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Fission yeast Stn1 maintains stability of repetitive DNA at subtelomere and ribosomal DNA regions

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ABSTRACT

Telomere binding protein Stn1 forms the CST (Cdc13/CTC1-STN1-TEN1) complex in budding yeast and mammals. Likewise, fission yeast Stn1 and Ten1 form a complex indispensable for telomere protection. We have previously reported that *stn1-1*, a high-temperature sensitive mutant, rapidly loses telomere DNA at the restrictive temperature due to frequent failure of replication fork progression at telomeres and subtelomeres, both containing repetitive sequences. It is unclear, however, whether Stn1 is required for maintaining other repetitive DNAs such as ribosomal DNA. In this study, we have demonstrated that *stn1-1* cells, even when grown at the permissive temperature, exhibited dynamic rearrangements in the telomere-proximal regions of subtelomere and ribosomal DNA repeats. Furthermore, Rad52 and γH2A accumulation was observed at ribosomal DNA repeats in the *stn1-1* mutant. The phenotypes exhibited by the *stn1-1* allele were largely suppressed in the absence of Reb1, a replication fork barrier-forming protein, suggesting that Stn1 is involved in the maintenance of the arrested replication forks. Collectively, we propose that Stn1 maintains the stability of repetitive DNAs at subtelomeres and rDNA regions.

INTRODUCTION

Progression of DNA replication forks along the genome during S phase can be impeded by a number of programmed and unprogrammed events. Such replication obstacles include tightly associated non-histone DNA-binding proteins, collisions with transcription complexes, and stable DNA secondary structures (e.g. G-quadruplex) (reviewed in (1)). When replication forks stall and collapse due to such replication barriers, cells commonly attempt to resume DNA replication by homologous recombination (HR). The eukaryotic ribosomal RNA genes (rDNAs) comprise clusters of transcription/replication units, which have been well-characterized for frequent replication fork stalling. Repeat units of rDNA in two phylogenetically distant yeast species, budding and fission yeasts, contain replication origins and unidirectionally replication-blocking DNA elements termed replication fork barriers (RFBs), in addition to ribosomal RNA (rRNA)-encoding genes (2,3). In budding yeast, RFB-binding protein Fob1 prevents replication fork progression (4). Similarly, in fission yeast, DNA-binding proteins Reb1 and Sap1 confer fork barrier activity to polar RFBs (5,6). Arrested replication forks at these RFBs potentially induce DNA double-strand break (DSB) formation (7). The 'programmed' fork arrest and subsequent DSB formation provoke DNA damage repair responses, which lead to HR between rDNA repeats, thereby changing rDNA copy numbers (8,9). RFBs at rDNA repeats are thus considered to be key factors in rDNA copy number changes.

Replication fork progression is also impeded at termini of linear chromosomes, telomeres. Telomeres are specialized chromatin complexes consisting of tandemly repeated

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short telomeric DNA sequences (the consensus sequence in fission yeast is 5′-GGTTACA-3′, (10)) together with associated proteins. The most distal end of telomeric DNA is a single-stranded (ss) guanine-rich 3′ extension, referred to as the G-overhang, while the adjoining telomeric DNA is double-stranded (ds). The guanine-rich telomeric DNA repeats can form higher order DNA structures, and thus are potentially inhibitory to replication fork progression (reviewed in (11)). The G-overhang invades into the telomeric dsDNA, forming a loop structure known as a t-loop (12). In addition, G-rich telomeric repeats are prone to form G-quadruplex structures (13). The telomeric ssDNA is associated with the CST (Ctc1-Stn1-Ten1 in higher eukaryotes, Cdc13-Stn1-Ten1 in budding yeast) complex (14–17). In mammals and fission yeast, the ss and ds regions of telomeric DNAs are bridged by the shelterin complex (18,19).

In fission yeast, Stn1 and Ten1 homologues have been identified (20), but it is not known whether this organism has a Ctc1 homologue. Recently, we and others revealed that Stn1 in fission yeast plays a crucial role in DNA replication at telomeres and subtelomeres adjacent to the telomeric repeats (21,22). Subtelomeres contain repetitive sequences, which are refractory to DNA replication and likely induce rearrangements in fission yeast (23,24). Temperature-sensitive *stn1* mutants display replication failure at subtelomeres, resulting in immediate loss of telomeric DNA upon shifting to restrictive temperatures (21,22). Although the precise mechanism of how Stn1 facilitates telomeric DNA replication has not yet been clarified, accumulating evidence suggests that there is a functional relationship between Stn1 and DNA polymerase α (Pol α). Pol α contributes to lagging strand synthesis by forming a complex with DNA primase (25). Overexpression of Pol α components partially prevented the loss of telomeres in *stn1* temperature-sensitive mutant cells (22). Stn1 interacts with Pol α (26–28) and promotes primase activity in various species (28–31). Taken together, it has been proposed that Stn1 facilitates replication fork progression at telomeric regions by regulating the Pol α-primase complex (22). Considering the essential role of the Pol α-primase complex in general DNA replication throughout the genome, we asked whether fission yeast Stn1 promotes replication fork progression in other genomic regions apart from telomeres and subtelomeres. We were particularly intrigued by rDNA regions because they contain replication-inhibitory elements and consist of repetitive DNAs as in the case of subtelomeres and telomeres. Indeed, we previously observed a moderate reduction in the amount of replication intermediates at rDNA loci in a fission yeast temperature-sensitive *stn1* mutant, *stn1-1*, at the restrictive temperature (21). While it has been shown that human STN1 facilitates restart of stalled replication forks at GC-rich repetitive sequences under replication stress (32,33), it is unknown whether Stn1 prevents the rearrangement of arrays of large repeats, such as rDNA loci.

In this study, we found that the *stn1-1* mutant is sensitive to the replication-inhibiting drug hydroxyurea (HU) at non-restrictive temperatures. Interestingly, the HU sensitivity is suppressed by depletion of Reb1, an RFB protein in fission yeast, suggesting a role of Stn1 in rDNA replication. *stn1-1* mutant cells exhibit a hyper-recombination phenotype and accumulation of HR proteins at rDNA and telomere-proximal regions of subtelomeres, suggesting HR-mediated DNA repair at these loci. Consistent with this notion, the *stn1-1* mutant shows severe growth retardation in genetic backgrounds inactivated for the HR-mediated DNA repair pathway. Collectively, our results identify a previously unrecognized function of Stn1 in the stable inheritance of repetitive DNAs.

