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Citation for published version:
Houseley, J & Tollervey, D 2011, 'Repeat expansion in the budding yeast ribosomal DNA can occur independently of the canonical homologous recombination machinery' Nucleic Acids Research, vol 39, no. 20, pp. 8778-8791., 10.1093/nar/gkr589

Digital Object Identifier (DOI):
10.1093/nar/gkr589

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher final version (usually the publisher pdf)

Published In:
Nucleic Acids Research

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Download date: 20. Feb. 2015

8778–8791 Nucleic Acids Research, 2011, Vol. 39, No. 20 Published online 17 July 2011
doi:10.1093/nar/gkr589

Repeat expansion in the budding yeast ribosomal DNA can occur independently of the canonical homologous recombination machinery

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Received February 12, 2011; Revised and Accepted June 30, 2011

ABSTRACT

Major eukaryotic genomic elements, including the ribosomal DNA (rDNA), are composed of repeated sequences with well-defined copy numbers that must be maintained by regulated recombination. Although mechanisms that instigate rDNA recombination have been identified, none are directional and they therefore cannot explain precise repeat number control. Here, we show that yeast lacking histone chaperone Asf1 undergo reproducible rDNA repeat expansions. These expansions do not require the replication fork blocking protein Fob1 and are therefore independent of known rDNA expansion mechanisms. We propose the existence of a regulated rDNA repeat gain pathway that becomes constitutively active in *asf1*Δ mutants. Cells lacking *ASF1* accumulate rDNA repeats with high fidelity in a processive manner across multiple cell divisions. The mechanism of repeat gain is dependent on highly repetitive sequence but, surprisingly, is independent of the homologous recombination proteins Rad52, Rad51 and Rad59. The expansion mechanism is compromised by mutations that decrease the processivity of DNA replication, which leads to progressive loss of rDNA repeats. Our data suggest that a novel mode of break-induced replication occurs in repetitive DNA that is dependent on high homology but does not require the canonical homologous recombination machinery.

INTRODUCTION

Repetitive sequences constitute a significant fraction of most eukaryotic genomes. They primarily occur at highly functionalized chromosome elements such as centromeres, telomeres and the ribosomal DNA (rDNA), and are therefore vital to cellular survival. However, their repetitive nature presents significant problems, as recombination events initiated in response to spontaneous DNA damage would naturally lead to large gains and losses of sequence. Such changes must normally be prevented or corrected since repetitive regions tend to have well-defined, stable repeat numbers; for example, the lengths of human rDNA repeat tracts are moderately heritable (1).

The yeast rDNA is a repetitive sequence that has long been studied as a model system for homologous recombination (2). The budding yeast genome contains a single rDNA cluster arranged as a linear array of 150–200 rDNA repeats on chromosome XII. Each repeat contains a 35S rRNA gene, a 5S rRNA gene, and two intergenic spacers containing multiple functional elements and non-coding RNAs (ncRNAs) (Figure 1A). A fragment of the rDNA repeat called *HOT1* containing both intergenic spacer regions has recombination stimulatory activity when transposed to ectopic sites within the genome (3). *HOT1* consists of two separate elements: the promoter and the enhancer for RNA pol I transcription of 35S (4), and transcription by RNA pol I is required for *HOT1*-stimulated recombination (4–6). Mutation or loss of a number of proteins dramatically reduces the frequency of recombination at *HOT1*, including the general recombination factors Rad52 and Rad1 (7), and the rDNA-specific protein Fob1 (8). Fob1 is a key component of the replication fork barrier (RFB – Figure 1A), which stalls replication forks travelling against the direction of pre-rRNA transcription (9). The ability of replication forks to displace barriers must be offset by multiple factors, and in *Saccharomyces cerevisiae* it has been shown that Csm3 and Tof1 stabilize the RFB but are dispensable for RFB activity in cells lacking the replicative helicase Rrm3 (10). In keeping with the tight connection between RFB activity and recombination, Tof1 is required for rDNA recombination in wild-type

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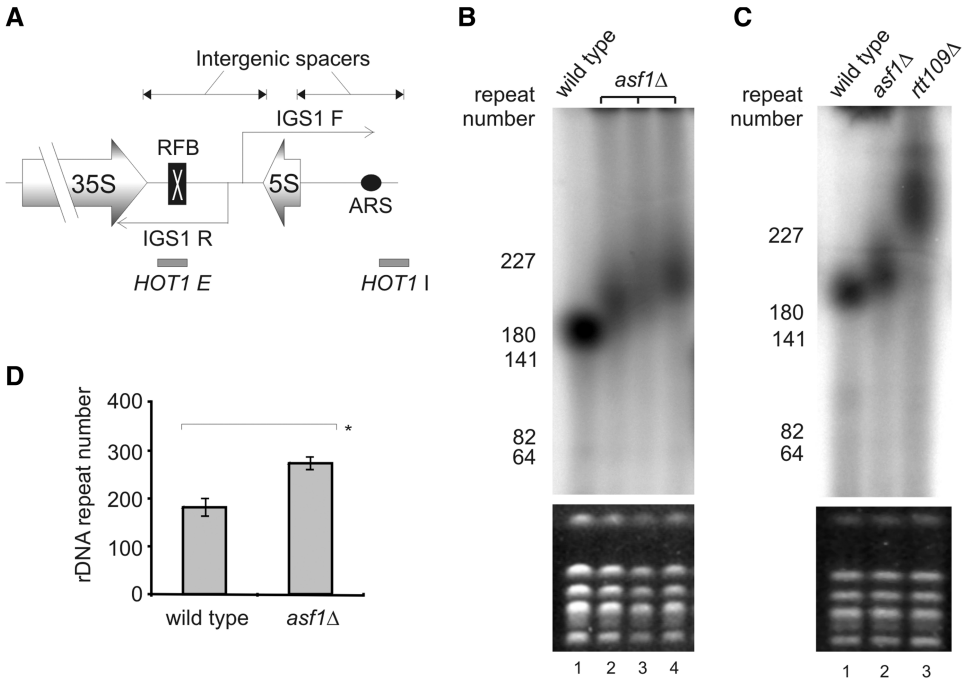

Figure 1. The Asf1-Rtt109 complex regulates rDNA stability. (**A**) Schematic of a single rDNA repeat showing the two ribosomal RNA genes (35S and 5S) separated by two intergenic spacers. The intergenic spacers contain an origin of replication (ARS), the replication fork barrier (RFB) and the promoter for two ncRNAs (IGS1 F and IGS1 R). *HOT1* regions E (pol I enhancer) and I (pol I promoter) are defined in (4). (**B**) PFGE analysis rDNA repeat number in wild-type and three independent clones of *asf1*Δ. Cells were grown to stationary phase in rich media, DNA was separated on a 0.8% pulsed field gel. Upper panel shows chromosome XII by hybridization with a probe against 18S rDNA. Ethidium staining of other chromosomes is shown in the lower panel, highest visible chromosome is IV. (**C**) PFGE analysis of *asf1*Δ and *rtt109*Δ cells performed as in (B). (**D**) Quantification of the rDNA repeat number based on the 18S:*ACT1* ratio in log phase cells. DNA was extracted from mid-log cells in YPD, digested with *Bgl*II and analyzed by Southern blotting. Error bars represent ±1 standard error, $*P < 0.05$ by Student's *t*-test.

yeast but dispensable in *rrm3* mutants (11). Although rDNA RFBs exist in many eukaryotes, Fob1 is not conserved outside budding yeast. In the fission yeast *S. pombe* analogous functions are performed by Reb1 and Sap1 (12,13), which bind directly to RFB sites but require additional factors to mediate fork arrest (14,15).

