The median value of recombination frequencies of nine cultures was used to calculate the rate of recombination (46).

Measurement of telomere length

Telomere length was measured by Southern hybridization according to the procedure described previously (21) by using an AlkPhos DirectTM kit module (Amersham Pharmacia Biotech). Briefly, chromosomal DNA, which was digested with ApaI and separated by electrophoresis on a 2% agarose gel, was probed with a 0.3 kb DNA fragment containing telomeric repeat sequences, which was derived from pNSU70 (47).

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay described by Takahashi *et al.* (48) was adopted with modification. Cells grown in 100 ml of YPAD medium at 30°C were fixed with formaldehyde. For immunoprecipitation, anti-Myc antibody (Cell Signaling Technology™) and protein G-coated dynabeads (Dynal) were used. Immunoprecipitated DNA was extracted and suspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA). The following primers were used in PCRs to amplify the telomeric DNA: TOP, 5′-CGGCTGACGGG-TGGGGCCCAATA-3′; BOTTOM, 5′-GTGTGGAATTGAG-TATGGTGAA-3′; and the *eno1*⁺ DNA: TOP, 5′-TGC-CCCGGGTTTAAAACTTAGCAGCCTT-3′; BOTTOM, 5′-CTTTCAACGTCTTGAACG-3′.

RESULTS

Construction of the *rad11-D223Y* mutant

To construct the *rad11-D223Y* mutant, we used the allele replacement method as described previously (49). First, the plasmid pT7-rad11-D223Y-ura4 (see Materials and Methods) was cut with AatII and transformed into haploid strain JY746 (*h*⁺ *leu1-32 ura4-D18 ade6-M210*) (Fig. 1). Next, the resultant cells were grown in YPAD medium to allow deletion of the *ura4*⁺ cassette by intrachromosomal recombination between the *rad11* allele and the *rad11-D223Y* allele. Then, *ura4*⁻ cells were selected on EMM plates containing 0.2% 5-fluoroorotic acid (5-FOA). Then, the *rad11-D223Y* mutation was confirmed by DNA sequencing.

We first examined the effect of the *rad11-D223Y* mutation on normal mitotic growth. The growth rate was not affected by the *rad11-D223Y* mutation (data not shown). Flow cytometric analysis of logarithmically growing *rad11-D223Y* cells showed that most of the *rad11-D223Y* cells had a DNA content of 2C, suggesting that *rad11-D223Y* cells are not arrested in G_1 or S phase (data not shown). Moreover, when the structure of *S.pombe* chromosomes was analyzed by pulsed-field gel electrophoresis, chromosomes from *rad11-D223Y* cells entered the gel and were separated into three chromosomes, indicating that DNA replication is completed in the *rad11-D223Y* cells (data not shown). Based on these analyses, we concluded that the mitotic cell cycle is not affected by the *rad11-D223Y* mutation.

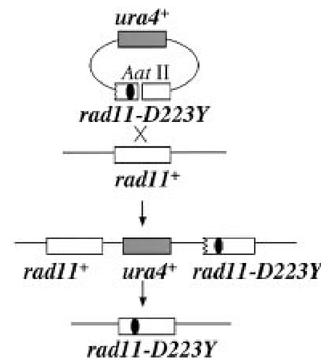

Figure 1. Schematic illustration of construction of the *rad11-D223Y* mutant by allele replacement method. The plasmid shown at the top (pT7-rad11-D223Y-ura4, see Materials and Methods) contains the *ura4*⁺ cassette and the *rad11-D223Y* allele. The position corresponding to the D223Y mutation is denoted by a solid circle. The plasmid linearized by digestion with AatII was transformed into haploid strain JY746 (*h*⁺ *leu1-32 ura4-D18 ade6-M210*). The *rad11-D223Y* mutant was obtained by spontaneous direct-repeat recombination between the *rad11*⁺ allele and the *rad11-D223Y* allele.

