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Investigation

Rad9-mediated checkpoint activation is responsible for elevated expansions of GAA repeats in CST-deficient yeast

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Abstract

Large-scale expansion of $(GAA)_n$ repeats in the first intron of the *FXN* gene is responsible for the severe neurodegenerative disease, Friedreich's ataxia in humans. We have previously conducted an unbiased genetic screen for GAA repeat instability in a yeast experimental system. The majority of genes that came from this screen encoded the components of DNA replication machinery, strongly implying that replication irregularities are at the heart of GAA repeat expansions. This screen, however, also produced two unexpected hits: members of the CST complex, *CDC13* and *TEN1* genes, which are required for telomere maintenance. To understand how the CST complex could affect intra-chromosomal GAA repeats, we studied the well-characterized temperature-sensitive *cdc13-1* mutation and its effects on GAA repeat instability in yeast. We found that in-line with the screen results, this mutation leads to ~10-fold increase in the rate of large-scale expansions of the $(GAA)_{100}$ repeat at semi-permissive temperature. Unexpectedly, the hyper-expansion phenotype of the *cdc13-1* mutant largely depends on activation of the G2/M checkpoint, as deletions of individual genes *RAD9*, *MEC1*, *RAD53*, and *EXO1* belonging to this pathway rescued the increased GAA expansions. Furthermore, the hyper-expansion phenotype of the *cdc13-1* mutant depended on the subunit of DNA polymerase δ, Pol32. We hypothesize, therefore, that increased repeat expansions in the *cdc13-1* mutant happen during post-replicative repair of nicks or small gaps within repetitive tracts during the G2 phase of the cell cycle upon activation of the G2/M checkpoint.

Keywords: DNA repeat; repeat expansions; DNA damage checkpoint; chromosomal fragility; genome instability; hereditary disease; Friedreich's ataxia; telomere; CST complex; DNA repair

Introduction

DNA repeat-instability causes upwards of 40 neurodegenerative diseases in humans. This repeat instability, namely expansion and contraction of trinucleotide sequences, can occur within different domains of a carrier gene (Mirkin 2007; Khristich and Mirkin 2020). The trinucleotide repeat GAA (and its corresponding triplet on the opposite DNA strand—TTC) is present within the intron of the Frataxin gene, *FXN*, and expansion beyond the length of 35–100 repetitions, to over 500 repetitions, leads to the onset of a neuromuscular disorder known as Friedreich's ataxia (Campuzano *et al.* 1996). GAA/TTC repeats are known to form a triplex H-DNA (Mirkin 2006), which is an impediment for DNA polymerization *in vitro* (Gacy *et al.* 1998) and *in vivo* (Krasilnikova and Mirkin 2004b). In a yeast experimental system, GAA repeats appear to expand and contract during the course of replication (Shishkin *et al.* 2009; Shah *et al.* 2012; Tsutakawa *et al.* 2017; Jedrychowska *et al.* 2019; Khristich *et al.* 2020). However, repeat instability can also occur in non-dividing cells in FRDA patients (Bourn *et al.* 2012), as well as in yeast quiescent cells (Neil *et al.* 2021) during DNA repair. The results from an unbiased genetic screen for GAA repeat instability in yeast (Zhang *et al.* 2012) were in agreement with these models. It gave, however, two unexpected hits: the *CDC13* and *TEN1* genes, which encode essential components of the trimeric CST complex (Cdc13-Stn1-Ten1) (Grandin *et al.* 2001). In the screen, the yTHC essential gene collection was used wherein essential genes were placed under the TET promoter and addition of doxycycline resulted in downregulation of the gene. In conditions where expression should be downregulated (TET-OFF), TET-*CDC13* and TET-*TEN1* strains led to roughly an order of magnitude increase in the repeat expansion rate.

The CST complex plays a major role in the end-protection of the 3′ G-rich telomeric overhang (Lin and Zakian 1996; Webb *et al.* 2013) and in the regulation of telomerase recruitment (Nugent *et al.* 1996). Cdc13, the central DNA-binding protein in the complex, binds to the single-stranded G-rich telomeric tail sequence-specifically and recruits telomerase in early S-phase (Taggart *et al.* 2002). At the end of S-phase, cyclin-mediated phosphorylation of Stn1, the second subunit in the complex, leads to Stn1

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binding to Cdc13, followed by recruitment of Ten1 and formation of the end-protecting cap. Upon capping, telomerase can no longer be recruited until the next S-phase (Liu *et al.* 2014). It is unknown whether the CST complex has functions outside its roles at the telomere in Saccharomyces *cerevisiae*. It was therefore of interest to elucidate why proteins that associate with repetitive $G_{2–3}(TG)_{1–6}$ tracts at a telomere would have an effect on the instability of GAA/TTC tracts located in the middle of the chromosome.