MATERIALS AND METHODS

Strains and media

Schizosaccharomyces pombe strains used in this study are listed in Supplementary Table S1. Media and basic techniques for yeast are described elsewhere (34). Cells growing in YES medium were used for all experiments in this paper. Hydroxyurea (Sigma-Aldrich) was included in YES as necessary.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described (35), with the following modifications. Briefly, cells were fixed with 1% formaldehyde for 25 min at room temperature. The fixation was quenched with 250 mM Glycine for 5 min, followed by three washes with TBS buffer (20 mM Tris–Cl pH 7.5, 150 mM NaCl). The washed cells were suspended in lysis buffer (50 mM HEPES–KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.1% sodium deoxycholate, Complete (Roche), 1 mM PMSF) and lysed with a Multi-beads shocker (YASUI KIKAI) with 0.5 mm zirconia balls (Nikkato). Chromatin was sheared by sonication using a Sonifier 250 (BRANSON) and the soluble fraction was recovered by centrifugation. Prior to immunoprecipitation, Dynabeads protein G (Thermo Fisher) were pre-incubated with antibodies. Antibodies used in this study are the following: myc, 9B11 (Cell Signaling Technology); phospho-H2A, Anti-Histone H2A (phospho S129) (Abcam). The soluble fraction was incubated with antibodies-Dynabeads protein G complexes for more than six hours at 4°C. After washing steps, the immunoprecipitates were eluted with TE buffer containing 1% SDS, incubated overnight at 65°C to reverse the crosslinks, and then treated with Proteinase K (Nacalai tesque) at 37°C. The coprecipitated DNA was recovered by phenol–chloroform extraction and RNase treatment followed by column purification using a QIAquick PCR purification kit (QIAGEN). The purified DNA was quantified by quantitative PCR (qPCR) using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Thermo Fisher). Primers used for qPCR are listed in Supplementary Table S2. The summary of statistical analysis is described in Supplementary Table S3.

Pulsed-field gel electrophoresis (PFGE)

Chromosomal DNA in agarose gel plugs was prepared as described previously (36) and digested with *Sfi*I (TOYOBO) or *Not*I (TOYOBO). The digested DNA was separated by PFGE with a CHEF-DR-III PFGE apparatus (BioRad) under the following conditions: 1% SEAKEM

Gold (LONZA) agarose gel in 0.5× TBE; electrode angle 120°; voltage gradient 6.8 V/cm; initiating switching time 40 s; final switching time 80 s; run time 15 h; temperature 10°C. The separated DNA was transferred onto Amersham Hybond-N + nylon membranes (GE Healthcare) under alkaline conditions and analyzed by Southern hybridization as described below.

Southern hybridization

Schizosaccharomyces pombe genomic DNA was obtained using the glass bead-phenol chloroform method (37) and digested with *Apa*I (Takara). Digested DNA was separated on a 1% agarose gel in 0.5× TBE buffer and transferred onto Amersham Hybond-XL nylon membranes (GE Healthcare) under alkaline conditions. Membranes were hybridized with probes labelled with ^{32}P-dCTP (Perkin-Elmer) by a Random Primed DNA labeling kit (Roche) or DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche). For TAS1 detection, an *Apa*I-*Eco*RI fragment excised from pNSU70 was used as a probe (38). For rDNA detection, a 3-kb *Hin*dIII-*Kpn*I fragment for the radioisotope labeling or a PCR product amplified with TM693 and TM694 primers for the DIG labeling within the rDNA repeat were used as a probe. For Supplementary Figure S1B, I, L, M and C probes were prepared and used as in a previous study (19). The membranes were exposed to an imaging plate (FUJIFILM), and signals were detected with a FLA 7000 system (FUJIFILM).

rDNA copy number analysis

Genomic DNA was obtained using the glass bead-phenol chloroform method (37). Genomic DNA was quantified by a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Thermo Fisher). Primers used for qPCR are listed in Supplementary Table S2. The summary of statistical analysis is described in Supplementary Table S3.

Yeast two-hybrid assay

Saccharomyces cerevisiae Y190 strain (*MATa ura3-52 his3-D200 lys2-801 ade2-101 trp1-901 leu2-3, 112gal4*Δ *gal80*Δ *LYS2::GAL-HIS3 URA3::GAL-lacZ cyh*^{r}) was transformed with Y2H plasmids expressing fusion proteins. The full-length Taz1 coding sequence was cloned into pGBKT7 (Gal4 DNA binding domain vector) (Clontech) and Reb1 (K54 to stop codon) was cloned into pGAD-GH. A β-gal assay was performed according to the manufacturers' instructions.

RESULTS

Temperature-sensitive *stn1-1* mutant is sensitive to hydroxyurea at a non-restrictive temperature

We previously isolated a high-temperature sensitive mutant allele of the *stn1*$^+$ gene termed *stn1-1*, which produces a Stn1 protein with two amino acid substitutions, I177M and M180I (21). 2D-gel analysis of replicative intermediate DNAs demonstrated that *stn1-1* mutant cells are defective in replication at subtelomeres when cultured at 36°C, the restrictive temperature. It was observed that approximately 30-kb subtelomeric DNAs were lost in a *stn1-1* mutant strain when cultured at 36°C (21). In analyzing the *stn1-1* mutant further at 30°C, a lower temperature than 36°C, we found that the *stn1-1* mutant grows normally in the control media but is sensitive to hydroxyurea (HU), a chemical that retards replication fork progression by depleting dNTP pools (39) (Figure 1A), suggesting that DNA replication is compromised in the *stn1-1* mutant even at a non-restrictive temperature, which is consistent with previous reports using a different *stn1* mutant (22).

Because Stn1 is localized at telomeric repeats and is required for telomere DNA replication and telomere protection (20–22), we asked whether the HU sensitivity of *stn1-1* was due to defective replication at telomeric repeat DNAs. It is known in fission yeast that the chromosomes undergo self-circularization when telomeric repeat DNAs are lost due to inactivation of telomerase (40,41). Importantly, those circular chromosomes lack any telomere repeat DNAs (42); accordingly, we tested whether the *stn1-1* HU-sensitive phenotype still manifested in a strain already lacking telomere repeats. We generated circular chromosome-containing cells by deleting *trt1*$^+$ which encodes the catalytic subunit of telomerase, and confirmed that they contained circular chromosomes devoid of any telomeric DNAs using telomere Southern analysis and pulsed-field gel electrophoresis (PFGE) (Supplementary Figure S1A and B). We next replaced endogenous *stn1*$^+$ with *stn1-1-Flag* in the *trt1*Δ clone to obtain *stn1-1-Flag trt1*Δ (*stn1-1* with a Flag tag sequence at its C terminus). We observed that *trt1*Δ cells containing circular chromosomes and the wild-type *stn1*$^+$ gene (Figure 1B, *trt1*Δ) showed higher HU sensitivity compared to wild-type *stn1*$^+$ cells with linear chromosomes at 30°C (Figure 1B, WT). The result is consistent with a previous study showing that cells harboring circular chromosomes are sensitive to replication-inhibitory drugs, such as MMS and HU (36,43). Importantly, *stn1-1 trt1*Δ cells maintained circular chromosomes (Supplementary Figure S1), and were more sensitive to HU than the *stn1*$^+$ *trt1*Δ cells (Figure 1B, *stn1-1 trt1*Δ and *trt1*Δ, respectively).