rad11-D223Y mutation has little effect on direct repeat recombination

The *S.cerevisiae rfa1-D228Y* mutant was originally isolated by screening for a mutant with a mutation that suppresses the decrease of the recombination rate in *rad1 rad52* strains (15). The *rfa1-D228Y* mutation on its own causes a 15-fold increase in direct repeat recombination (15,50). Therefore, we examined the recombination frequency of *rad11-D223Y* cells. We used strains containing a non-tandem direct repeat of *ade6*⁻ heteroalleles to measure the *ade6*⁺ recombination frequency (Fig. 2A). The rate of *ade6*⁺ formation was not significantly increased in *rad11-D223Y* cells (Fig. 2B). These results indicate that, unlike the *S.cerevisiae rfa1-D228Y* mutation, the *rad11-D223Y* mutation has little effect on the frequency of direct-repeat recombination.

rad11-D223Y mutant is methylmethane sulfonate and hydroxyurea sensitive

Although the *rad11-D223Y* mutation does not affect the mitotic cell cycle, it may affect the viability when DNA replication is disturbed. Thus, we examined the HU and MMS sensitivity of the *rad11-D223Y* mutant. As shown in Figure 3A, the growth of *rad11-D223Y* cells was strongly inhibited in the presence of MMS or HU, indicating that *rad11-D223Y* cells are MMS and HU sensitive. MMS alkylates the DNA. These DNA can block DNA synthesis and stall the replication fork. Indeed, DNA replication is slowed down in the presence of 0.01% MMS in *S.pombe*, suggesting that the DNA damage caused by MMS causes

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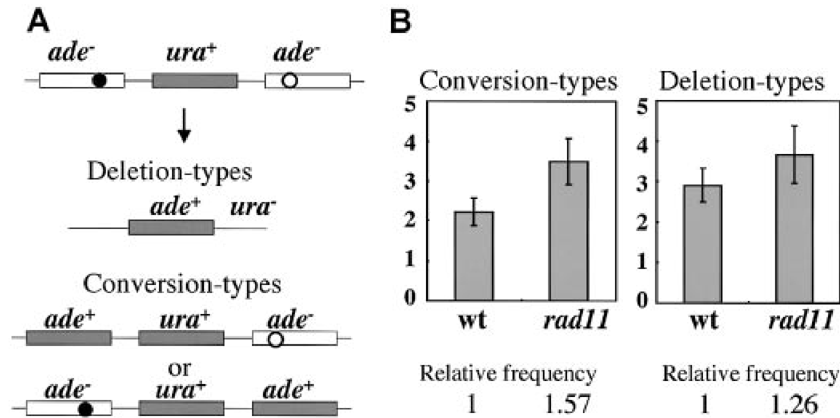

Figure 2. Spontaneous recombination between the *ade6B::ura4⁺::ade6X* direct repeats. (**A**) Schematic illustration of intrachromosomal recombination substrate and recombination products. The strain contains *ade6B* (solid circle) and *ade6X* (open circle). The *ade6* repeats are separated by a 1.8 kb HindIII region containing *ura4⁺* marker. *ade6⁺* and *ura4⁺* alleles are denoted by a filled-in box. *ade⁺ ura⁺* recombinants are referred to as conversion-type, and *ade⁺ ura⁻* as deletion-type recombinants. (**B**) Effect of *rad11-D223Y* mutation on the spontaneous rate of adenine prototroph formation. The rates of *ade⁺* formation and *ade⁺ ura⁺* formation were experimentally determined using isogenic strains of the wild type (TNF79) and *rad11* (YO006). The rate ($\times 10^{-5}$) of the formation of *ade⁺ ura⁺* recombinants (conversion) and the rate ($\times 10^{-5}$) of the formation of *ade⁺ ura⁻* recombinants (deletion) are shown. The rate of *ade⁺ ura⁻* formation was calculated by subtracting the *ade⁺ ura⁺* rate from the *ade⁺* (*ura±*) rate. The recombination rate per cell division was determined using the median value of the recombination frequency of nine cultures. The bar shows the mean value of three sets of experiments. Standard deviations are shown by error bars.