Several possibilities could be considered to explain elevated repeat instability in CST hypomorphs. In the study by Zhang *et al.* (2012), we hypothesized that telomere uncapping in those strains could sequester the fork-stabilizing complex (Tof1-Mrc1-Csm3) to long single-stranded telomeric tails in CST hypomorphs, complicating replication through the GAA repeat (Krasilnikova and Mirkin 2004b; Shishkin *et al.* 2009). The CST complex has also been shown to coordinate replication at telomeres by stimulating DNA polymerase α priming activity in C-strand fill-in (Lue *et al.* 2014). More recently, the human CST complex was implicated in the recruitment of repair proteins to the replication fork, helping it progress through hard-to-replicate regions (Chastain *et al.* 2016). Finally, it was conceivable that the Rad9-dependent G2/M checkpoint, which is activated in Cdc13-deficient strains (Weinert and Hartwell 1993) could be responsible for the elevated repeat instability.

To unravel the role of Cdc13 in GAA instability, we decided to concentrate on the well-characterized Cdc13 mutant allele, *cdc13-1* (P371S). This proline to serine substitution occurs within the second oligonucleotide-binding fold (OB2) of the protein, which is very near to the nuclear localization signal and renders yeast cells temperature-sensitive. At temperatures over 27°C, this mutation leads to cell death. Recently, it was shown that the *cdc13-1* mutation leads to decreased levels of Cdc13 in the nucleus at elevated temperatures (Mersaoui *et al.* 2018). The decreased levels of Cdc13 are still sufficient for telomerase recruitment, resulting in the G-rich strand being extended by telomerase in the *cdc13-1* mutant. However, the level of Cdc13 is insufficient for Cdc13 dimerization and binding of Ten1 and Stn1 proteins to form the telomeric cap (Mason *et al.* 2013), resulting in aberrant resection of the telomeric C-strand by Exo1 and other nucleases (Garvik *et al.* 1995; Zubko *et al.* 2004). Altogether, these events lead to the hallmark of the *cdc13-1* phenotype—the presence of long single-stranded telomeric G-rich overhangs (Garvik *et al.* 1995; Nugent *et al.* 1996).

The presence of such telomeric overhangs triggers Rad9-mediated G2/M checkpoint activation and cell-cycle arrest (Garvik *et al.* 1995). Rad9 is recruited to telomeres upon the following cascade of events. First, Mec1 is partially activated upon its recruitment to RPA-coated ssDNA (Zou and Elledge 2003). Second, the "9-1-1" complex (Ddc1-Mec3-Rad17) is loaded to single-double-strand DNA junctions by Rad24 (Majka *et al.* 2006). Third, Dpb11 recruitment by Ddc1 leads to the full activation of Mec1 and phosphorylation of Rad9 (Pfander and Diffley 2011). Rad9 then recruits Rad53 (Navas *et al.* 1996). The DNA damage checkpoint (DDC) culminates in Rad53 phosphorylation and downstream signaling, and ultimately arrests the cell-cycle via blocking the metaphase to anaphase transition. In addition, specific to the DDC in cells with uncapped telomeres, is the inhibition of mitotic exit (Valerio-Santiago *et al.* 2013; Matellán and Monje-Casas 2020). Altogether this could provide additional time for post-replicative repair of nicks and small gaps that may be present at the GAA repeat locus.

Using our yeast experimental system for measuring genetic instability caused by the $(GAA)_n$ repeat (Shishkin *et al.* 2009; Shah *et al.* 2012), we found that the *cdc13-1* mutation leads to an over 10-fold increase in the rate of large-scale expansions of the $(GAA)_{100}$ repeat at the semi-permissive temperature, which is in-line with the earlier screen results (Zhang *et al.* 2012). This increase in expansions is due to the activation of the Rad9-mediated checkpoint pathway, since it is abolished by mutations in genes of this pathway, including *MEC1*, *EXO1*, and *RAD53*. It also depends on the Pol32 subunit of DNA polymerase δ, suggesting that filling in ssDNA gaps via DNA polymerization is necessary. We suggest, therefore, that G2/M checkpoint activation in response to telomeric uncapping in the *cdc13-1* mutant is a key step in driving repeat expansion at GAA repeats during the G2 phase of the cell cycle, where post-replicative repair of nicks or small gaps may incur further instability.

Materials and methods

Yeast strains and plasmids

Strains used in this study were made in the CH1585 background, isogenic to commonly used lab strain S288c, with the *UR-*GAA_{100}*-A3* cassette integrated into chromosome III, of the genotype *MATa leu2-Δ1 trp1-Δ63 ura3-52 his3-200 bar1Δ::HIS3 ChrIII(75423-75715)::UR--*GAA_{100}*-A3-TRP1*.

The *cdc13-1* mutation (P371S) was made using CRISPR with the bRA90 plasmid (gift from the Haber Lab), containing *CAS9*, a *LEU* selection marker, and into which the appropriate guide RNA had been cloned. The plasmid, along with an 80-mer containing the *cdc13-1* mutation as the repair template, was transformed into the KS001 strain containing GAA repeats (Shah *et al.* 2012). Yeast cells that had successfully taken up the plasmid were selected on media lacking leucine and further screened for the desired repair using the temperature-sensitive phenotype of *cdc13-1* cells (Nugent *et al.* 1996). The *cdc13-1* mutation was confirmed using Sanger sequencing and the plasmid expelled by growing on complete media, streaking for singles and confirming loss of plasmid in desired clones by observing no growth on media lacking leucine.