Next, to further verify the HU-hypersensitivity induced by Stn1 dysfunction, we disrupted the *stn1*$^+$ gene in the *trt1*Δ background, because the *stn1*$^+$ gene is essential for cell growth and can be deleted only in cells maintaining circular chromosomes (20). We generated two independent *stn1*Δ *trt1*Δ clones and confirmed chromosomal circularization (Supplementary Figure S1C). Both clones showed increased HU sensitivity compared to *trt1*Δ cells (Figure 1C). We further tested HU sensitivity of seven single colonies derived from each clone, and found that all the clone A-derived colonies were slightly more sensitive to HU than those derived from clone B (Supplementary Figure S2A), which is also noticeable in Figure 1C, suggesting that HU sensitivity can vary between the *stn1*Δ *trt1*Δ clones, while this phenotype may be stably maintained through several generations (see Discussion for more details). Taken together, Stn1 is required for survival under replication stress even in telomere-less strains.

In addition to telomeric repeats, Stn1 is also crucial for replication at subtelomeres (21,22). Next we asked whether

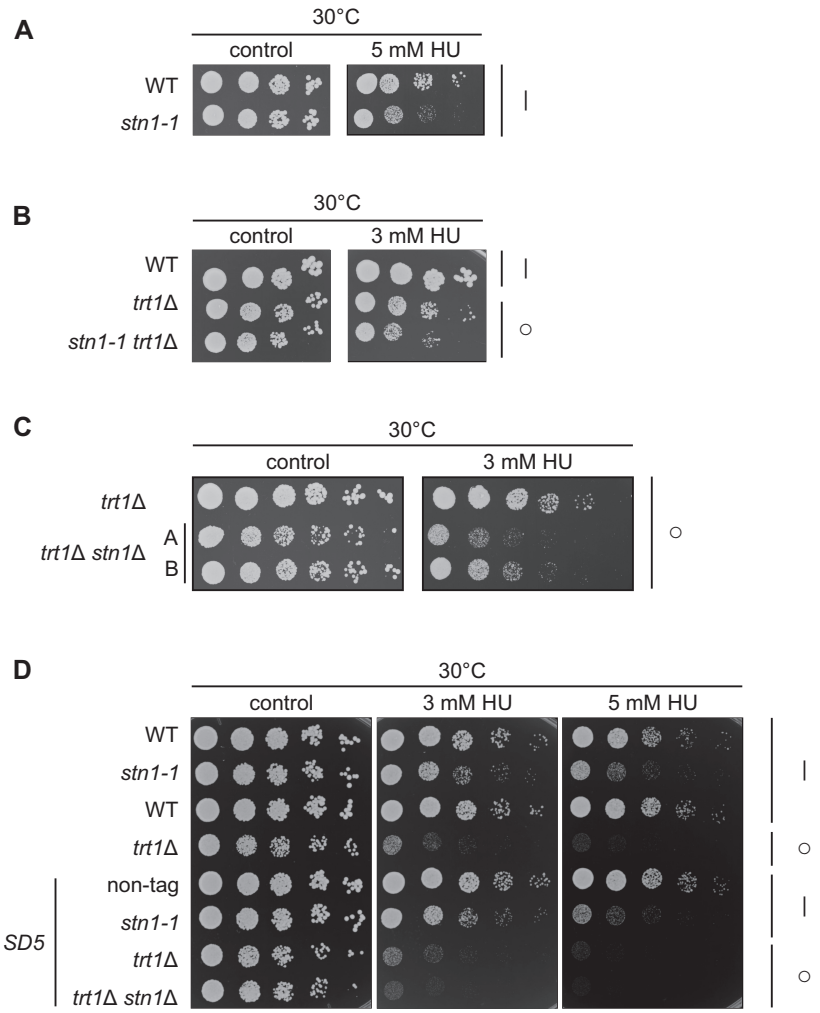

Figure 1. *stn1-1* is sensitive to replication stress. (**A**) Sensitivity to HU of wild-type and *stn1-1*. Ten-fold serial dilutions of the indicated strains were spotted onto YES plates containing 5 mM HU or without HU (control), and incubated for 3 days at 30°C. (**B**) Sensitivity to HU of wild-type, *trt1*Δ and *trt1*Δ *stn1-1*. Ten-fold serial dilutions of the indicated strains were spotted onto YES plates containing 3 mM HU or no HU (control), respectively, and incubated for 3 days at 30°C. (**C**) Sensitivity to HU of *stn1*Δ. Five-fold serial dilutions of the cells were spotted onto YES plates containing 3 mM HU, or no HU (control), and incubated for 4 days at 30°C. For *trt1*Δ *stn1*Δ, two independent clones (A and B) were examined. (**D**) Sensitivity to HU of strains that lack subtelomeres (*SD5* background). Five-fold serial dilutions of the cells were spotted onto YES plates containing 3 mM HU, 5 mM HU or no HU (control), and incubated for 4 days at 30°C. Lines or circles shown at the right side of each panel indicate cells harboring linear chromosomes or circular chromosomes.

the HU-hypersensitivity of *stn1-1* was caused by defective replication at subtelomeres. Fission yeast contains three chromosomes, hence six telomeres. Subtelomeres in Chromosomes I and II, ∼100 kb regions adjoining the telomeric repeats, consist of repetitive sequences shared by the four respective subtelomeres (24,44). Chromosome III is unique in that long arrays of rDNA repeats are closely located in the vicinity of both telomeres, whereas chromosomes I and II do not have rDNA repeats. In this paper, subtelomeres refer to the non-rDNA sequences adjacent to telomere repeats in Chromosome I and II, and not to rDNA arrays. It is known that subtelomere structures vary in different strains; strains may contain subtelomere sequences between telomere repeats and an rDNA array at none, one, or both termini of chromosome III (24,44). Accordingly, different strains contain four to six subtelomere regions in total. In a strain called JP1225, where the subtelomere structures have been extensively analyzed, the four subtelomeres derived from chromosomes I and II, and one subtelomere at the left arm of chromosome III consist of regions called *SH* (subtelomeric homologous) sequences, ∼15–60 kb in length, immediately adjacent to the telomere repeats (44) (Supplementary Figure S2D). The *SH* sequences characteristically consist of multiple repeat sequences shared by multiple subtelomeres with high sequence identities (>98%) (24,44). A strain referred to as *SD5* (*SH* deletion five) has been constructed by deleting all five of the *SH* regions in JP1225. Since the *SD5* strain was deleted for the 30-kb subtelomere DNAs, it allowed us to investigate whether the HU-sensitivity of *stn1* mutants was caused by a failure of replication at the *SH* regions of subtelomeres. We replaced endogenous *stn1*$^{+}$ with *stn1-1-Flag* in the *SD5* strains to obtain *stn1-1-Flag SD5*. The sensitivity to HU was higher in *stn1-1-Flag SD5* than *SD5* (Figure 1D). Furthermore, starting with *SD5 trt1*Δ,

which was obtained by deleting *trt1*$^+$ in *SD5* and shown to harbor circular chromosomes (44), we disrupted *stn1*$^+$ to produce *SD5 trt1*Δ *stn1*Δ with circular chromosomes (Supplementary Figure S1C). Interestingly, *SD5 trt1*Δ cells became more sensitive to HU upon deletion of *stn1*$^+$ (Figure 1D). These results suggested that the HU-hypersensitivity associated with Stn1 dysfunction is partly due to defective replication outside of telomeres and *SH* regions of subtelomeres.