Outside the *HOT1* region, rDNA recombination has been detected at the 3′-end of the 35S gene, particularly in cells lacking Fob1, showing that non-Fob1-mediated recombination mechanisms must also be active in the rDNA (16). Since the head-on collision of replication and transcription machinery can lead to replication fork pausing (17), it seems likely that in the absence of the RFB, replication forks stall where they encounter RNA pol I transcription at the 3′-end of 35S and this can functionally substitute for the RFB in recombination. However such effects are dependent on the nature of the RNA polymerase; RNA pol III causes robust replication pausing (17), whereas the effects are more variable for RNA pol II (18), and the outcome of collisions between the replication machinery and RNA pol I remains unclear.

Stalled replication forks are associated with recombination in prokaryotes, where recombination is involved in replication fork restart [reviewed in (19)], and in Eukaryotes where stalling during replication of fragile sites is closely linked to chromosomal translocations (20,21). Replication fork stalling is clearly a dangerous process—it prevents timely replication and can cause chromosome rearrangements. However, rDNA RFBs are found across evolution (22–27) suggesting that induced replication fork stalling is an important process. Replication forks stalled at defined barriers are handled differently to those induced by replication stress. Notably, replication forks stalled due to stress require the S-phase checkpoint for stability (28,29), whereas replication forks stalled at defined barriers do not (30,31).

The outcome of recombination initiated at the RFB is controlled by transcription. Cells lacking Sir2 lose silencing in the rDNA (32,33) and undergo rDNA hyper-recombination resulting in extreme destabilization of repeat number (34). However, the recombination rate in *sir2*Δ strains is not actually increased (35). Rather, ncRNA transcription within the rDNA disrupts sister chromatid cohesion allowing recombination between the broken DNA end and an unmatched rDNA repeat (36), a process known as unequal sister chromatid exchange. Although expression of rDNA-encoded ncRNAs allows the cell to change the frequency at which sister chromatid exchange occurs between unequal repeats, there is little evidence that this process is directional, indicating that it cannot be entirely responsible for precise repeat number control.

In a previous analysis, we noted that loss of the RNA surveillance factor Trf4 biases recombination towards

repeat loss, suggesting the existence of directional repeat change mechanisms (37). We therefore decided to screen known genetic interaction partners of *TRF4* for mutations with an opposing repeat gain phenotype.

MATERIALS AND METHODS

Yeast strains, media, plasmids and drug treatments

Yeast deletion strains were created by standard methods using the oligonucleotides in Supplementary Table S1; strains are listed in Supplementary Table S2. Cells were grown on YPD except for plasmid assays for which synthetic media was used (0.69% YNB with ammonium sulfate, amino acids, 2% glucose). For 5 FOA selections, synthetic media lacking histidine was supplemented with 1 mg/ml 5 FOA (Zymo Research) and 50 μg/ml uracil. Plasmids are described in Supplementary Table S3. Hydroxyurea from a 2 M stock was used at 125 mM or 200 mM, camptothecin from a 4 mg/ml stock in DMSO was used at 10 μg/ml in plates containing 25 mM HEPES, pH 7.2, control plates contained DMSO.

PFGE

Stationary phase cells (2.5×10^7) were washed and re-suspended in 50 μl of PFGE wash (10 mM Tris pH 7.5, 50 mM EDTA) containing 17 U lyticase, and then solidified in blocks with 1% LE agarose (SeaKem). These were digested with 340 U/ml lyticase in PFGE wash for 1 h at 37°C, then with 1 mg/ml Proteinase K in 100 mM EDTA, 0.1% sodium deoxycholate, 1% sodium lauryl sarcosine at 55°C overnight. After four washes with PFGE wash, plugs were run on 0.8% gels on a CHEF-DR II system (BioRad) in 1× TBE at 3 V/cm, 300–900 s switch time, 12°C for 68 h, with *H. wingii* ladder (BioRad). Gels were stained with ethidium bromide or SYBR Safe prior to treatment with 0.25 N HCl for 30 min, 0.5 N NaOH for 30 min, and 0.5 M Tris pH 7.5 1.5 M NaCl for 15 min twice. DNA was transferred to HyBond N+ in 6× SSC and UV cross-linked before hybridization with a random primed probe in Church Hyb overnight at 65°C and washed twice for 20 min with 0.5× SSC 0.1% SDS at 65°C.

Genomic DNA preparation and Southern blotting

Stationary phase cells (2 ml) were spheroplasted in 250 μl Buffer A (1.2 M sorbitol, 50 mM EDTA, 10 mM DTT, 340 U/ml lyticase) for 30 min at 37°C. After 5 min centrifugation at 1000 *g*, cells were re-suspended in 400 μl Buffer B (0.3% SDS, 50 mM EDTA, 100 μg/ml RNase A), for 30 min at 37°C, 4 μl 20 mg/ml Proteinase K was added and cells incubated for 30 min at 65°C. 160 μl 5 M KOAc was added and samples placed on ice for 30 min, centrifuged at 10 000 *g* for 15 min, and supernatant poured into a new tube containing 500 μl Phenol:Chloroform pH 8. After mixing on a wheel for 15 min, centrifugation at 10 000 *g* for 5 min and extraction with cut tips, DNA was precipitated with 1 ml ethanol, washed with 70% ethanol and re-suspended in 50 μl TE overnight at 4°C. 8 μl DNA was digested in 20 μl total volume with 10–20 U enzyme and separated on 1% TBE-agarose gels. Blotting and probing were as above, except that depurination was for 15 min.

Marker loss assay

A *MET25-GFP* marker was inserted randomly in the IGS2 region of the rDNA as previously described (37), and strains were maintained on plates lacking methionine. Two separate colonies from two independent clones of each genotype (total four colonies) were grown overnight in YPD to allow marker loss, then 500 log-phase cells were plated on MLA plates (0.3% peptone, 0.5% yeast extract, 0.02% NH_4SO_4, 0.1% $Pb(NO_3)_2$).

Other methods

Northern blots were performed as described (37), probes are listed in Supplementary Table S4. Images were prepared for publication using ImageJ; they were smoothed to reduce noise, contrast adjusted and had a Gamma Shift of 3 applied.

RESULTS

Cells lacking Asf1 undergo rDNA repeat expansion

The rDNA array accounts for ~40% of chromosome XII, which is by far the largest chromosome in the yeast genome. In consequence, chromosome XII is readily visualized by pulsed field gel electrophoresis (PFGE), and its mobility provides a direct measurement of rDNA repeat number. To search for mutants that alter rDNA repeat number in a directional manner, we used PFGE to measure the rDNA repeat number of a selection of mutants having overlapping synthetic lethal profiles with *trf4*Δ. The rationale was that proteins that function in the same pathway as Trf4 would show related patterns of synthetic lethal interactions. In an initial screen of 22 factors (Supplementary Table S6), we found that *asf1Δ* cells have a highly reproducible rDNA repeat gain phenotype (Figure 1B).

Asf1 is a histone chaperone involved in multiple replication and repair processes (38). Asf1 associates with the histone H3 lysine 56 acetyl transferase Rtt109 (39,40), which is required for the function of Asf1 in double-strand break (DSB) repair and histone delivery to replication forks (41–44). Consistent with this, *rtt109*Δ strains show very similar phenotypes to *asf1*Δ confirming the role of this complex in rDNA maintenance (Figure 1C). The larger expansion in *rtt109*Δ compared to *asf1*Δ likely reflects clone-to-clone variability rather than the mutation having a stronger effect.