Deletion strains used for fluctuation test studies were made using standard PCR and transformation procedures (Gietz and Woods 2006) of either *hphMX4* or *natMX6* gene fragments.

The *rad53-K227A* mutation was constructed using CRISPR with the pRCC-N plasmid, containing the *CAS9* gene and *NAT* selection marker, amplified guide RNA to the target gene (GENEROSO et al. 2016) and transformed along with the repair template into wild-type and *cdc13-1* strains. Transformants were screened for increased sensitivity to 100 mM hydroxyurea and confirmed using Sanger Sequencing.

For fragility studies, the arm loss strain was made by integrating the *UR-*GAA_{100}*-A3* cassette into the non-essential arm of chromosome V as in McGinty *et al.* (2017). The DDRA strain was made by integrating the *AD-URA3-*GAA_{100}*-DE2* cassette into the endogenous *ADE2* location on chromosome XV (Polleys and Freudenreich 2020).

Yeast media

Standard yeast media were used. For fluctuation tests used to calculate GAA expansion rates, 5-fluoro-orotic acid (5-FOA) media was used with 1.75 g of 5-FOA per liter (1.75% 5-FOA) and 50 mg of uracil per liter (50 μg/ml final concentration) in synthetic dextrose (SD) complete media. To calculate GAA contraction rates, cells were selected on SD lacking uracil. For arm loss experiments using 5-FOA +canavanine media, 60 mg per liter of L-canavanine was used in SD media lacking tryptophan. For direct duplication and recombination fragility experiments, SD media lacking adenine and containing 5-FOA was used.

Fluctuation tests

Wild-type and *cdc13-1* strains containing the *UR-GAA*$_{100}$-*A3* cassette were incubated for 48 h and 72 h, respectively at the semi-permissive temperature of 27°C, or for 72 h for both strains at the permissive temperature of 23°C. Strains were grown on complete media supplemented with uracil (final concentration 50 μg/ml). At least 12 individual colonies with a confirmed initial tract length of GAA$_{100}$ (i.e. non-expanded) for at least two independent isolates of each strain, were serially diluted in sterile water such that for wild-type, a 10-fold dilution, and for *cdc13-1*, a 100-fold dilution of the resuspended colony was plated onto 5-FOA media. For other mutants, strains were grown between 48 h and 96 h on complete media supplemented with uracil at 27°C to achieve similarly sized colonies to wild-type. Colonies were serially diluted to achieve appropriate density of cells on 5-FOA media. A 10^5-fold dilution was plated onto complete media as a measure of total cell count. Yeast was grown at 23°C for 4 days and then colonies were counted. Rate calculations were made as described in (Radchenko *et al.* 2018). DNA from at least 96 colonies for each strain was isolated and PCR using primers A2 and B2 was conducted (see Supplementary Table S2) followed by gel electrophoresis to confirm expansion events.

For the contraction assay, wild-type and *cdc13-1* strains were grown at the indicated temperature to obtain single colonies (as for the expansion assay) and then plated onto either media lacking uracil (SD-Ura) or complete media, for a total cell count. Yeast was grown for 6 days at 23°C. Rate calculations were done as described above. Rates were considered to be significantly different if 95% confidence intervals for the rate values did not overlap between two samples (Foster 2006).

For the arm loss assay, wild-type colonies were grown on complete media for 3 days and *cdc13-1* colonies were grown on complete media for 5 days at 27°C. Both wild-type and *cdc13-1* strains were grown for 4 days at 23°C. Colonies were plated undiluted onto 5-FOA+ canavanine, and diluted 10^5-fold and plated on complete media. *CAN1* encodes an arginine permease and canavanine encodes a toxic arginine analog—loss of *CAN1* prevents uptake of this toxic analog and allows cells to survive on media containing canavanine. Yeast was grown at 23°C for 6 days and colonies were then counted. Rate calculations were done as described above.

For the DDRA assay, wild-type colonies were grown on complete media for 5 days and *cdc13-1* colonies were grown on complete media for 6 days at 27°C. Both wild-type and *cdc13-1* strains were grown for 5 days at 23°C. Colonies were plated at a 10^2-fold dilution onto 5-FOA -Ade, and diluted 10^5-fold and plated on complete media. Yeast was grown at 23°C for 5 days and colonies were counted. Rate calculations were done as described above. Rates were considered to be significantly different if 95% confidence intervals for the rate values did not overlap between two samples (Foster 2006).

Rad53 phosphorylation western blots

Strains were grown in YPD to log phase (OD_{600} = 0.6) and subsequently synchronized in G1/S by adding 3 μM α-factor (Zymo Research) for 90 min and released into the cell cycle with 50 μg/ml Pronase E (537088-10KU, Sigma Aldrich). Protein extraction (5 OD) was conducted at the indicated time-points after release as previously described (Foiani *et al.* 1994). Total protein extracts were normalized using a Non-Interfering Protein Assay™ Kit (Millipore), separated on a 10% gel, transferred to a PVDF membrane, and probed with an anti-Rad53 antibody (Abcam ab166859, 1:1000). Rad53 phosphorylation is observed as band retardation.