Stn1 supports rDNA repeat stability

We previously detected a mild reduction in the amount of replication intermediate DNA at rDNA loci in *stn1-1* cells at 36°C compared to 25°C (21), suggesting that rDNA replication is impaired in the *stn1-1* strain at the restrictive temperature. This previous finding together with the current data (Figure 1B–D) suggest that rDNA is a candidate for the aberrant DNA replication in the *stn1-1* in the presence of HU at the non-restrictive temperature. Fission yeast possesses approximately 100–150 copies of a 10.9-kb rDNA repeat unit, which is tandemly repeated at both ends of chromosome III (45,46). Each unit includes the three *Ter* elements that function as RFBs (5,47). Interestingly, upon disruption of *reb1*$^+$, which is required for two out of the three *Ter*-mediated RFB activities at rDNA (5,47), the HU sensitivity of *stn1-1* at 30°C was partially but reproducibly suppressed (Figure 2A). This result suggests that the RFBs at rDNA are responsible, at least partly, for the colony growth retardation of *stn1-1* in the presence of HU, and that inactivation of the RFB activities restores robust rDNA replication. Taken together, these results are indicative of the role of Stn1 in proper replication of the rDNA repeats.

Given that Stn1 physically interacts with Pol α (26–28), we hypothesized that the association of the Pol α-primase complex with chromatin at rDNA is affected by the *stn1-1* mutant. We analyzed the association of Pol1, a catalytic subunit of Pol α, in *stn1-1* by ChIP. It is known that the replication fork pauses at RFBs and collides with the transcription machinery at RFP4 in rDNA repeats (47). We observed that Pol α association was reduced at rDNA in *stn1-1* cells, but not at *gal1*$^+$ and non-origin loci (within the ORF of *tas3*$^+$ gene on chromosome II (48)), where no replication fork block has been reported (Figure 2B and Supplementary Figure S3B). Thus, the association of Pol α was specifically impaired at rDNA in *stn1-1* cells.

Dissociation of the replisome has been proposed to induce resection of DSBs at RFBs, resulting in Rad52-mediated rearrangement of rDNA repeats, since they comprise a large number of tandem repeats (8,9). Given our observation that Stn1 is implicated in the replication of rDNA, we hypothesized that the instability of rDNA repeats would be further aggravated in the *stn1-1* mutant due to the increased replication failure at the rDNA arrays. To test this, we first measured the length of rDNA regions as a proxy for rDNA repeat integrity. We isolated several single clones of *stn1*$^+$*-Flag* (the endogenous *stn1*$^+$ gene was tagged with a C-terminal Flag tag sequence) and *stn1-1-Flag* (the endogenous *stn1*$^+$ gene was replaced by *stn1-1-Flag*) strains. Both *stn1*$^+$*-Flag* and *stn1-1-Flag* strains grow normally as well as wild-type at 25°C (21). Individual clones were allowed to grow continuously at 25°C for ten days by subculturing each day so that they underwent many rounds of S phase. After that, genomic DNA of these cells was isolated and treated with *Sfi*I endonuclease, which cleaves outside of the respective rDNA arrays located at the ends of chromosome III, thus generating two large DNA fragments encompassing the two rDNA arrays (Figure 2C, schematic diagram). The digested DNA was then analyzed by PFGE followed by Southern blotting using an rDNA fragment as a probe. Typically, two characteristic rDNA repeat-specific *Sfi*I fragments were observed in wild-type and *stn1*$^+$*-Flag* (denoted as A and B, originating from the left and right arm of chromosome III, respectively, in Figure 2C schematic diagram; (36,49)). In the wild-type strain and two independent clones of *stn1*$^+$*-Flag* strains, fragments A and B were approximately 700–900 and 300–400 kb, respectively, at Day 1 of the culture. The two fragments were recognizable as distinct bands as shown in signal distributions, although the sizes were moderately changed after ten days of continuous culture (Figure 2C). In three independently isolated clones of *stn1-1-Flag* strains, fragments A and B also appeared as two distinct bands at Day 1. At Day 10, however, highly smeared signals for both fragments were detected, and quantification revealed an overall increase in the fragment sizes (Figure 2C). In contrast, these *Sfi*I fragments were relatively stable in *stn1*$^+$*-Flag* strains, both in terms of size and signal distributions. Consistent with the PFGE-Southern blotting analysis, the overall rDNA copy numbers in *stn1-1-Flag* strains estimated by quantitative PCR increased after the prolonged culture, but remained constant in *stn1*$^+$*-Flag* strains (Figure 2D). These results indicate that the copy number of the rDNA repeat is unstable in *stn1-1* cells even at 25°C. We concluded that Stn1 is required for stable maintenance of rDNA copy number during cell divisions.

It is known that rDNA repeat instability is induced by RFB activity in budding yeast (8). Given that a *reb1*$^+$ deletion partially suppressed the reduced viability of *stn1-1* cells in the presence of HU (Figure 2A), we predicted that the rDNA repeat instability in *stn1-1* would also be suppressed by *reb1*$^+$ deletion. Indeed, the increase in the copy number of rDNA repeats in continuously cultured *stn1-1* cells was not observed in *stn1-1* cells lacking *reb1*$^+$ (Figure 2D). Consistently, we also confirmed by Southern blotting that the smeared signals observed in *stn1-1* cells remained as two distinct bands by disruption of *reb1*$^+$, suggesting that rearrangement of rDNA in *stn1-1* cells is partially dependent on Reb1 (Supplementary Figure S4A). This result supports the idea that aberrant amplification of rDNA repeats in the *stn1-1* strain is due to the replication fork barrier activity.

Stn1 suppresses recombination at rDNA repeats

Our observations that Stn1 is required for proper replication and repeat stability at rDNA arrays [(21); this study (Figure 2)] led to the notion that compromised replication of rDNA in *stn1-1* cells causes repetitive DNA instability through frequent HR among rDNA repeats. To test this possibility, we carried out a chromatin immunoprecipitation (ChIP) assay based on Rad52, which is recruited to chromatin during the early steps of the recombination