Replication intermediates and topologically distorted chromosomes do not migrate correctly in PFGE gels (45,46). To minimize these effects, PFGE analyses were performed on non-dividing, stationary phase cells. However, to confirm that the observed repeat number changes are real and also present in growing cells, Southern blots were performed on DNA extracted from log-phase cells. Relative repeat numbers were calculated from the rDNA:*ACT1* signal ratio, which confirmed that

the *asf1*Δ cell populations carry significant rDNA expansions compared to wild-type (Figure 1D). Curiously, Southern blotting consistently reports a larger value for repeat expansion than inspection of the PFGE gels. We suspect that the PFGE assay under-represents repeat number in *asf1*Δ; in cultures that have undergone the least expansion relative to wild-type, Chr. XII migrates in an intense band, whereas those that have undergone larger expansions show a dispersed population of Chr. XII that is less readily detected above the lane background.

rDNA repeat expansion is not dependent on the RFB

The best characterized recombination pathway allowing rDNA expansion requires the RFB and therefore Fob1. Loss of Fob1 stabilizes rDNA repeat number in otherwise hyper-recombinant *sir2*Δ mutants (47) (see also Figure 2A—compare smeared signal in lane 3 with sharp bands in lanes 4–6), but the rDNA repeat number changes seen in *asf1*Δ strains occurred largely independently of Fob1 (Figure 2A—compare smeared signal in lane 7 with lanes 8–10), and hence of RFB function.

Repeat expansion quantification by PFGE is difficult (e.g. Figure 2A lane 10 has an indeterminate phenotype) so we quantified the effect of *ASF1* deletion in wild-type and *fob1*Δ cells by Southern blot (Figure 2B). The average rDNA repeat number increased on deletion of *ASF1* by the same amount irrespective of the presence or absence of Fob1 (Figure 2B); therefore, Fob1 does not contribute to the repeat expansion pathway in *asf1Δ* cells.

Even though Fob1 is not required for repeat expansion, deletion of *ASF1* may affect recombination initiated from replication forks stalled at the RFB. To test this, *ASF1* was deleted in a *sir2*Δ background in which cells undergo Fob1-mediated hyper-recombination. Because *sir2*Δ clones undergo very frequent rDNA repeat number changes, they show massive clone-to-clone and experiment-to-experiment variation (compare *sir2*Δ samples in Figure 2A, 2C and 2E) complicating analyses of rDNA stability changes. However, multiple clones of the *asf1*Δ *sir2*Δ double mutant showed a strongly enhanced repeat expansion phenotype relative to *asf1*Δ single mutants (Figure 2C), with the rDNA band always present at the resolution limit of the gel, far above any observed *sir2*Δ clone. rDNA hyper-recombination in *sir2Δ* strains is triggered by overexpression of rDNA ncRNAs, and ncRNA overexpression was further increased in the *asf1*Δ *sir2*Δ double mutant (Figure 2D). When quantified and normalized to repeat number, the *asf1*Δ *sir2*Δ cells were found to produce 2- to 3-fold more ncRNA than *sir2*Δ cells. To ensure that the enhanced repeat expansion phenotype observed in *sir2*Δ *asf1*Δ cells is directly attributable to effects on Fob1-mediated recombination, *ASF1* was deleted in a *sir2*Δ *fob1*Δ double mutant. The triple mutants displayed rDNA repeat profiles similar to an *asf1*Δ single mutant (Figure 2E compare lanes 7–9 to lane 2), showing that the increased expansion in *asf1*Δ *sir2*Δ mutants requires recombination initiated at the RFB.

rDNA expansion is progressive and of high fidelity

To further characterize the Fob1-independent repeat expansion pathway in *asf1*Δ cells, we tested the rate, accuracy and directionality of rDNA repeat number change. To aid these experiments, we constructed a strain in which the *asf1*Δ mutation was complemented with a plasmid-borne wild-type copy of the gene that can be selectively removed. This strain had a normal rDNA copy number, but after expulsion of the plasmid it underwent a slow increase in repeat number across multiple sequential platings (each representing 15–20 generations) (Figure 3A). This shows that the repeat expansions are the result of many small, cumulative changes. These changes appear to have high fidelity as we were unable to detect rDNA repeats of abnormal size in *asf1*Δ cells, even after many generations (Supplementary Figure S1A). Although this does not rule out small sequence changes, it is clear that large insertions or deletions do not occur at a substantial rate in this process.

Repeat expansion may result from events that exclusively cause repeat gain, or from a general instability with repeat gain outweighing repeat loss. To distinguish these possibilities, strains carrying a single copy *MET25* marker gene randomly integrated into the rDNA were used; if loss of *ASF1* causes general instability then loss of the *MET25* marker will occur more frequently, but if *asf1*Δ mutants only undergo repeat gain, then the rate of *MET25* marker loss would be unchanged. In this assay, *asf1*Δ cells showed a low rate of marker loss, equivalent to wild-type cells based on colony color (Supplementary Figure S1B), suggesting that loss of *ASF1* causes only repeat expansion as opposed to expansion-biased instability. No significant difference in marker loss rate was observed in *fob1*Δ cells, suggesting that the primary mechanism of repeat loss in this assay is single-strand annealing (48,49). Surprisingly, duplication of the *MET25* marker was not detected in *asf1*Δ strains. The strains shown in Figure 2B carried the rDNA *MET25* marker, and although the absolute repeat number of the rDNA is significantly increased in these strains, probing the same Southern blot for *MET25* revealed no evidence of marker amplification (Supplementary Figure S1C). Therefore, rDNA expansion events in *asf1*Δ mutants are restricted to perfect repeats and are not accompanied by general rDNA instability. In contrast, marker duplication is readily detected in rDNA recombination events initiated by Fob1 (50).

rDNA expansion is independent of the homologous recombination machinery

Because the heat shock required to delete *ASF1* using standard transformation protocols also alters rDNA repeat number, we used the *asf1*Δ p*ASF1* strain shown in Figure 3A for further experiments. In this strain, the plasmid carrying the wild-type *ASF1* gene is lost naturally during cell division under drug selection. In each experiment, the *asf1*Δ p*ASF1* strain was transformed with a tester mutation and three clones selected. Due to the transformation step, these clones often displayed varying repeat