Telomeric G-overhang assay

To determine the amount of ssDNA present at the telomere, telomeric G-overhang assays were done as previously described (Bertuch and Lundblad 2003). Briefly, cells were grown to on OD_{600} of 1.0 in YPD at the permissive temperature (23°C), split and grown at either the permissive (23°C) or restrictive (30°C) temperature for an additional 4 h. Genomic DNA was isolated using phenol: chloroform and quantified using a Qubit (Thermo Fisher; Qubit dsDNA BR Assay kit #Q32853). DNA was normalized to 4 μG of DNA per sample. Following overnight *Xho*I digestion, the DNA was precipitated and resuspended in 12 μl of NEB buffer 2.1 (New England Biolabs, Buffer 2.1 #B7202S; 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 μg/ml BSA pH 7.9). The oligo 5′ CCC ACC ACA CAC ACC CAC CAC ACA CAC CCA CCA CAC 3′ was 5′ dATP γ^{32}p-end-labeled using T4 polynucleotide kinase (New England Biolabs, T4 PNK #M0201S). Labeled probe was added to DNA samples and hybridized for 10 min at 37°C followed by 1 h on ice. The samples were loaded into a 0.75% agarose gel and electrophoresed at 45 V for 16 h. The gel was dried and imaged using a phosphorimager (GE Amersham Typhoon). Following detection of the single-strand telomeric signal, the gel was denatured, neutralized, and rehybridized with the same oligonucleotide probe in order to detect the total amount of telomeric DNA. Quantification of G-overhangs was done using ImageQuant, where the signal in the native gel was measured and divided by the total signal in the denatured gel of each indicated sample.

Analysis of replication intermediates by 2D N/N gel-electrophoresis

100 GAA repeats were cloned between *BamH*I and *Xba*I of pYES6 plasmid. Yeast cells were transformed with the constructed plasmid by lithium acetate method (Gietz and Woods 2006) and selecting on YPD containing blasticidin (50 μg/ml). Cells were grown at 23°C (*cdc13-1*) or 30°C (wild-type) in YPD with blasticidin until OD_{600} = 1.5. The length of repetitive tracts was confirmed, before and after the cell culture, by yeast colony PCR as described in Radchenko *et al.* (2018) with primers flanking the inserts, and amplicons were resolved on 1.5% agarose gels. Replication intermediates were isolated according to the "QIAGEN genomic DNA Handbook," using genomic-tip 100/G columns. DNA was digested by *Nde*I and *Ps*iI (New England Biolabs) for 7 h at 37°C. First-dimension gels (0.4% agarose in TBE 1×) were run at 1 V/cm for 22 h at room temperature, while second-dimension gels (1% agarose in TBE 1×) were run at 5 V/cm for 9 h at 4°C in the presence of ethidium bromide (0.3 μg/ml). Gels were washed 15 min in 0.25 N HCl before an overnight transfer to a charged nylon membrane (Hybond-XL, GE Healthcare) in 0.4 N NaOH. Hybridization was performed overnight with a 413 bp randomly primed probe, corresponding to the Gal promoter region of pYES6 plasmid. Membranes were washed twice with washing solution I (SSC 2×, 1% SDS) pre-heated at 65°C and twice with washing solution II (SSC 0.1×, 0.1% SDS) pre-heated at 42°C. Membranes were exposed on IR-sensitive screens for 2-5 days and detection was performed on a Pharos FX PhosphorImager (Bio-Rad). Densitograms were done with ImageJ (NIH) and quantification analysis was done as described in (Krasilnikova and Mirkin 2004a). Statistical analysis was performed with GraphPad Prism software. Mean percentage of replication fork slowing values was compared by unpaired *t*-test.

Results

GAA expansions and contractions of an expanded GAA tract are increased in the *cdc13-1* mutant

Previously, a screen to detect genes important in preventing fragility and expansion of an expanded GAA trinucleotide repeat (Zhang *et al.* 2012) identified the essential genes *CDC13* and *TEN1*—which encode proteins that form the telomeric cap in *S. cerevisiae*, known as the CST complex (Cdc13-Stn1-Ten1). Specifically, a TET-OFF version of *CDC13* showed a 14-fold increase in GAA expansion rate over the wild-type in the downregulated state, suggesting that reduced levels of Cdc13 were responsible. We confirmed and added to this finding by showing that GAA expansion rate increases in a doxycycline dose-dependent manner in a strain carrying the TET-*CDC13* allele (Supplementary Figure S1). Even in the absence of doxycycline, there is a slight (2-fold) increase in repeat expansions, which could be due to leaky downregulation or out-of-register expression of the TET-*CDC13* allele.