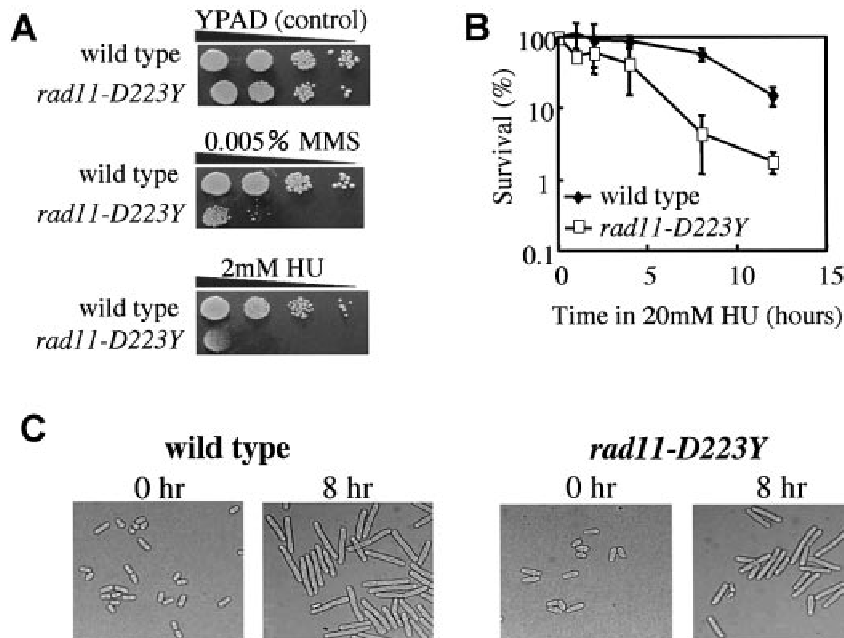

Figure 3. *rad11-D223Y* cells are MMS and HU sensitive. (**A**) The sensitivities of wild-type cells (JY746) and *rad11-D223Y* cells (YO001) to MMS and HU determined in a spot test. (**B**) Viability of the wild-type cells and the *rad11-D223Y* cells in YPAD medium in the presence of 20 mM HU. Standard deviations are shown by error bars. (**C**) Both the wild-type cells and the *rad11-D223Y* cells were elongated after 8 h of incubation in YPAD medium in the presence of 10 mM HU. For genotypes, see Table 1.

problems during replication and is not repaired until S phase (27). HU also blocks DNA replication by depleting deoxynucleotides. Therefore, our results suggest that the *rad11-D223Y* mutant has a defect in the recovery from stalled replication forks.

Next, we examined the response to HU in more detail. First, we examined the viability loss in 20 mM HU in liquid medium. In human cells and *S.cerevisiae*, RPA is required for recruitment of the ATR–ATRIP complex and Mec1–Ddc2 complex, which correspond to the *S.pombe* Rad3–Rad26 complex, to sites of DNA damage (51). Similarly, if the *rad11-D223Y* mutant had a defect in the recruitment of the Rad3–Rad26 complex to sites of DNA damage in *S.pombe*, the mutant would lose viability very quickly in the presence of HU. Only 0.1% of *rad3-d* cells survived after 4 h in 10 mM HU (52). In contrast, the cell viability of the *rad11-D223Y*

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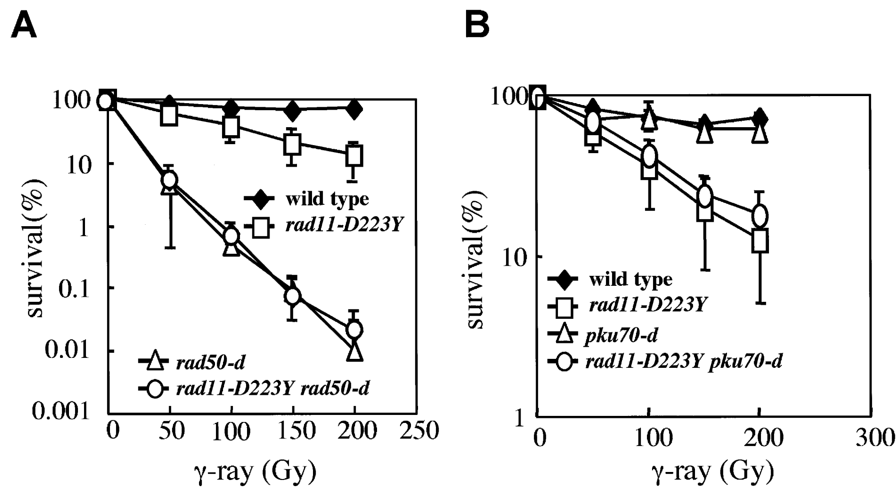