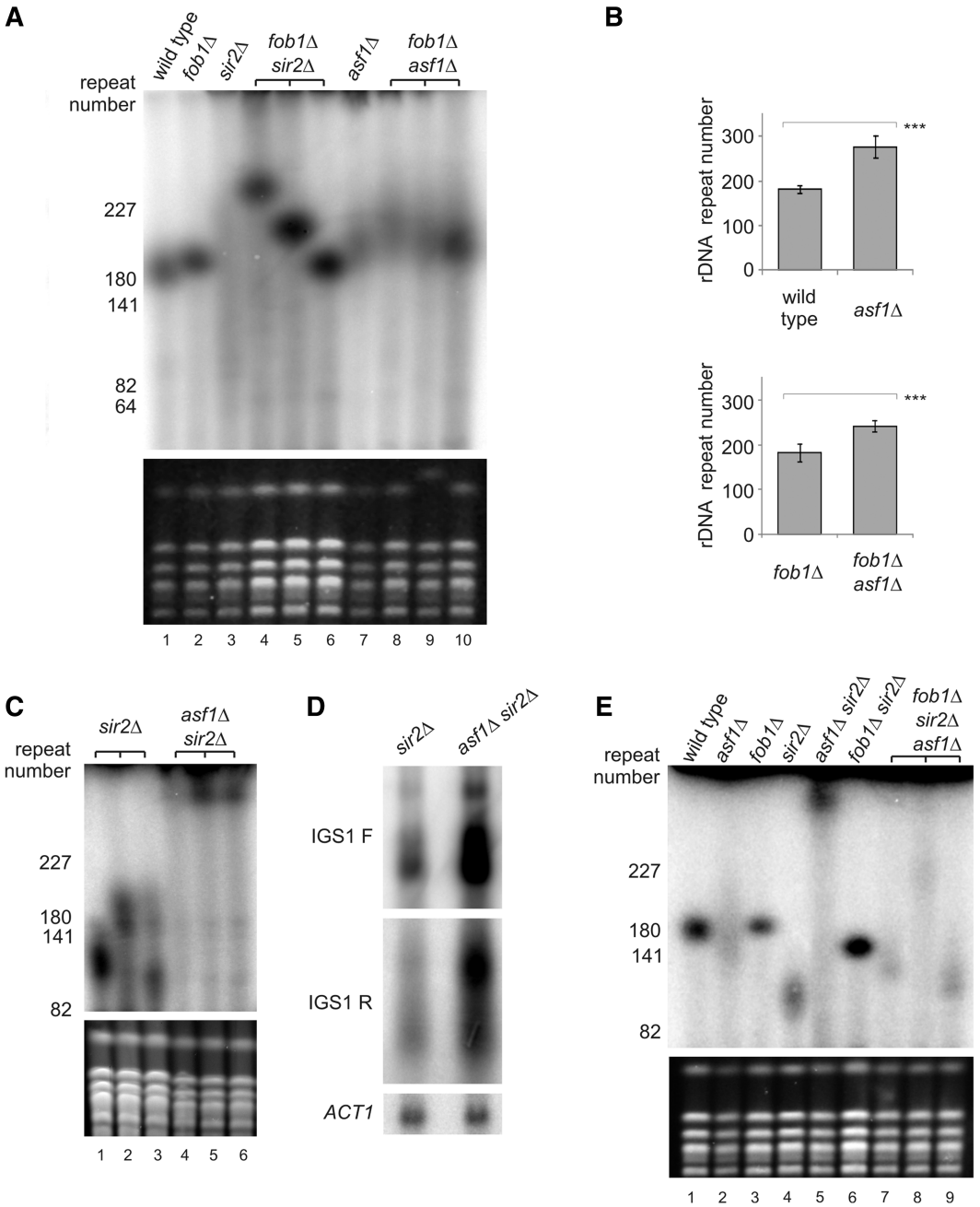

Figure 2. Fob1 is not required for rDNA expansion in *asf1*Δ cells. (**A**) PFGE analysis of the rDNA recombination phenotypes of *sir2*Δ and *asf1*Δ mutations introduced into wild-type and *fob1*Δ backgrounds, performed as in Figure 1B. The change in size of chromosome IV in lane 9 appears to be a sporadic event unrelated to the loss of *ASF1*. (**B**) Quantification of repeat gain in *asf1*Δ strains in *FOB1* and *fob1*Δ backgrounds. *ASF1* was deleted in wild-type and *fob1*Δ strains carrying a randomly integrated MET25 marker in the rDNA, and cells were spread on four successive YPD plates prior to DNA extraction from 12 colonies of each genotype. Genomic DNA was digested with *Sal*I and *Spe*I, and rDNA repeats were quantified by Southern blot relative to *ACT1*. Error bars represent ±1 standard error, $***P < 0.01$ by Student's *t*-test. (**C**) PFGE analysis of *sir2*Δ and *sir2*Δ *asf1*Δ cells performed as in Figure 1B. (**D**) Northern analysis of intergenic spacer ncRNAs in *sir2* and *asf1*Δ *sir2*Δ. Cells were grown to mid-log in rich media; RNA was extracted and separated on a 1.2% glyoxal gel, and probed for IGS1 F, IGS1 R and *ACT1*. (**E**) PFGE analysis of *asf1*Δ in wild-type, *sir2*Δ and *sir2*Δ *fob1*Δ cells performed as in Figure 1B.

number; e.g. see Figure 3C lanes 3–5, showing three independent clones of *asf1*Δ p*ASF1 rad59*Δ *rad52*Δ derived from transformation of the strain shown in Supplementary Figure S3 lane 11. Each clone was plated four times successively on media that selects for maintenance or loss of the plasmid and then analyzed by PFGE. In each case, the strain that has been selected for plasmid loss can be directly compared to its age-matched parental strain. For example, in Figure 3B, lanes 1 and 2 represent the same clone of *asf1*Δ p*ASF1* plated on media