We then concentrated on the effect of the well-studied temperature-sensitive *cdc13-1* (P371S) mutation on GAA repeat expansions. This mutant was chosen due to its decreased level of Cdc13 in the nucleus (Mersaoui *et al.* 2018), similarly to the hypomorphic TET-*CDC13* strain. As a result of decreased levels in the nucleus in the *cdc13-1* strain, the mutant Cdc13 protein is still able to recruit telomerase but is unable to dimerize and recruit Stn1 and Ten1 and form the telomere-capping complex CST (Mason *et al.* 2013).

Our expansion cassette contains a *URA3* gene, artificially split by an intron carrying 100 GAA repeats, which is placed into chromosome III adjacent to the early-firing origin ARS306 (Shishkin *et al.* 2009) (Figure 1A), which is ~80 kb away from the nearest telomere. Due to the inability of yeast to splice introns over one kilobase in length (Yu and Gabriel 1999), the expansion events adding more than 10 repeats preclude *URA3* splicing, making yeast resistant to 5-fluoroorotic acid (5-FOA). We have previously found that, on average, ~50 repeats are added at a single step (Shah *et al.* 2012).

The *cdc13-1* strain containing the *UR*-GAA_{100}-*A3* cassette was grown either at the permissive temperature of 23°C or the semi-permissive temperature of 27°C on non-selective media and then individual colonies were plated on selective media containing 5-FOA. Repeat expansions among 5-FOA-resistant clones were confirmed via single-colony PCR and the rate of expansions was calculated using FluCalc—a webtool using a maximum-likelihood estimator algorithm (Radchenko *et al.* 2018). The *cdc13-1* strain showed an 18-fold increase in GAA expansion events over the wild-type at the semi-permissive temperature of 27°C (Figure 1B). No difference in expansion rates between the mutant and the wild type was observed at the permissive temperature of 23°C.

Our experimental system can also be tuned to study GAA repeat contractions (Khristich *et al.* 2020). To this end, we use a cassette bearing 150 GAA repeats in the *URA3* intron, which renders the cell URA^-. Large-scale (~60 repeats) contractions restore *URA3* splicing making cells URA^+. The *cdc13-1* mutation resulted in a 7-fold increase in the contraction rate at the semi-permissive temperature, while no difference from wild-type at the permissive temperature was observed (Figure 1C). Taken together, at the semi-permissive temperature, the *cdc13-1* mutant increases GAA repeat instability.

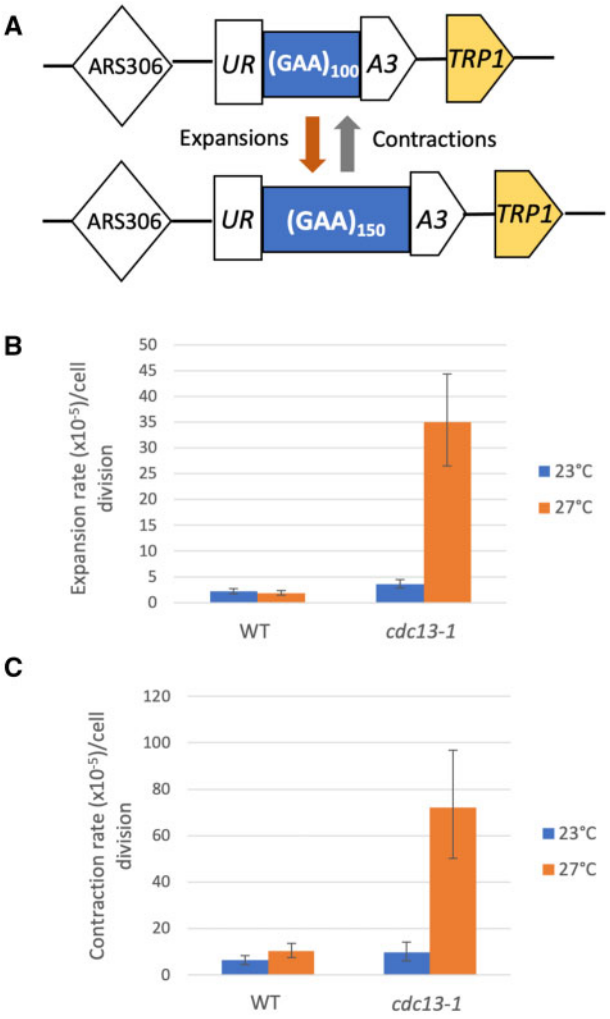

Figure 1 Cdc13 counteracts GAA repeat expansions and prevents contractions. (A) Diagram of large-scale expansions or contractions of $(GAA)_n$ repeats in yeast (see text for details). (B) The *cdc13-1* mutation increases expansion rate of a GAA_{100} repeat at the semi-permissive temperature. Wild-type and *cdc13-1* strains containing 100 repeats (*UR*-GAA_{100}-*A3*) were grown on complete media followed by plating on 5-FOA media. Bars represent 95% confidence intervals; number of experiments (n) ≥2. (C) The *cdc13-1* mutation increases contraction rate of the GAA_{150} repeat at the semi-permissive temperature. Wild-type and *cdc13-1* strains containing 150 repeats (*UR*-GAA_{150}-*A3*) were grown on complete media as for the expansion assay, followed by plating on media lacking uracil. Bars represent 95% confidence intervals; $n \geq 2$. ARS, autonomous replicating sequence; *TRP1*, tryptophan biosynthesis gene.