Figure 4. Epistasis analysis between *rad11-D223Y* cells and *rad50-d* cells or *pku70-d* cells for γ-ray sensitivity. (**A**) The sensitivities to γ-rays of wild-type cells, JY746 (diamonds), *rad11-D223Y* cells, YO001 (squares), *rad50-d* cells, KT120 (triangles) and *rad11-D223Y rad50* double mutants, YO003 (circles). (**B**) The sensitivities to γ-rays of wild-type cells, JY746 (diamonds), *rad11-D223Y* cells, YO001 (squares), *pku70-d* cells, Pku70L (triangles), and *rad11-D223Y pku70* double mutants, YO002 (circles). For genotypes, see Table 1. Standard deviations are shown by error bars.

mutant was still 40% after 4 h in 20 mM HU (Fig. 3B). These results suggest that the *rad11-D223Y* mutation does not cause a severe S-phase checkpoint defect. Consistent with these data, most of the *rad11-D223Y* cells became elongated after treatment with 10 mM HU for 8 h (Fig. 3C). These results suggest that cell cycle arrest mediated by the S-phase checkpoint appears to be normal in *rad11-D223Y* cells.

Epistasis analysis between *rad11-D223Y* cells and *rad50-d* cells or *pku70-d* cells for γ-ray sensitivity

The *S.cerevisiae rfa1-D228Y* mutant is not γ-ray sensitive. However, the *S.pombe rad11-D223Y* mutant might show a different phenotype in DNA repair ability. Thus, we examined the γ-ray sensitivity of *rad11-D223Y* cells. Unlike the *S.cerevisiae rfa1-D228Y* mutant, *rad11-D223Y* cells were more γ-ray sensitive than wild-type cells (Fig. 4A). DNA DSBs caused by γ-rays are mainly repaired by HR in *S.pombe*. Therefore, our result suggests that *rad11-D223Y* cells have a defect in HR repair ability.

We next examined the γ-ray sensitivity of the *rad50 rad11-D223Y* double mutant. If the *rad11-D223Y* mutation affected some DNA repair activity other than Rad50-dependent HR repair activity, *rad50 rad11-D223Y* double mutants should become more γ-ray sensitive than each single mutant. However, the γ-ray sensitivity of the *rad50 rad11-D223Y* double mutant was almost the same as that of the *rad50* single mutant, indicating that the *rad11-D223Y* mutant has a defect in the Rad50-dependent HR repair pathway (Fig. 4A).

In *S.cerevisiae*, it is reported that the *rfa1-t11* mutation does not affect the degradation rate of HO-induced DSB ends (53). However, it is unknown whether the *S.pombe rad11-D223Y* mutation affects the processing of DSB ends. We have shown that the γ-ray sensitivity of *rad50-d* cells is suppressed by deletion of *pku70*⁺. Based on this and other genetic data, we have concluded that the Rad50 complex is required for the processing of DSB ends; however, in the absence of the Rad50 complex, a second nuclease (Exo1) can resect DSB ends, but this nuclease activity of Exo1 is inhibited by Ku heterodimer (44). Similarly, if *rad11-D223Y* mutation affected the efficiency of Rad50-dependent DSB end processing, the γ-ray sensitivity of *rad11-D223Y* cells might be suppressed by deletion of *pku70*⁺. However, the γ-ray sensitivity of *rad11-D223Y* cells was not suppressed by deletion of *pku70*⁺ (Fig. 4B). This result suggests that the *rad11-D223Y* mutation does not affect the efficiency of Rad50-dependent DSB end processing. We assume that the *rad11-D223Y* mutation affects some DNA repair activities that function after DSB ends are processed by the Rad50 complex.