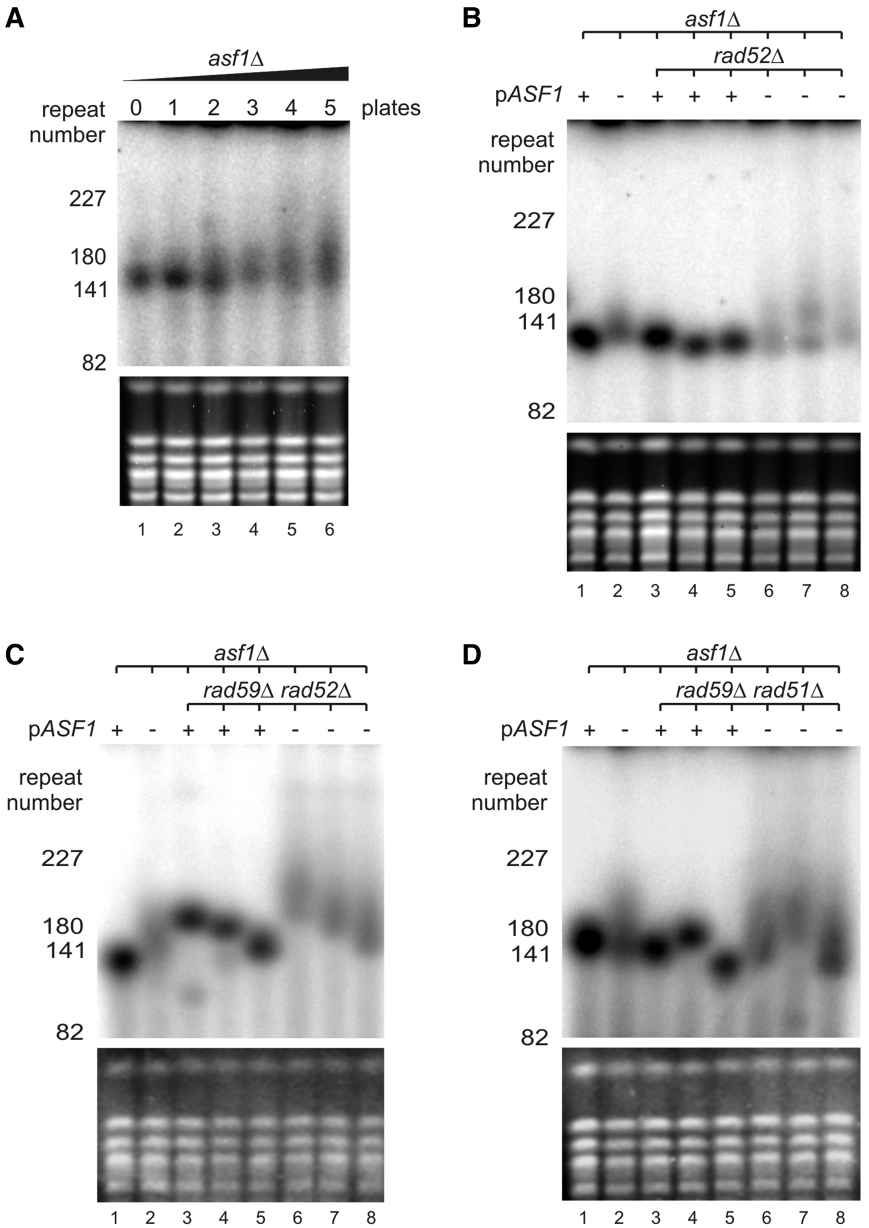

Figure 3. *asf1*Δ rDNA expansions are not dependent on the HR machinery. (**A**) Time course of rDNA repeat gain in an *asf1*Δ strain. An *asf1*Δ mutant was complemented with a plasmid (p*ASF1*) carrying wild-type *ASF1* and a *URA3* selectable marker, which restored wild-type rDNA repeat number. In this strain, *ASF1* can be removed by negative selection for plasmid loss, avoiding the harsh conditions involved in transformation. This *asf1*Δ p*ASF1* strain was plated on 5 FOA to select for plasmid loss and samples taken after the indicated number of platings (each representing 15–20 generations), the triangle denotes increasing time after loss of *ASF1*. PFGE analysis as in Figure 1B. (**B**) Analysis of *ASF1* deletion in wild-type and *rad52*Δ backgrounds. *RAD52* was deleted in the *asf1*Δ p*ASF1* strain described in (A), after which the *ASF1* plasmid was removed from the parental strain and three clones of *rad52*Δ. PFGE analysis as in Figure 1B was performed after four platings (each representing 15–20 generations). rDNA expansion is assessed by comparing each individual clone grown on media that selects for plasmid loss to the same clone grown on media that selects for plasmid retention. Lanes 1 and 2 represent a single *asf1*Δ p*ASF1* clone plated on media that selects for plasmid retention and plasmid loss respectively; lanes 3 and 6, lanes 4 and 7, lanes 5 and 8 represent equivalent pairs of single clones grown on media selecting for plasmid retention or loss. (**C**) Analysis of *ASF1* loss in a *rad59*Δ *rad52*Δ background. Experiment was performed as described in (B), *RAD52* was deleted in *asf1*Δ p*ASF1 rad59*Δ clone 1 (Supplementary Figure S3 lane 11) to generate three clones of *asf1*Δ p*ASF1 rad59*Δ *rad52*Δ (lanes 3–5), followed by plasmid removal (lanes 6–8). (**D**) Analysis of *ASF1* loss in a *rad59*Δ *rad51*Δ background. Experiment was performed as described in (B), *RAD51* was deleted in *asf1*Δ p*ASF1 rad59*Δ clone 1 (Supplementary Figure S3 lane 11) to generate three clones of *asf1*Δ p*ASF1 rad59*Δ *rad51*Δ (lanes 3–5), followed by plasmid removal (lanes 6–8).

that selects for plasmid retention or loss, respectively. Similarly, lanes 3–5 and lanes 6–8 show the same clones plated on media that selects for plasmid retention or loss, respectively. Using this rigorous procedure, we could directly assess the effect of *asf1*Δ in mutant backgrounds by comparing multiple clones to their parent *ASF1* strain.

To determine whether repeat gain in *asf1*Δ involves the homologous recombination (HR) machinery, the key

recombination protein Rad52 [see (51) and references therein] was deleted in the *asf1*Δ p*ASF1* strain, followed by plasmid elimination. Repeat expansion in *asf1*Δ occurred readily in the absence of Rad52, suggesting that expansion does not proceed by a standard HR mechanism (Figure 3B). This observation was unexpected, as non-HR-based mechanisms of repeat expansion with high fidelity are rare. A separate assay was used to ensure the *rad52*Δ strains were truly defective in HR: *rad52* mutants are hypersensitive to hydroxyurea as they are unable to repair broken replication forks (52), so we ensured that growth of *asf1*Δ p*ASF1 rad52*Δ was inhibited by 125 mM hydroxyurea (Supplementary Figure S2A). Furthermore, although Figure 3B shows three independent *rad52*Δ clones, we constructed a fresh a*sf1Δ pASF1* strain from wild-type, deleted *RAD52* and repeated the experiment, giving the same result (data not shown).

We noticed that a second discrete rDNA allele formed in one *rad52*Δ clone after loss of *ASF1* (Figure 3B lane 7), which we had not observed in *asf1*Δ mutants. To investigate this further, we performed an extended time course with *asf1*Δ and *asf1*Δ *rad52*Δ clones (Supplementary Figure S2B). We noted that although repeat gain occurred with or without *RAD52*, the *asf1*Δ *rad52*Δ mutants accumulated shorter deleted rDNA alleles at later time points, suggesting that although Rad52 is not required for rDNA expansion, it is required to maintain rDNA stability in *asf1*Δ mutants. We again tested for suppressor mutations by ensuring that at the end of this experiment, the *asf1*Δ *rad52*Δ strains were hypersensitive to hydroxyurea when transformed with an *ASF1* plasmid, and hypersensitive to DSB formation when transformed with an inducible *GAL::HO* plasmid (Supplementary Figure S2C and D).

We then analyzed other members of the *RAD52* epistasis group, Rad51 and Rad59, which each interact with Rad52 but can participate in different recombination mechanisms (53–55). Deletion of *ASF1* in *rad51*Δ and in *rad59*Δ cells lead to equivalent or greater repeat expansions to those observed in *asf1Δ* single mutants (Supplementary Figure S3), consistent with the suggestion that repeat expansion in *asf1*Δ cells is fully independent of the canonical HR machinery.

Rad59 is homologous to Rad52 (53) and hence potentially functionally redundant in some processes; therefore, we tested repeat expansion in *asf1*Δ strains lacking both proteins and found that repeat expansion could still occur (Figure 3C). Degeneracy between Rad51 and Rad59 has been demonstrated both in telomerase-independent telomere lengthening (56) and in break-induced replication (BIR) (53,57) so we tested for repeat expansion in *rad59*Δ *rad51*Δ *asf1*Δ mutants, which also occurred readily (Figure 3D).

Finally, we tested the involvement of non-homologous end joining (NHEJ) in the process by deleting *ASF1* in cells lacking Dnl4 or Rad50 (58,59). Neither of these mutations had any effect on the observed repeat expansions (Supplementary Figure S4), as expected since NHEJ pathways should only mediate repeat loss.

DNA polymerase mutations impair rDNA expansions

BIR at telomeres and Rad52-independent copy number variation and have both been ascribed to a replication-based mechanism that requires the Pol32 component of DNA polymerase δ (60,61). *POL32* deletion in *asf1*Δ p*ASF1* caused a loss of rDNA repeats (Figure 4A compare lane 1 to lanes 3–5), and removal of the *ASF1* plasmid caused further repeat loss instead of the repeat gain seen in wild-type cells (Figure 4A; compare the three clones in lanes 3–5 to the same clones in lanes 6–8, in which lane 6 shows the same clone as lane 3 with the plasmid cured, the same is true for lanes 7 versus 4 and 8 versus 5). This demonstrates that Pol32 is important for the rDNA repeat expansions seen in *asf1*Δ cells and suggests that this mechanism is also required for normal rDNA repeat number maintenance.

BIR at telomeres is dependent on DNA polymerase ε, and a mutation in the core catalytic component, *pol2-11*, blocks BIR at a later stage than *pol32*Δ (60). The *pol2-11* mutation caused large rDNA contractions, and completely inhibited the repeat expansions caused by the loss of *ASF1* (Figure 4B), showing that normal function of DNA polymerase ε is required for rDNA repeat stability and expansion. The *pol2-11* mutation also inhibits the function of the S-phase checkpoint (62,63), which could potentially be involved in repeat expansion, so multiple checkpoint mutants (*tel1*Δ, *sml1*Δ *mec1*Δ, *mrc1Δ, rad9*Δ) were tested for rDNA phenotypes. These mutations did not cause changes in rDNA repeat number or suppress the repeat expansion in *asf1*Δ cells (data not shown), showing rDNA expansion to be S-phase checkpoint independent.

It might be anticipated that DNA polymerase mutations would lead to genome-wide instability. However, the observed effects were apparently specific for the rDNA, with no detectable alterations to other chromosomes in any analyzed clone of *pol32*Δ or *pol2-11* (Figure 4A, B; lower panels where all chromosomes except XII are shown). Occasional changes in the lengths of other chromosomes were seen during this research (e.g. Figure 2A lane 9), but these were seemingly random and not restricted to any particular genotype.