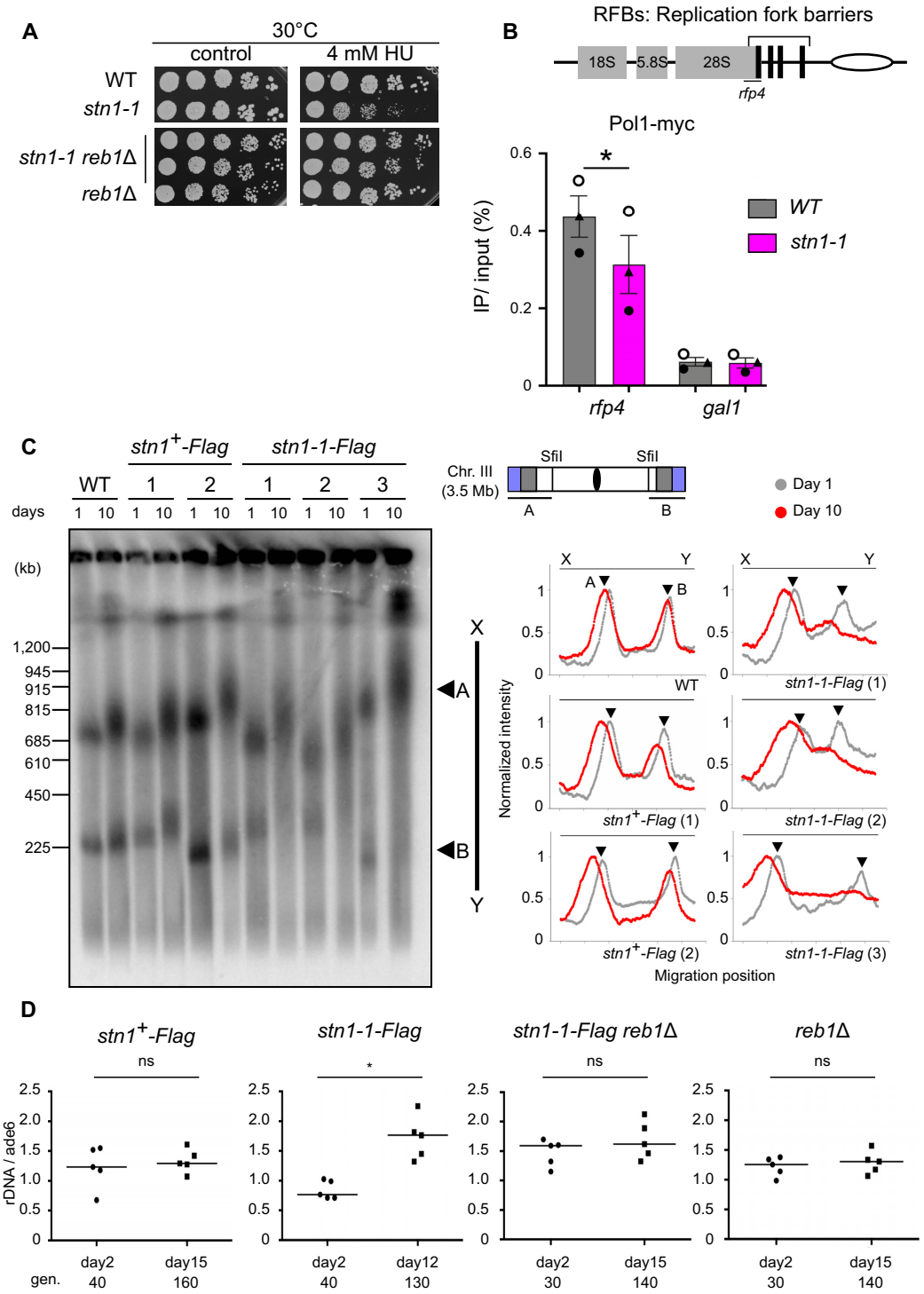

Figure 2. Rearrangements at rDNA and subtelomeres are incurred in *stn1-1*. (**A**) Sensitivity to HU of wild-type, *reb1*Δ, and *stn1-1 reb1*Δ. Five-fold serial dilutions of the indicated strains were spotted onto YES plates containing 4 mM HU or no HU (control), and incubated for 4 days at 30°C. Two independent clones of *stn1-1 reb1*Δ were examined. These separated images (top and bottom) were taken from a single photo of the respective plate. (**B**) Pol1-myc localization at *rfp4* and *gal1*$^+$ loci tested by ChIP. *gal1*$^+$ was used as a control locus. Each symbol indicates independent experiments. Error bar indicates SEM ($n = 3$). *P*-value was calculated by a paired two-tailed Student's *t*-test (*$P < 0.05$). Three biological replicates were tested. (**C**) Instability of rDNA repeats in prolonged cultures of *stn1-1*. Independent clones were cultured in liquid YES for the indicated days at 25°C, and then genomic DNA was digested by *Sfi*I endonuclease and analyzed by PFGE followed by Southern hybridization with a radiolabelled rDNA probe (left panel). The position of *Sfi*I sites, telomeric repeats (blue boxes), rDNA repeats (shaded boxes) and centromeres (oval) are shown at the top right schematic diagram. Signal intensity was quantified along each lane (the quantified range is indicated by a line at the right side of the gel) and normalized to the maximum value for each lane. The density plot for every clone is shown at the right side. Gray and red colors represent Day1 and Day10, respectively. (**D**) The copy number of rDNA repeats relative to that of the *ade6*$^+$ gene was determined by qPCR. Each dot represents the result from an individual colony ($n = 5$). Horizontal lines indicate the median value. A Mann–Whitney test was used for statistical analysis (*$P < 0.05$; ns, not significance).

process (50). We constructed strains expressing myc-tagged Rad52 from its endogenous locus, and tested the association of Rad52 with telomeric ends, as well as two loci that are present in every rDNA repeat: *ars3001*, a unique early-replicating origin of DNA replication within rDNA repeats, which is located adjacent to RFBs; and *rfp4*, the fourth replication fork barrier (Figure 3A). To effectively observe the consequence of replication fork arrest at the early origin, we arrested the cell cycle with a high concentration of HU (12 mM). Consistent with a previous report (21), we observed greater accumulation of Rad52 at the loci adjacent to telomeric repeats in *stn1-1* than in wild-type when cells were cultured at 25°C, in the absence or presence of HU, as indicated by calculating the relative enrichment of ChIP signals at the tested loci compared to a control locus, *his1*$^+$ (Figure 3B). Notably, the accumulation of Rad52 at *ars3001* was significantly enhanced in *stn1-1* compared to wild-type, even in the absence of HU. This observation was not limited to *ars3001*, since a similar Rad52 accumulation was observed at another region of rDNA, *rfp4*. In the presence of HU, although Rad52 accumulated particularly at *ars3001* even in wild-type, a further accumulation of Rad52 was observed in *stn1-1*. These data suggested that Stn1 suppresses HR-mediated repair at rDNA. We also examined phosphorylated serine 129 of histone H2A, termed γH2A, which is formed at DSB sites by ATM/ATR kinases (51,52). Similar to Rad52, even in the absence of HU, a significantly elevated accumulation of γH2A was observed at rDNA (*ars3001* and *rfp4*) in the *stn1-1* cells at 25°C, compared to wild-type (Figure 3C). In the presence of HU, while γH2A accumulated at *ars3001* in wild-type, consistent with a previous report (53), further accumulation of γH2A was observed in *stn1-1* (Figure 3C). We confirmed that such accumulation of γH2A was not observed at a negative-control locus (Supplementary Figure S5). These data provide evidence for increased DSB induction at rDNA in *stn1-1* cells. Because DSBs at rDNA are induced by RFBs in budding yeast (7), it is likely that the frequent HR and DSB induction at rDNA in *stn1-1* is due to RFBs. Consistently, accumulation of γH2A at rDNA in *stn1-1* cells was suppressed by disruption of the RFB factor *reb1*$^+$ (Supplementary Figure S4B). These results are in accordance with our hypothesis that compromised replication at rDNA in *stn1-1* cells, particularly due to RFB activities, aberrantly activates the HR repair pathway, leading to the repeat DNA instability at these loci.