Epistasis analysis between *rad11-D223Y* cells and *rad50-d* cells for UV sensitivity

To further elucidate the effect of the *rad11D223Y* mutation on DNA repair, we examined UV sensitivity. *rad11-D223Y* cells were also sensitive to UV light (Fig. 5). However, unlike the situation for γ-ray sensitivity, *rad11-D223Y rad50* double mutants became more UV sensitive than each single mutant (Fig. 5). These results suggest that *rad11-D223Y* mutants have a defect in the repair of UV-induced DNA damage that is independent of Rad50.

rad11-D223Y mutation causes telomere shortening

The telomere length of the *S.cerevisiae rfa1-D228Y* mutant itself is normal, but mutations in both *yku70* and *rfa1-D228Y* cause synergistic telomere shortening, suggesting that RPA plays a role at telomere ends in the absence of Ku heterodimer (50). Thus, we examined the telomere length of the *rad11-D223Y* mutant and *rad11-D223Y pku70* double mutant (Fig. 6A–C). Unlike the *S.cerevisiae rfa1-D228Y* mutation, the *rad11-D223Y* single mutation itself caused significant telomere shortening (Fig. 6B, lane 2). This result indicates that RPA is required for telomere length regulation in wild-type *S.pombe* cells. Mutations in both *rad11*⁺ and *pku70*⁺ caused synergistic telomere shortening (Fig. 6B, lanes 2 and 4), indicating that RPA functions independently of Ku heterodimer for telomere length regulation. We also examined the telomere length of the *rad11-D223Y rad50* double mutant.

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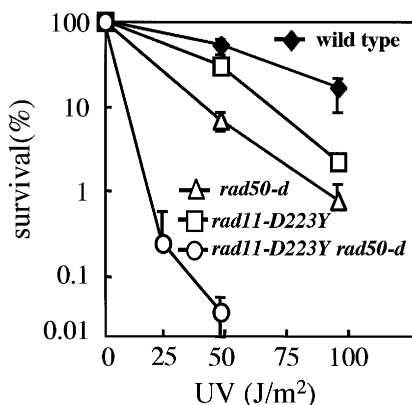

Figure 5. Epistasis analysis between *rad11-D223Y* cells and *rad50-d* cells for UV sensitivity. The sensitivities to UV light of wild-type cells, JY746 (diamonds), *rad11-D223Y* cells, YO001 (squares), *rad50-d* cells, KT120 (triangles) and *rad11-D223Y rad50* double mutants, YO003 (circles). For genotypes, see Table 1. Standard deviations are shown by error bars.

Synergistic telomere shortening was not observed in the double mutant (Fig. 6C), suggesting that RPA and Rad50 are included in the same epistatic group for telomere length regulation.

Rad11 binds to telomere ends

Requirement of *rad11*⁺ for telomere length regulation implies that RPA binds to telomere ends. Therefore we tested the binding of Rad11 to telomeres by the ChIP assay (Fig. 7A and B). We tagged the C-terminus of Rad11 and Rad11-D223Y with Myc-tag. Growth and DNA damage sensitivity were not affected by tagging of Rad11 with Myc-tag (data not shown). Anti-Myc antibody was used for immunoprecipitation and the precipitated DNA was amplified by PCR with primers for the telomeric region or *eno1*⁺as a control. Telomere DNA was significantly amplified in cells, which expressed Myc-tagged Rad11 protein from their own promoter (Fig. 7B). These results indicate that Rad11 (and probably RPA complex) binds to telomere DNA. For unknown reasons, the binding of Rad11 to telomeres was increased by *rad11-D223Y* mutation (Fig. 7B). Since the protein expression level of Rad11-D223Y-Myc and Rad11-Myc were almost the same (data not shown), the stronger binding of Rad11-D223Y-Myc to the telomere is not due to the increased protein level of Rad11-D223Y in *rad11-D223Y* cells.

DISCUSSION

RPA has been suggested to play a role in telomere maintenance in *S.cerevisiae yku70-d* cells. However, the role of RPA in telomere maintenance in wild-type cells remains unclear. We found that the *rad11-D223Y* mutation itself caused telomere shortening (Fig. 6). Moreover Rad11-Myc bound to telomere ends by ChIP assay (Fig. 7). These results strongly suggest that RPA is directly required for telomere length regulation.