Effects of the *cdc13-1* mutation on GAA repeat instability are not due to increased fork stalling at the repeat

A priori, increases in repeat expansions and contractions could be attributed to general defects in replication in the *cdc13-1* mutant. For example, several studies in humans suggest that CTC1, the ortholog of yeast Cdc13, has a role in DNA replication outside of telomeres (Miyake *et al.* 2009; Chastain *et al.* 2016). In addition, in the original genetic screen paper (Zhang *et al.* 2012), we hypothesized that telomere uncapping in CST hypomorphs could lead to sequestering the fork-stabilizing complex (Tof1-Mrc1-Csm3) to long single-stranded telomeric tails, leading to problematic

replication fork progression through the GAA repeat and replication fork stalling (Krasilnikova and Mirkin 2004b; Shishkin *et al.* 2009). Elevated fork stalling at the repeat could then cause an increase in repeat expansions and contractions, as was previously shown by us and others (Pelletier *et al.* 2003; Krasilnikova and Mirkin 2004b; Kerrest *et al.* 2009; Voineagu *et al.* 2009b; Gadgil *et al.* 2017; Gellon *et al.* 2019).

To test whether the *cdc13-1* mutation causes replication defects, we compared replication fork stalling in the wild-type and *cdc13-1* strains at the GAA repeat using 2-dimensional (2-D) neutral/neutral gel-electrophoresis of replication intermediates—an approach that is well established from our earlier studies of repeat-mediated fork stalling (Krasilnikova and Mirkin 2004a). A high copy-number plasmid containing a 2 μm-plasmid replication origin, a blasticidin selection marker, and the $(GAA)_{100}$ repeat was transformed into both the wild-type and the *cdc13-1* mutant strains (Figure 2A), which allowed us to analyze a large amount of replication intermediates. While the epigenetic context of the repeat may differ between this plasmid and our cassette on chromosome III, we have previously demonstrated that there is no difference, in either the strength or the orientation dependence of the repeat-mediated fork stalling, between the multicopy plasmid and chromosome III (Shishkin *et al.* 2009).

Yeast cultures were grown at either the permissive (23°C) or the semi-permissive (27°C) temperature followed by the isolation of replication intermediates, their separation by 2-D gel electrophoresis and Southern blot hybridization to visualize plasmid replication. In this approach, the appearance and intensity of the bulge on the otherwise smooth replication arc reflects the appearance and the strength of the replication fork stalling. Figure 2 shows repeat-mediated fork stalling in the wild-type and *cdc13-1* strains at both temperatures. Quantification of the strength of the replication fork stalling—the radioactivity in the bulge normalized to the smooth replication arc (Krasilnikova and Mirkin 2004a)— shows that the *cdc13-1* mutation does not cause an increase in fork stalling at the repeat at the semi-permissive temperature. If anything, it slightly (1.5×) decreases it (Figure 2, C and D), but this decrease is not statistically significant. We conclude, therefore, that the observed increase in repeat instability in the *cdc13-1* mutant is not caused by an increase in impaired replication through the GAA repeat.