HR repair is crucial for *stn1-1* mutant cell viability

Our data indicative of frequent Rad52 accumulation at rDNA in the *stn1-1* strain (Figure 3B) led us to examine whether HR is required for sustained viability of the *stn1-1* mutant cells. We found that the *stn1-1 rad51*Δ double mutant displayed a severe synthetic growth defect even in the absence of HU at two permissive temperatures, 25 and 30°C (Figure 4A). We also examined the *mus81*$^+$ gene, which encodes a Holliday-junction resolvase that is involved in the recombinogenic repair of stalled replication forks (54). It was reported that the stability of the rDNA repeat number requires Mus81 in budding yeast (55). Similar to the case of the *rad51*$^+$ gene deletion, cell growth was synthetically retarded when both the *stn1-1* mutation and the *mus81*$^+$ deletion were introduced (Figure 4B). These results indicate that the *stn1-1* cells are unable to efficiently process replication-associated DNA damage without the functional HR repair machinery.

Given that replication in *stn1-1* is compromised at rDNA and subtelomeres, both of which consist of repetitive sequences, and that HR plays a vital role in maintaining growth of the *stn1-1* mutant, we asked whether replication forks are unstable at these repetitive DNAs in *stn1-1* cells. To investigate the effect of impaired replication fork stability in *stn1-1*, we disrupted *swi1*$^+$, encoding a subunit of the fork protection complex (FPC), which associates and translocates together with the replisome on chromatin during replication to stabilize replication forks (56,57). It is known that DNA damage at rDNA and subtelomeres is provoked by *swi1*Δ, and that the replication fork stalling at rDNA caused by HU treatment is enhanced in *swi1*Δ cells (57,58). Indeed, we found that the *stn1-1 swi1*Δ double mutant showed synthetic growth defects, and that these growth defects were greatly pronounced in the presence of HU (Figure 4C). This result suggests that, consistent with our hypothesis, replication fork progression is impaired and HR-mediated repair is activated in *stn1-1* cells.

Subtelomeric instability caused by *stn1-1* is rescued by *reb1*Δ

Fission yeast subtelomeres also contain repetitive DNA sequence elements that frequently undergo rearrangements (24,44). We examined the stability of repetitive DNA at telomere-proximal regions of subtelomeres in *stn1-1* cells by Southern blot analysis using a TAS1 (telomere-associated sequences) probe, which recognizes sequences located ∼1 kb-centromeric from the most distal ends of chromosomes (38,41). We detected aberrant TAS1 signals forming a ladder in the high-molecular weight range in the *stn1-1* strain (shown by an asterisk in Figure 5A and Supplementary Figure S6), suggesting amplification of the repeat units due to hyper-recombination in *stn1-1*. Remarkably, the ladder signals became significantly fainter upon deleting *reb1*$^+$ (Figure 5A), indicating that the subtelomeric recombination is suppressed by *reb1*Δ. Because localization of Reb1 at subtelomeres by direct DNA binding has not been demonstrated in fission yeast, we examined the possibility of protein-mediated localization mechanisms. Indeed, as shown in Figure 5B, we found that Reb1 interacted with Taz1, a DNA-binding component of the telomeric shelterin complex (59). Considering the well-established fact that Taz1 localization spreads towards subtelomeres (60), the interaction between Reb1 and Taz1 could account for the function of Reb1 at subtelomeres. Intriguingly, γH2A accumulation at subtelomeric regions in the *stn1-1* strain was reduced in *stn1-1 reb1*Δ cells (Figure 5C). Taken together, our results support a model in which Stn1 is crucial for maintaining the integrity of repetitive sequences with an intrinsic condition of hard-to-replicate conferred by Reb1 (Supplementary Figure S7).

DISCUSSION

In this study, we have demonstrated that Stn1 maintains the stability of repetitive genomic regions, telomere-proximal regions of subtelomeres and rDNA regions. Cells

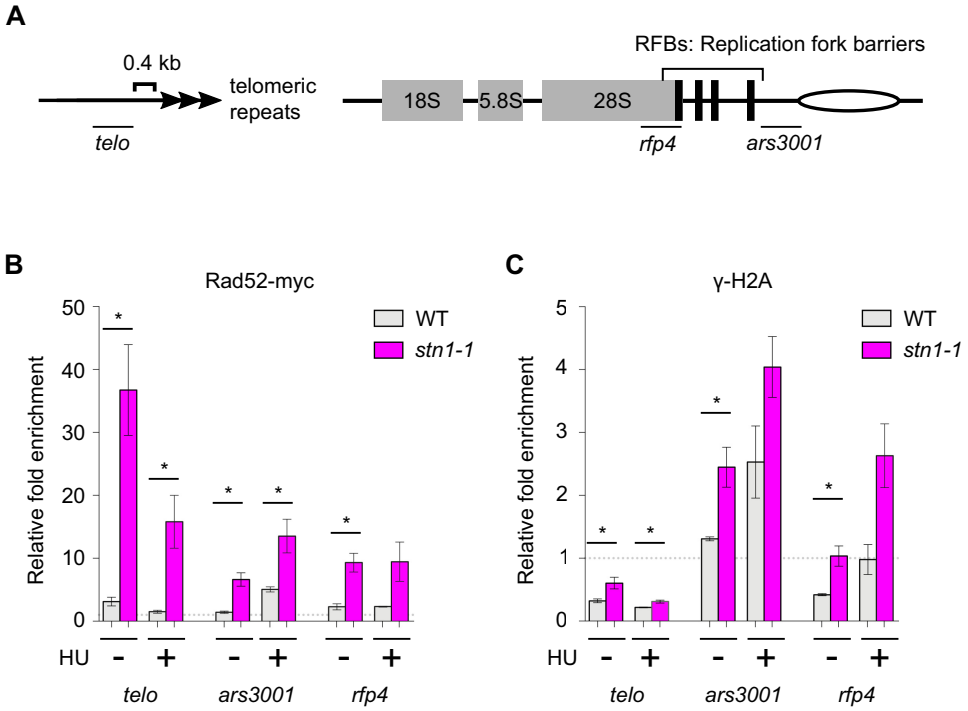

Figure 3. DNA damage markers accumulate at rDNA in *stn1-1* cells. (**A**) Location of primers used for qPCR. (**B** and **C**) Rad52-myc and γH2A localization at telomeres and rDNA tested by ChIP. The indicated strains were cultured in YES liquid medium with or without 12 mM HU for 4 h at 25°C. Enrichments relative to the signal at the *his1*$^+$ locus are shown. Error bars indicate the SEM ($n = 3$). *P*-value was calculated by a ratio paired two-tailed Student's *t*-test (*, $P < 0.05$). Three biological replicates were tested.

possessing a temperature-sensitive mutant allele of *stn1-1* were vulnerable to HU-induced replication stress. Interestingly, the sensitivity was partially rescued by reducing the RFB activity at rDNA arrays by deleting *reb1*$^+$ (Figure 2). We propose that fission yeast Stn1 is required for completion of DNA replication in hard-to-replicate regions, particularly those comprising repetitive sequences, and protects their integrity. Interestingly, it was reported that pathogenic mutations in human CTC1, which forms the trimeric CST complex together with STN1 and TEN1, lead to spontaneous chromosome breaks at fragile sites, typical of hard-to-replicate regions (61,62). Although our current work focuses on rDNA and subtelomeres, we do not exclude the possibility that Stn1 functions at other loci where DNA replication forks are prone to be arrested, as has been suggested for the mammalian CST complex (32,33).