How does RPA regulate telomere length? Rad11-D223Y-Myc also bound to telomeres, indicating that telomere shortening in the *rad11-D223Y* mutant is not due to loss of DNA-binding ability of Rad11-D223Y protein (Fig. 7). In *S.pombe*, G-rich single-stranded overhang is increased in S phase (37). Although the binding of Rad11 to telomere could be mediated by interaction with other telomere-binding proteins, it is plausible that RPA binds to the G-rich overhang because RPA is a single-stranded DNA-binding protein. Pot1 and Est1 are thought to bind to the G-rich overhang and these proteins are required for telomere elongation (25,20,54). Therefore, RPA might be required for the recruitment of these proteins or other proteins that are required for telomere length regulation. Another possible role of RPA on telomeres is to remove a secondary structure (possibly G-quartet) formed by the G-rich single-stranded overhang (55). Such a structure may prevent the binding of Pot1 and Est1 to telomere ends. Indeed, RPA is thought to remove secondary structure formed by 3′ single-stranded tails at DSB ends to promote the formation of a Rad51 filament (56). At this time, the exact roles of RPA on telomere are still unclear. More detailed investigation is necessary to understand the exact roles of RPA in telomere length regulation.

rad11-D223Y cells were γ-ray, UV, MMS and HU sensitive (Figs 3–5). Epistasis analysis suggested that *rad11-D223Y* cells have a defect in at least two repair pathways. *rad11-D223Y* cells were epistatic to *rad50-d* cells for γ-ray sensitivity, suggesting that the *rad11-D223Y* mutant has a defect in HR repair. In *rad50-d* cells, the efficiency of the processing of DSB ends would be very low (44,53). Since RPA is thought to bind to 3′ single-stranded overhangs at DSB ends, mutation in *rad11*⁺ would not cause a further problem if 3′ single-stranded overhangs do not exist at DSB ends in *rad50-d* cells. This would be the reason why the *rad11-D223Y* mutation does not increase the γ-ray sensitivity of *rad50-d* cells. This idea is consistent with our assumption that the *rad11-D223Y* mutation affects some DNA repair activities that function after DSBs are processed by the Rad50 complex. Since RPA stimulates the strand exchange activity of Rad51 *in vitro* (13), the *rad11-D223Y* mutation may affect the filament formation ability and/or strand exchange activity of Rhp51 (*S.pombe* Rad51 homolog) protein.

The second defect in DNA repair ability in *rad11-D223Y* cells is suggested to be independent of the HR repair pathway, because the *rad11-D223Y rad50* double mutant became more UV sensitive than each single mutant. Fission yeast has at least two pathways to repair photolesions in DNA, namely, nucleotide excision repair (NER) and UV-damaged DNA endonuclease-dependent excision repair (UVER) (57). Repair downstream of the UVER pathway is divided into the Rad2 sub-pathway and recombination sub-pathway. As RPA is involved in NER repair *in vitro* (58), *rad11-D223Y* cells might have a defect in the NER pathway. However, since RPA controls Rad2 activity during Okazaki fragment processing (59), it is possible that *rad11-D223Y* cells have a defect in the Rad2 sub-pathway in the UVER pathway. Alternatively, if pyrimidine dimers produced by UV light were not removed, they would cause replication fork arrest. The HU sensitivity of the *rad11-D223Y* mutant suggests that this mutant has a defect in the recovery from stalled replication forks. Therefore, we cannot rule out the possibility that the UV sensitivity of *rad11-D223Y* cells could be due to a defect in the recovery from stalled replication forks.

As discussed above, the phenotypes of telomere length, direct-repeat recombination, and γ-ray sensitivity of *S.pombe*