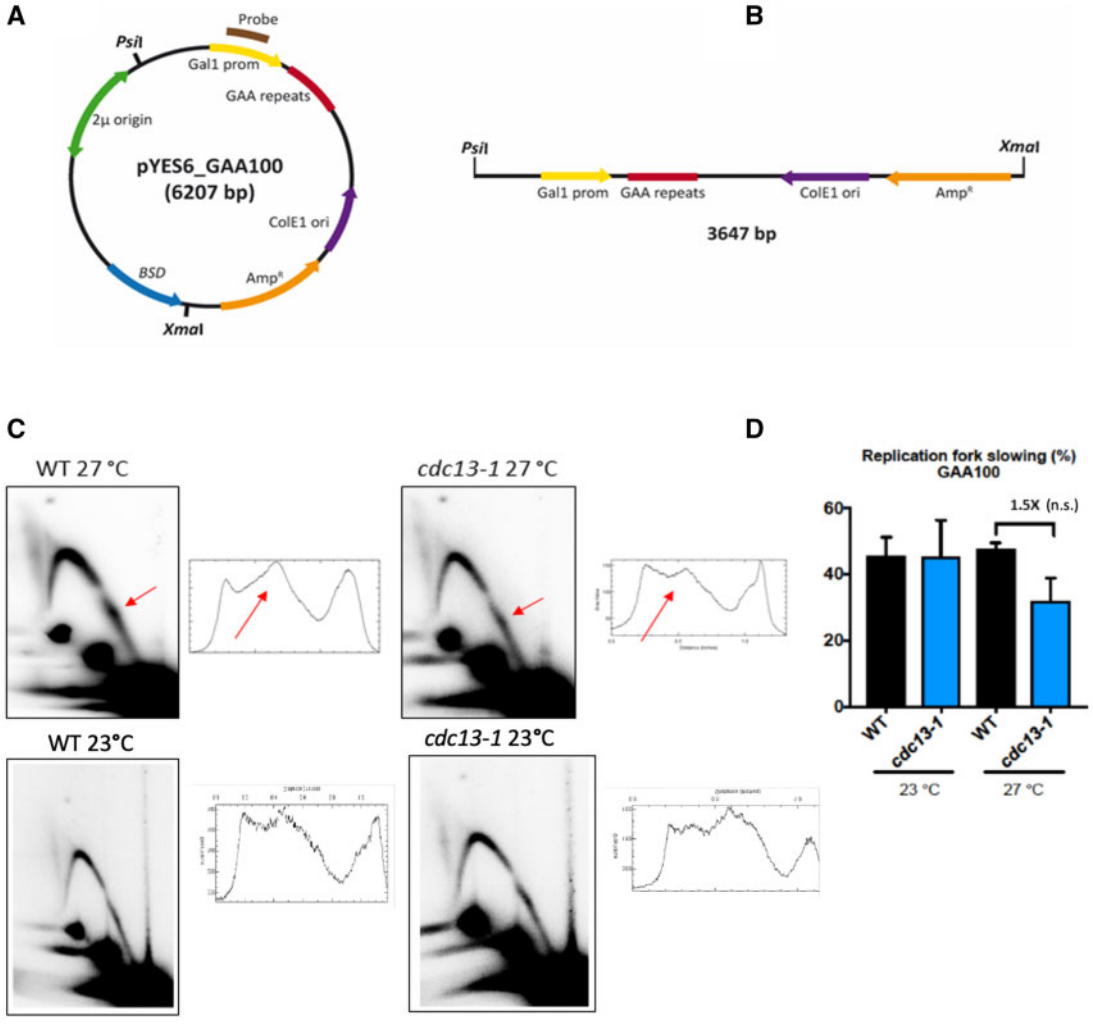

Figure 2 The *cdc13-1* mutant displays no significant effect on fork stalling at GAA repeats. (A) A high-copy 2 μm plasmid containing a blasticidin marker for selection in yeast and a probe site near the $(GAA)_{100}$ repeat tract was transformed into both wild-type and *cdc13-1* strains. (B) Restriction sites *PsiI* and *XmaI* allow for repeats to be placed about one third of the distance from start of probed fragment (3647 bp in length). (C) *cdc13-1* does not show increased fork stalling at GAA repeats. Wild-type and *cdc13-1* strains were grown at either 23°C or 27°C to $OD_{600} = 1.5$ and replication intermediates isolated. Analysis of replication intermediates using 2D gel electrophoresis was conducted as described in Methods. (D) Quantification of fork-slowing. Mean percentage of replication fork slowing values was compared by unpaired *t*-test ($n = 2$). *BSD*, blasticidin resistance gene; n.s., non-significant.

The *cdc13-1* mutation does not have a *bona-fide* effect on GAA fragility

The TET-*CDC13* allele was initially identified in a screen for GAA fragility resulting in chromosome V arm loss (Zhang *et al.* 2012). We, thus, conducted a similar experiment by introducing the *UR*-GAA_{100}-*A3* cassette (described above) into the non-essential arm of chromosome V (McGinty *et al.* 2017) in the *cdc13-1* strain. The cassette was placed centromere-proximal to the *CAN1* gene, so that the breakage at the GAA tract and consequent arm loss would make survivors resistant to both 5-FOA and canavanine (Figure 3A). The rate of these events at the permissive and non-permissive temperatures was then calculated using FluCalc. Similarly, to the observations from the screen, the *cdc13-1* mutant showed a 50-fold increase in GAA-mediated arm loss as compared to the wild-type at the semi-permissive temperature (Figure 3B). Importantly, however, the rate of arm loss is a composite of two variables: the rate of breakage at the repeat and the efficiency of telomere addition to the broken chromosome, and both processes could be affected by the *cdc13-1* mutation. As replication fork progression through the GAA repeats is similar to wild-type (Figure 2), it is unlikely that the *cdc13-1* mutant (directly or indirectly) complicates DNA replication through the repeat resulting in breakage. However, the increased arm loss in the *cdc13-1* mutant could be explained by more efficient telomere addition owing to the telomere-capping deficiency (Nugent *et al.* 1996), which we found would not answer whether more breaks are happening at the GAA tract in the *cdc13-1* mutant.

To answer this question, we instead decided to use an alternative fragility assay that does not involve telomere addition called the **d**irect **d**uplication and **r**ecombination **a**ssay (DDRA) (Freudenreich *et al.* 1998; Polleys and Freudenreich 2020). In this assay, the endogenous *ADE2* gene is broken into two halves with overlapping regions of 968 bp of homology, which are separated by the functional *URA3* gene and the repeat of interest (in our case the $(GAA)_{100}$ repeat). Yeast containing this construct are ADE- and URA+ (Figure 3C). Breakage at the repeat tract, followed by end-resection and single-strand annealing of the *ADE2* homologies, leads to the appearance of ADE+ and 5-FOA-resistant clones (Figure 3C). The rate of those events is a proxy for repeat-mediated fragility. Using this assay, we found no increase in the rate of fragility in the *cdc13-1* mutant at the semi-permissive temperature (Figure 3D).