The alteration of chromosome configuration induced by dysfunctional Stn1

To deepen our understanding of the function of Stn1 at non-telomeric regions, we generated *trt1*Δ *stn1*Δ strains and examined their chromosomal structures (Figure 1C, Supplementary Figure S2A and B). Electrophoretically mobile *Sfi*I-digested rDNA-containing fragments were hardly detected with *trt1*Δ cells probably due to complex DNA structures such as replication or recombination intermediates, consistent with the previous observation (43,44). In contrast, large fragments resolved in the PFGE gel were detected by the rDNA probes in almost all of the *trt1*Δ *stn1*Δ strains. This result suggests that further alterations of chromosome configuration can be induced at rDNA loci by disruption of *stn1*$^+$ in the *trt1*Δ background. It is also possible that dysfunction of *stn1*$^+$ may reduce such aberrant DNA structures accumulated at rDNA loci, probably through frequent DNA breaks at arrested replication forks, thereby allowing the DNA fragments from *trt1*Δ *stn1*Δ cells to enter the gel. The accumulation of Rad52 and DSB marker γH2A at rDNA in the *stn1-1* mutant strain would support this idea (Figure 3).

Two independent isolates of the *trt1*Δ *stn1*Δ strain (clone A and B) showed a slight difference in sensitivity to HU (Figure 1C and Supplementary Figure S2A). Although the correlation between the HU sensitivity and the patterns of rDNA rearrangements stated above were not apparent, we found that the total number of rDNA copies in clone A was slightly lower than in clone B, while the subtelomeric DNA copy numbers were almost identical to each other (Figure 1C and Supplementary Figure S2C). This might be explained by a previous study where budding yeast cells with a low rDNA copy number were more sensitive to DNA damaging agents than those with a high copy number (63).

The recombinational repair and DNA damage checkpoint in *stn1-1*

Regulation of rDNA copy number generally involves DNA repair of broken replication forks at the respective locus. For example, naturally occurring fork breaks at RFBs are thought to be repaired by an MRX-dependent but HR-independent pathway that maintains a constant copy number (9). Repeat amplification observed in an *asf1*Δ

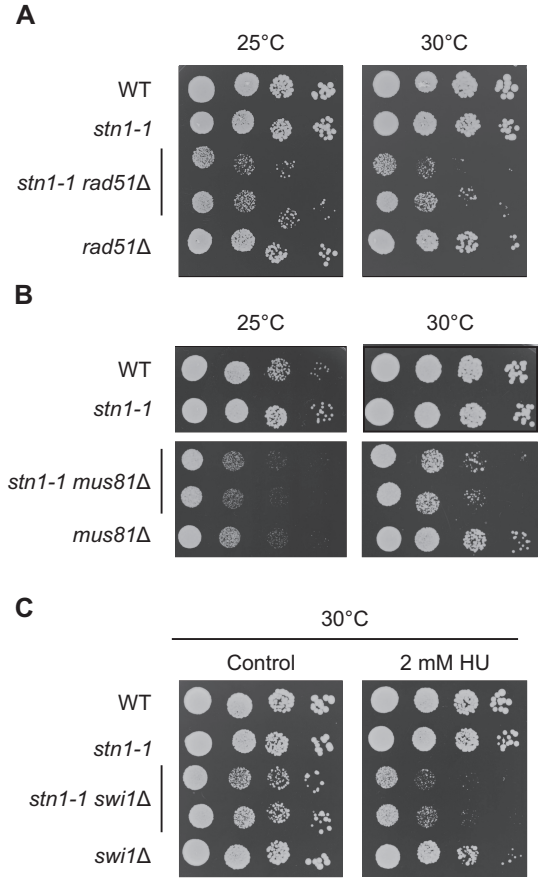

Figure 4. Homologous recombination contributes to survival of *stn1-1*. Growth retardation of the *stn1-1* mutant cells with deletion of HR-related genes: *rad51*Δ (**A**), *mus81*Δ (**B**) or *swi1*Δ (**C**). Ten-fold serial dilutions were spotted onto YES plates and incubated for 3–5 days at the indicated temperatures. In(B), these separated images (top and bottom) were taken from a single photo of the respective plate. In (C), WT, *stn1-1*, and the respective *swi1*Δ mutant cells were also spotted onto YES plates containing 2 mM HU.

background is also shown to be independent of HR and RFB proteins (64). These mechanisms, however, cannot fully explain the RFB (Reb1)-dependent repeat amplification observed in the *stn1-1* strain. Instead, we prefer a mechanism called unequal sister chromatid recombination (USCR), where a broken replication fork is repaired using the other replicating sister chromatid as a template for recombination (65). Although the USCR can result in both expansion and contraction of the repeats, our data indicated an overall increase in the rDNA copy numbers in *stn1-1* cells. This might be explained by a slight fitness advantage for higher rDNA copy numbers, since ribosomes are crucial for biomass growth (66).

Remarkably, the *stn1-1* cells grew normally at the permissive temperature, despite the accumulation of the DNA damage response factors, suggesting that the DNA damage checkpoint is not activated, consistent with our previous observation (21). One possible explanation for this is that the amount of γH2A is below the threshold to trigger the downstream signaling. Alternatively, cells may have evolved specific mechanisms to suppress the checkpoint activation at rDNA, where DSBs are regularly generated in each cell cycle. Indeed, Sir2-dependent heterochromatinization of the rDNA locus has been suggested to be one such mechanism in budding yeast (67).

The situation at subtelomeres/telomeres is similar but not identical to the case of rDNA loci. The amount of γH2A at telomeres was less than that at a non-telomeric control locus, which led us to be skeptical about the idea that the accumulated γH2A contributed to the checkpoint activation. In contrast, we observed significant enrichment of Rad52 at telomeres, which is consistent with our previous report (21). It is also worth mentioning that the extended telomeric tracts in *stn1-1* cells might provide more binding sites for Rad52 (Supplementary Figure S1). A number of studies have suggested that telomeres can escape detection by the checkpoint machinery due to the specialized nucleosome composition and telomeric chromatin that inhibit recruitment of the checkpoint mediator proteins (53,68,69). Collectively, our results suggest that the recombinogenic DNA ends generated in *stn1-1* cells do not elicit the checkpoint activation.