The smeared rDNA distribution in *pol32*Δ mutants suggested an ongoing contraction process, and it is possible that the lack of rDNA expansion seen in *asf1*Δ *pol32*Δ mutants is due to expansion and contraction processes acting in equilibrium. To test for competing expansion and contraction processes, we attempted to block ongoing repeat contraction in *pol32*Δ and *pol2-11* mutants by deleting *RAD52* (Figure 4C and D). Loss of *RAD52* stabilized the repeat number in *pol32*Δ cells, and on removal of *ASF1* limited rDNA expansion was observed. Therefore, the suppression of repeat expansion in the absence of Pol32 is not complete and competing expansion and contraction does partially explain the lack of rDNA expansion in *asf1*Δ *pol32*Δ cells. However, *pol2-11* and *pol2-11 rad52*Δ cells appear identical (compare Figure 4B lanes 3–4 with Figure 4D lanes 3–5), and no repeat expansion occurred when *ASF1* was deleted in a *pol2-11 rad52*Δ background (Figure 4D

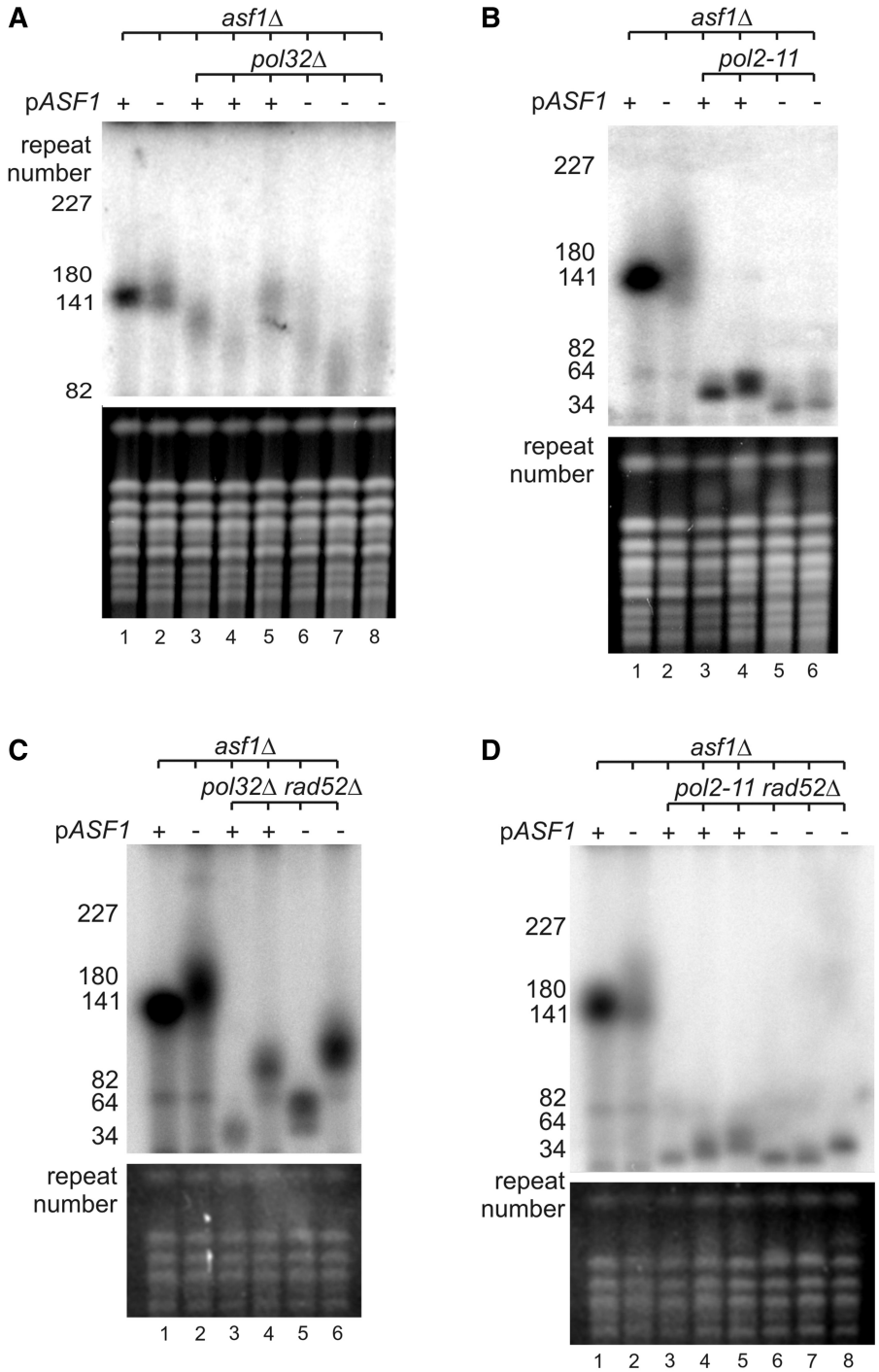

Figure 4. Repeat expansion in *asf1*Δ requires highly processive replication. (**A**) Analysis of *ASF1* loss in a *pol32*Δ background, performed as in Figure 3B. The full set of *S. cerevisiae* chromosomes (excluding XII) is shown by SYBR staining in the lower panel to demonstrate that replication defects in this strain do not cause general chromosome instability. (**B**) Analysis of *ASF1* loss in a *pol2-11* background, performed as in Figure 3B. Only two clones were analyzed due to low viability of *pol2-11* cells in this background. Note that the contracted Chromosome XII is visible between the top two chromosomes in the SYBR stained gel. As in A, the full set of chromosomes excluding XII is shown by ethidium staining. (**C**) Analysis of *ASF1* loss in a *pol32*Δ *rad52*Δ background. Experiment was performed as described in Figure 3B, *RAD52* was deleted in *asf1*Δ p*ASF1 pol32*Δ clone 1 (A lane 3) to generate 2 clones of *asf1*Δ p*ASF1 pol32*Δ *rad52*Δ (lanes 3–4), followed by plasmid removal (lanes 5–6). (D) Analysis of *ASF1* loss in a *pol2-11 rad52*Δ background. Experiment was performed as described in Figure 3B, *RAD52* was deleted in *asf1*Δ p*ASF1 pol2-11*Δ clone 1 (B lane 3) to generate three clones of *asf1*Δ p*ASF1 pol2-11*Δ *rad52*Δ (lanes 3–5), followed by plasmid removal (lanes 6–8).

compare lanes 3–5 to lanes 6–8). In fact, the *pol2-11 rad52Δ asf1Δ* strains displayed sharply defined rDNA bands rather than the smears that would be the inevitable result of competing expansion and contraction processes acting across a population of cells.

rDNA expansion requires highly processive replication of repetitive DNA

The effects of *pol32Δ* and *pol2-11* mutations on rDNA expansion indicate a BIR mechanism. However, in previous analyses BIR was largely Rad51 or Rad59-dependent (53,54,57,64) and almost invariably Rad52-dependent (53,54,57,64,65), leading us to investigate other mechanisms. Both *pol32Δ* and *pol2-11* mutations inhibit DNA polymerase processivity (66,67), so we tested the effects of other DNA replication inhibitors on rDNA repeat number.

Hydroxyurea is an inhibitor of ribonucleotide reductase (RnR) and inhibits replication by nucleotide depletion. Growth of wild-type cells on hydroxyurea caused some loss of rDNA repeats (Figure 5A) although the effect was weak and variable. Stronger and more reproducible effects were seen after treatment with camptothecin, a topoisomerase I inhibitor that stalls and breaks replication forks (Figure 5B). Therefore, reducing replication processivity by drug treatment produced similar effects to polymerase mutation. Suppression of rDNA expansion could not be tested due to hypersensitivity of *asf1Δ* strains to these drugs

As a more subtle method for reducing replication processivity, we tested *dun1Δ* mutants, in which the RnR inhibitor Sml1 is not degraded at the appropriate time. This leads to RnR inhibition in S phase and reduced deoxynucleotide supply to the replication machinery. In *dun1Δ* cells, the rDNA repeat loss phenotype was identical to *pol32Δ* mutants (Figure 5C). To ensure that the observed phenotype was indeed due to RnR inhibition, we repeated the experiment in an *sml1Δ* background in which RnR cannot be inhibited. This completely ameliorated the effects of *dun1Δ* on rDNA stability and restored *asf1Δ*-dependent expansion (Figure 5D).