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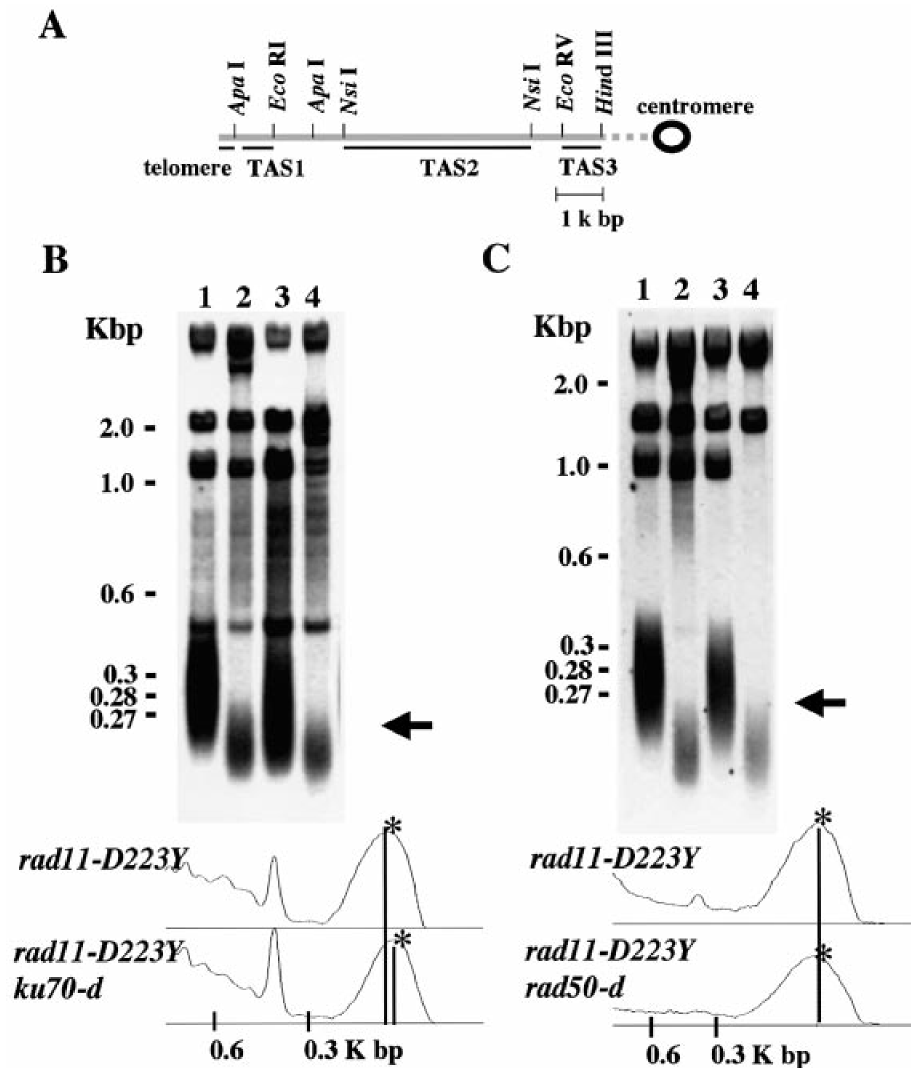

Figure 6. *rad11*+ is involved in telomere length maintenance. (**A**) Schematic presentation of the telomeric and telomere-associated sequences (TAS) of one chromosome arm cloned in the plasmid pNSU70 (47). The positions of telomere and telomere-associated sequences, TAS1, TAS2 and TAS3, are underlined. ApaI-digested telomere sequence was used as a probe for Southern hybridization assays. The chromosome arm is shown by the long gray bar. Restriction enzyme sites are shown above the long gray bar. (**B** and **C**) The telomere length of *rad11-D223Y*, *rad11-D223Y pku70-d* double mutants and *rad11-D223Y rad50-d* double mutants was evaluated by Southern hybridization. (B) Lane 1, wild-type cells (JY746); lane 2, *rad11-D223Y* (YO001); lane 3, *pku70-d* (PKU70L); lane 4, *rad11-D223Y pku70-d* double mutants (YO002). (C) Lane 1, wild-type cells (JY746); lane 2, *rad11-D223Y* (YO001); lane 3, *rad50-d* (KT120); lane 4, *rad11-D223Y rad50-d* double mutants (YO003). Telomeres are indicated by arrows. For genotypes, see Table 1. Peaks and distributions of the telomeric DNA-derived bands analyzed using NIH image 1.62 software are shown below. Telomere peaks are indicated by asterisks.

rad11-D223Y were very different from those of the *S.cerevisiae rfa1-D228Y* mutant, even though the amino acid sequences are highly conserved between *S.pombe* Rad11 protein and *S.cerevisiae* Rfa1 protein (37% identity for the full-length proteins). The amount of Rfa1 protein in *rfa1-D228Y* cells is reduced ~2-fold compared with that in the wild-type cells (15,60). In contrast, the amount of Rad11-D223Y-Myc protein is almost the same as that of Rad11-Myc protein (data not shown). These differences might be the reason for the phenotypic differences between these two mutants. Indeed, the protein level of Rfa1 affects the recombination frequency and DNA repair ability in *S.cerevisiae* (15,60). However, the different γ-ray sensitivities of these two mutants cannot be explained by the different protein expression levels, because the *S.pombe rad11-D223Y* mutant is γ-ray sensitive but the protein level is not affected by the mutation. This fact suggests that the *rad11-D223Y* mutation affects the enzymatic activity of Rad11 protein rather than the protein expression level.