Regulation of the replisome by Stn1

How does Stn1 facilitate DNA replication at hard-to-replicate regions? One possible mechanism is that Stn1 directly facilitates the association of the replisome with chromatin via the interaction between Stn1 and the Pol α-primase complex (26–28). Recently, it has been shown that mammalian STN1 interacts with AND-1/CTF4, which acts as a hub connecting Pol α and the replicative DNA helicase in the replisome (70). Interestingly, deletion of *CTF4* in budding yeast leads to resected DSBs at an RFB in rDNA (9). The resultant ssDNA initiates HR and eventually amplifies rDNA repeats. Since the disruption of *CTF4* leads to the dissociation of the replisome from chromatin (71), the replisome has been proposed to prevent resection of DSBs at RFBs (9). Similar to budding yeast *ctf4*Δ cells, fission yeast *stn1-1* cells showed a decreased occupancy of Pol α at rDNA (Figure 2B). Thus, it is possible that impaired function of the Pol α-primase complex in *stn1-1* cells induces the resected DSB-mediated recombination of rDNA repeats (Supplementary Figure S7). Recently, it has been shown that lack of Ssu72 phosphatase abolished the interaction between Pol α and Stn1 and induced the loss of the replication fork progression at rDNA (27), which supports our hypothesis that Stn1 facilitates replication at rDNA via regulating Pol α. Future study is necessary to understand the exact functions of Stn1 in replication.

Interplay between Stn1 and Reb1

We have shown that the *stn1-1* allele renders cells prone to generate recombinogenic DNA ends at rDNA and subtelomeres. Strikingly, the phenotype at both loci was suppressed by the deletion of *reb1*$^+$ (Figures 2, 5 and Supplementary Figure S4). One possible interpretation is that replication fork stalling at these repetitive regions is dependent on Reb1, and Stn1 is required to prevent the forks from collapse. This is particularly plausible for rDNA, as the role of Reb1 in the programmed replication fork arrest through

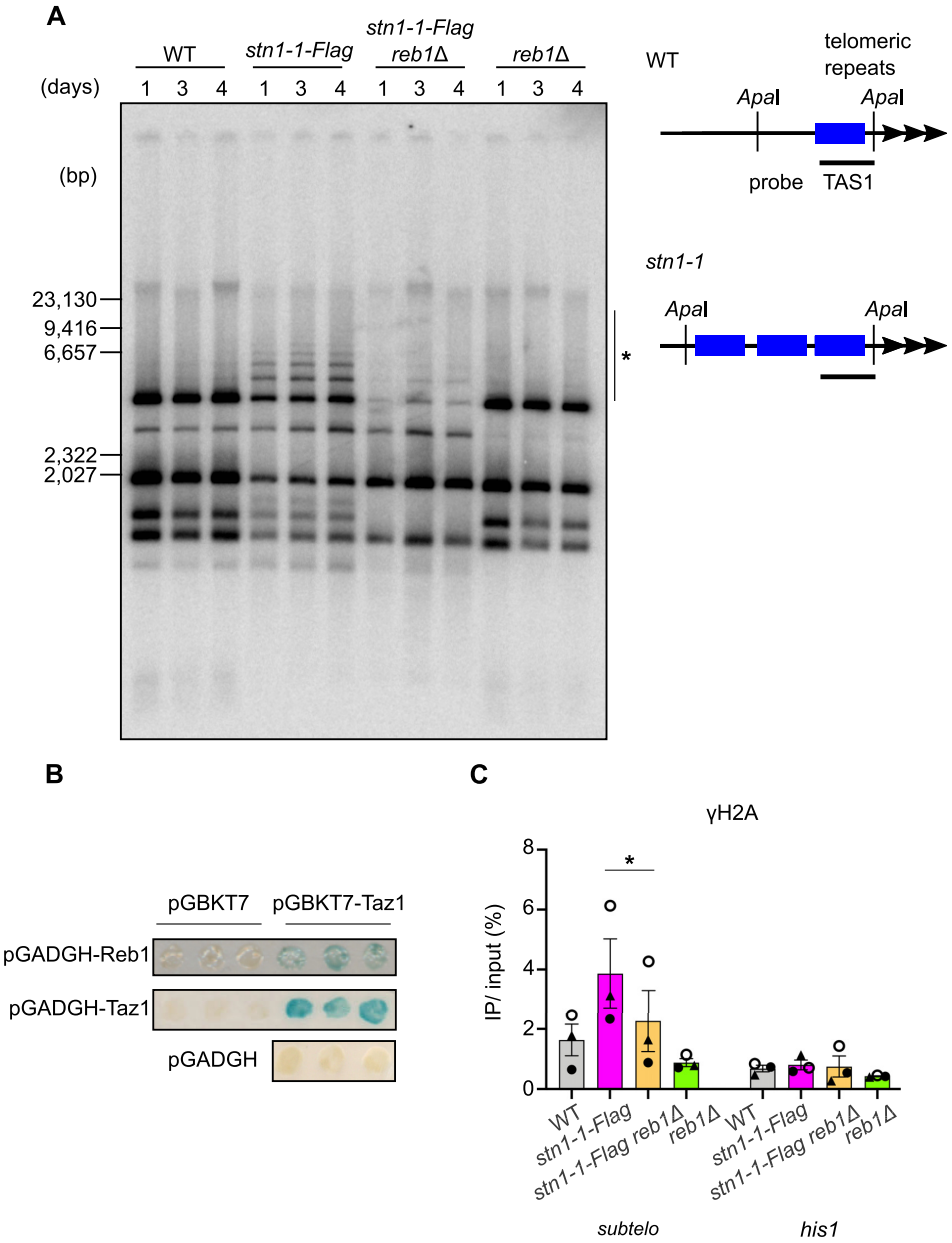

Figure 5. Deletion of *reb1*$^{+}$ suppresses subtelomeric instability caused by the *stn1-1* allele. (**A**) Cells were cultured in liquid YES for the indicated days at 25°C, and then genomic DNA was digested by ApaI endonuclease and analyzed by Southern blotting with the TAS1 DNA probe. (**B**) The interaction between Taz1 and Reb1 tested by the yeast two-hybrid assay. Taz1 served as a positive control. (**C**) γH2A localization at subtelomeres (around 45 kb-centromeric from the telomeric ends) and *his1*, assayed by ChIP. Indicated strains were cultured in YES liquid medium at 25°C. Each symbol indicates independent experiments. Error bars indicate the SEM ($n = 3$). Three biological replicates were tested. *P*-value was calculated by ANOVA with Tukey's test (*$P < 0.05$).

direct binding to *Ter2-3* sites within the repeat unit is well-established. Although *Ter*-like sequences do exist at subtelomeres, direct binding of Reb1 to the subtelomeric DNA has not been demonstrated. From this perspective, the interaction between Reb1 and Taz1 is intriguing and suggestive of Taz1-dependent localization of Reb1 at subtelomeres (Figure 5B). It is interesting that the budding yeast ortholog Reb1p directly binds to subtelomeres (72,73), implying a biological significance for the presence of Reb1 at the chromosomal ends. Regardless of the localization mechanisms, it is tempting to speculate that Reb1-induced replication fork stalling and its protection by Stn1 are coordinated regulatory mechanisms that ensure genome stability.

DATA AVAILABILITY

All strains and materials are available upon request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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