A potential reason for the lack of rDNA expansion when *ASF1* is deleted in polymerase mutants is that expansion requires a certain number of rDNA repeats and the reduced repeat number in the polymerase mutants is below this threshold. To test the effect of reduced repeat number on expansion, we deleted *ASF1* in strains with approximately 190, approximately 25 and 2 rDNA repeats (Figure 5E). Expansion readily occurred in the approximately 25 rDNA repeat strain, which has a similar number of repeats to *pol2-11*, showing that lack of expansion in *pol2-11* is not due to low repeat number. However, the 2 rDNA repeat strain showed no expansion on *ASF1* deletion, indicating that expansion requires multiple rDNA repeats. To confirm that the *asf1Δ* mutants in this experiment (particularly the 2 rDNA repeat strain) had not developed suppressor mutations and were not mis-genotyped, we confirmed that they were hypersensitive to hydroxyurea (Supplementary Figure S5).

Taken together, these data show that in an *asf1Δ* background, cells undergo rDNA repeat expansion by a mechanism independent of Fob1, and of Rad52 and other known HR proteins. The repeat expansion process requires ongoing processive replication and only occurs in repetitive regions.

DISCUSSION

The accurate maintenance of repeated genomic regions presents a difficult problem for any cell as recombination processes naturally alter repeat number. A number of proteins are known to repress excision recombination in the yeast rDNA and hence prevent loss of repeats [such as linker histone Hho1 and topoisomerase-related protein Hpr1 (68,69)]. However, mutagens such as hydroxyl radicals and ionizing radiation introduce DNA DSBs that are excellent substrates for single-strand annealing in repetitive DNA regions (70,71). This pathway by definition causes repeat loss, and should therefore decrease rDNA repeat number with time, whereas repeat numbers are actually very stable, implying the existence of compensating mechanisms for repeat expansion. One candidate mechanism involves the initiation of recombination from replication forks stalled at the RFB (72), combined with cohesin removal by ncRNA expression in the intergenic spacer (35,36). This mechanism is capable of generating both expansions and contractions but there is no evidence that it is directionally controlled. In cell populations forced to transcribe ribosomal RNA using RNA pol II, colonies that carry very large rDNA expansions do arise in a replication fork-dependent manner, suggesting expansion biased unequal sister chromatid exchange (73). However, this is actively selected for, because pol II transcription of the rDNA is relatively weak, so cells carrying more repeats have a growth advantage.

Here, we report the existence of a pathway in budding yeast that acts exclusively to increase rDNA repeat number. This pathway is constitutively active in strains lacking either Asf1 or Rtt109, which form a complex with a key role in directing broken replication forks into the HR pathway (74,75). Notably, all mutations that blocked the expansion pathway also caused rDNA repeat loss, strongly suggesting that the ongoing activity of this pathway is required for rDNA maintenance. This indicates that regulated Asf1-Rtt109 activity may control rDNA expansion by directing fork recovery into either HR or the alternative non-HR expansion pathway.

Much of our data on factors required for rDNA expansion is consistent with a BIR mechanism, including dependence on Pol32 and Pol2. However, we cannot find any evidence for the involvement of Rad52, which other studies found to be vital for BIR as it is for other homologous recombination reactions in yeast. Rad52 has multiple biochemical activities important for various modes of recombination; it can stimulate Rad51-mediated removal of RPA from single-stranded DNA regions allowing Rad51 to initiate strand displacement, and it can promote annealing of complementary strands in SSA, an activity enhanced by Rad59. In the absence of