The different phenotypes in *S.pombe rad11-D223Y* cells and *S.cerevisiae rfa1-D228Y* cells emphasize the importance of the investigation of *S.pombe* RPA, even though extensive mutational analysis of the *S.cerevisiae rfa1* gene has been performed. In *S.pombe*, only one *rad11* mutant (*rad11A*) has been reported so far (41). Both *rad11A* cells and *rad11-D223Y* cells are UV and γ-ray sensitive. However, unlike *rad11A* cells, *rad11-D223Y* cells are not temperature sensitive (data not shown), indicating that these two mutations affect the function of the RPA complex in different ways. Although RPA is thought to be involved in DNA replication,

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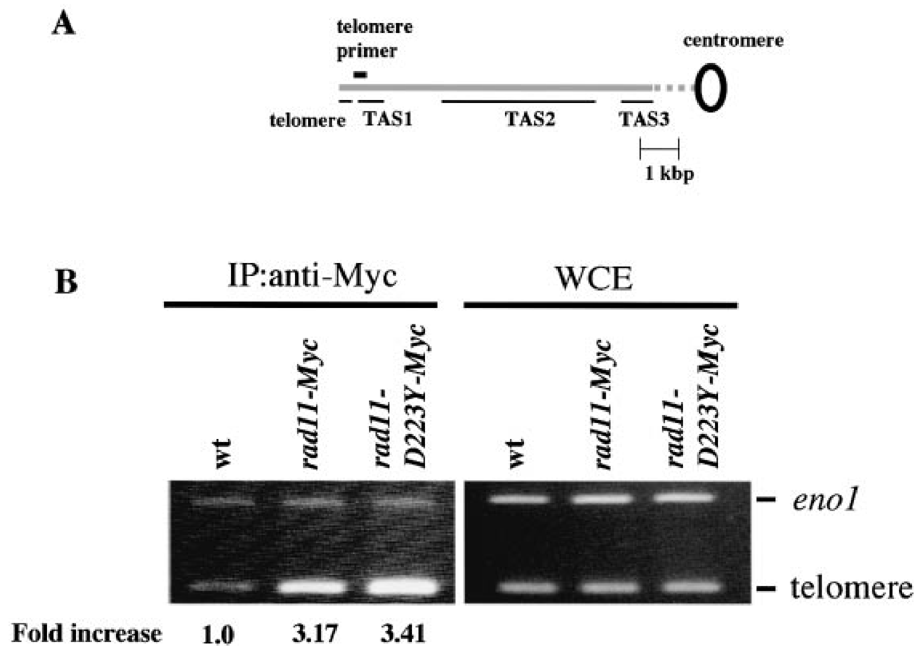

Figure 7. Rad11-Myc is bound to telomere DNA in the ChIP assay. (**A**) Schematic presentation of the location of the primer set used for the ChIP assay. The position of the primer set used for amplification of telomere DNA is shown above the long gray bar. The positions of telomere and telomere-associated sequences, TAS1, TAS2 and TAS3, are underlined. The chromosome arm is shown by the long gray bar. (**B**) The ChIP assay of rad11-Myc and rad11-D223Y-Myc. Untagged wild-type cells (JY746), rad11-Myc (YO004) cells and rad11-D223Y-Myc (YO005) cells were used. PCR was performed on whole-cell extracts (WCE) and on chromatin immunoprecipitates (IPs with anti-Myc) using primers to amplify telomere DNA (telomere) and primers to amplify DNA from the *eno1*$^+$ gene (*eno1*). The relative precipitated fold enrichment is shown underneath each lane. Ratios of telomere signals and *eno1*$^+$ signals were used to calculate relative precipitated fold enrichment.

recombination and repair, the exact roles of RPA in these aspects of DNA metabolism are not fully understood. Further investigation of the *rad11-D223Y* mutant and isolation of additional *rad11* mutants will provide useful information for elucidating the roles of RPA in DNA metabolism.

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