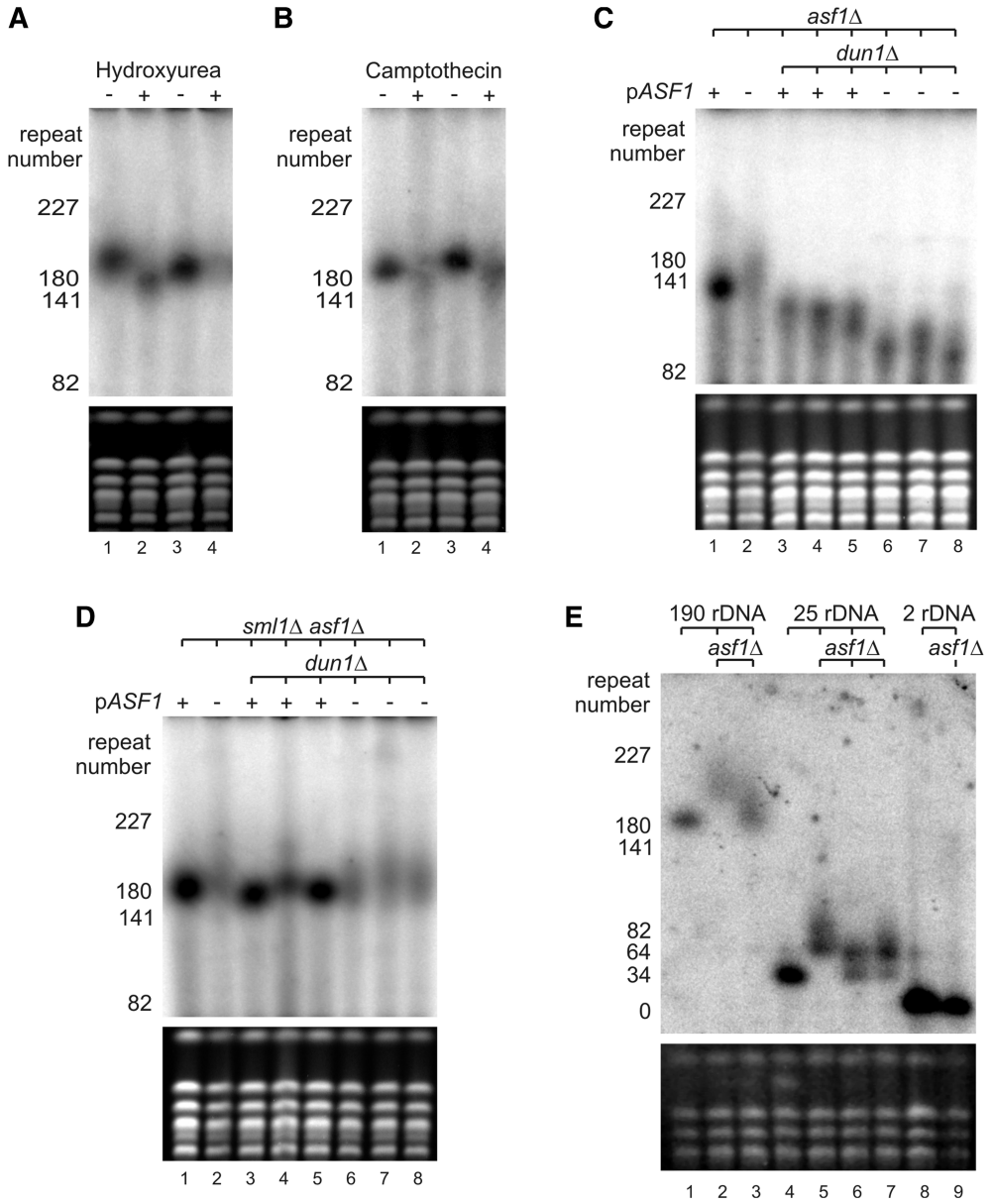

Figure 5. Repeat expansion requires processive replication of multiple repeats. (**A**) Hydroxyurea treatment. Wild-type cells were plated twice successively on plates containing 200 mM hydroxyurea followed by growth in YPD and PFGE analysis as in Figure 1B. (**B**) Camptothecin treatment performed as in (**A**) with 10 μg/ml camptothecin. (**C**) Analysis of *ASF1* loss in a *dun1*Δ background, performed as in Figure 3B. (**D**) Analysis of *ASF1* loss in an *sml1*Δ *dun1*Δ background, performed as in Figure 3B. The *asf1*Δ p*ASF1* strain was reconstructed in an *sml1*Δ background, followed by *DUN1* deletion and selection for plasmid loss. (**E**) Effect of *asf1*Δ in reduced rDNA repeat-number strains. *ASF1* was deleted in previously described strains carrying approximately 190, approximately 25 or 2 rDNA repeats and lacking *FOB1* (80,81), followed by four rounds of platings and PFGE analysis as in Figure 1B. Because growth of the 2-repeat strain is supported by GAL-driven rDNA expression from a plasmid with a *TRP1* marker, all manipulations of this strain were performed on synthetic GAL plates lacking Tryptophan, and to maintain consistency in the experiment all manipulations of the approximately 190 and 25 repeat strains were performed on synthetic GAL plates. Unlike other PFGEs shown in this work, this membrane was probed for *GAL2*, a single copy sequence on Chr. XII to avoid problems caused the widely different rDNA copy number of the strains in this experiment. The approximately 190 repeat strains give a weaker signal as the larger chromosomes transfer less efficiently onto the membrane.

Rad52, any single-stranded DNA that would initiate BIR is likely coated with RPA and so could neither initiate Rad51-dependent strand displacement nor efficiently anneal to other single-stranded regions.

Nonetheless, Rad52-independent BIR is not unknown. Microhomology-mediated BIR (MMBIR) (76) has been invoked to explain the formation of complex non-recurrent rearrangements underlying human gene copy number disorders, where break points in repeated sequences do not show the homologous sequences that are a hallmark of HR (77,78). This mechanism resembles BIR, except that it is initiated with only a few nucleotides

homology to the target sequence, leading to rearrangements with only microhomology detectable at the breakpoints. As for the rDNA expansions reported here, Rad51 and Rad52 are not required for MMBIR in yeast but Pol32 is necessary (61). Since this mechanism is Rad51-independent, it is likely that the free single strand that initiates MMBIR does so by annealing to another single-stranded region, albeit one with very limited homology. A source of single-stranded targets is other replication forks, as proposed for the fork stalling and template switching model (FoSTeS), in which the free DNA end at one broken replication fork can invade an adjacent fork, resulting in replication fork restart and DNA rearrangement (77). We propose that a FoSTeS-type mechanism is involved in rDNA expansion, with the single-stranded end from a collapsed replication fork invading an adjacent fork (Figure 6). This would form an alternative repair pathway that acts when BIR with the sister chromatid is blocked.

We found no evidence of partial rDNA repeats in *asf1*Δ strains, which would be clearly predicted if expansion events occurred at sites of microhomology. A key feature of the rDNA is the repetitive nature of the substrate, which turned out to be crucial for rDNA expansion since expansion was abolished in strains carrying only two rDNA repeats. Even in the context of ongoing rDNA expansion, a marker embedded inside the rDNA was not amplified. Within the rDNA, there is an unusually high density of homologous sequences undergoing replication, and, given that DNA replication appears to occur in discrete factories [reviewed in (79)], a free single-stranded end from one broken replication fork would rapidly be in very close proximity to a highly homologous single-stranded region at another fork. If higher homology leads to increased efficiency of annealing or fork restart, recombination would be strongly biased towards perfect repeat expansion. The problem remains of how a single-stranded region, which should be rapidly coated with RPA in the absence of the Rad52, can anneal to single-stranded regions at another replication fork, which should also be coated with RPA. It seems that there must be another factor capable of mediating strand annealing in the absence of Rad52, but the identity of this factor remains unknown. In summary, rDNA expansion in *asf1*Δ mutants appears to occur by a BIR-type mechanism, but represents a very unusual form of homologous recombination that works in the absence of key homologous recombination proteins.

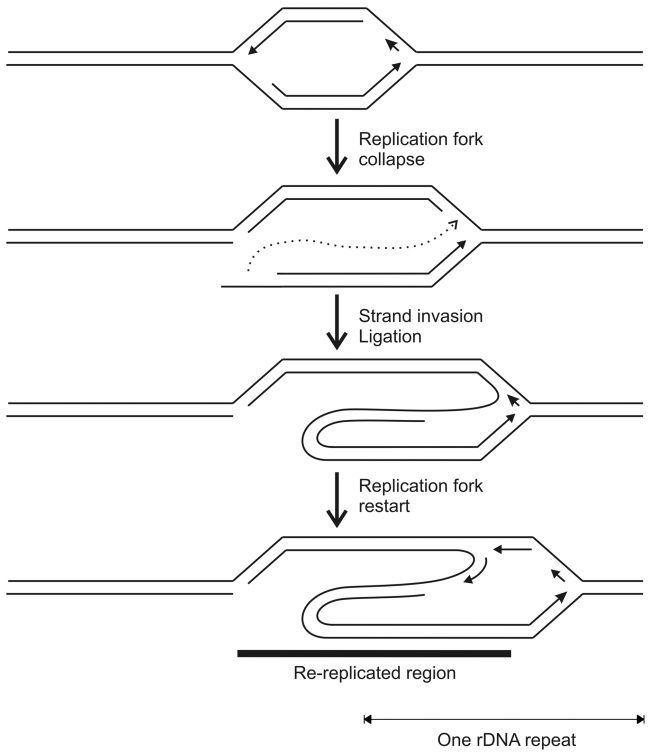

Figure 6. Proposed mechanism for rDNA expansion without the HR machinery. A replication fork collapses at one end of a replication bubble, leaving a single-stranded end from lagging strand synthesis. This single-stranded end anneals to the single-stranded region of another replication fork, and is extended and ligated to the newly synthesized strand by the same mechanism as an Okazaki fragment. The lagging strand synthesis from the invaded fork primes leading strand synthesis in the new fork, and replication fork restart occurs by an as yet unknown mechanism. This allows replication of the chromosome to the left of the diagram to be replicated, but with an additional rDNA repeat included.

One clear prediction of our model is that it is highly dependent on the freedom of the broken DNA end to move away and find another replication fork, which would be severely curtailed by cohesin binding in the rDNA. In keeping with this, in a *sir2*Δ background where rDNA cohesion is lost due to elevated ncRNA transcription, the repeat expansions that occur in *asf1*Δ cells are massively amplified. It should be noted that the mechanism proposed in Figure 6 is not innately expansion biased, as it would also be possible for the single-stranded region shown in Figure 6 to invade a converging replication fork (i.e. one approaching from the left of this image). This would cause rDNA contraction; however, replication fork restart would lead to partial chromosome XII re-replication and aneuploidy that would most likely be lethal, so such contractions would not be observed.

Human MMBIR-type genome rearrangements are medically important in generating copy number variations in germline and somatic cells, but are difficult to study because their occurrence is both temporally and spatially unpredictable. The yeast rDNA potentially provides a useful model for this process since the recombination events occur at reproducible and predictable rates and are limited to a well-defined genomic region.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We would like to thank Mike Tyers, Hille Tekotte and Takehiko Kobayashi for yeast strains; James Haber and Jorge B. Schvartzman for plasmids; Maria Vogelauer, Lea Harrington and David Leach for advice; Sveta Markovits

for insightful comments and Carmen Jack and Alex Murray for critical reading of the manuscript. Experiments were designed by J.H. and D.T., and performed by J.H. Manuscript was written by J.H. and D.T.

FUNDING

The Wellcome Trust (077248; 088335). Funding for open access charge: Wellcome Trust, via Tollervey lab.

Conflict of interest statement. None declared.

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