An apparent increase in repeat-mediated chromosome arm loss in Figure 3B could be due to the lack of capping and profound telomere elongation in the *cdc13-1* mutant, which might facilitate de-novo telomere addition. The same reasoning can be used to explain the elevated repeat-mediated arm-loss in TET-*CDC13* and TET-*TEN1* hypomorphs observed in Zhang *et al.* (2012). As the DDRA assay does not require *de novo* telomere addition, it is a better measure of repeat associated fragility in the *cdc13-1* mutant. Taken together, these data imply that the *cdc13-1* mutation does not cause an increase in *bona-fide* chromosomal fragility at the GAA repeat.

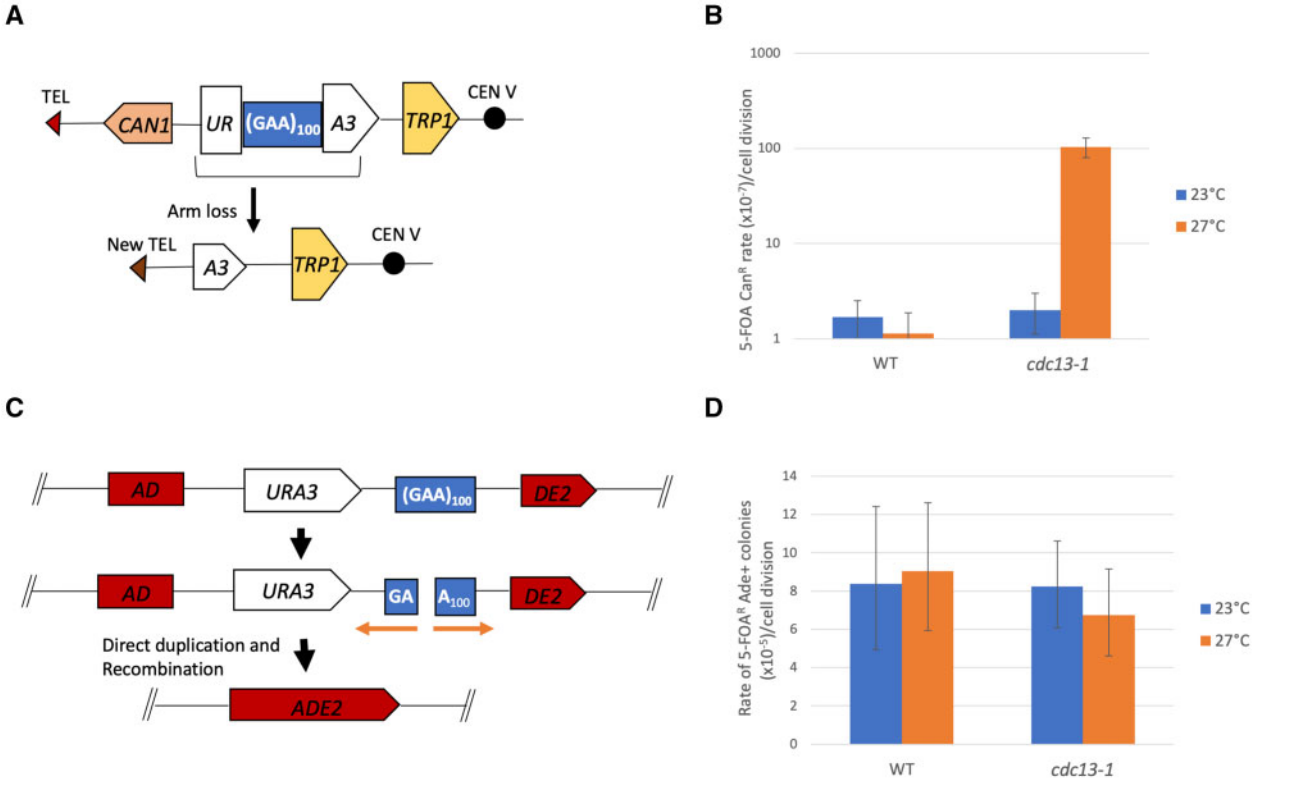

Figure 3 The *cdc13-1* mutation has no *bona-fide* effect on GAA repeat fragility. (A) Experimental system to study chromosomal arm loss caused by the GAA_{100} repeat (see text for details). Breakage at the GAA repeats will lead to the loss of *URA3* and *CAN1* genes, followed by repair through de-novo telomere addition, allowing growth on 5-FOA + canavanine media. (B) The *cdc13-1* mutation leads to an increase in arm loss at the semi-permissive temperature. Bars represent 95% confidence intervals; number of experiments (n) ≥2. (C) Experimental system to study fragility via a **d**irect **d**uplication and **r**ecombination **a**ssay (DDRA) (see text for details). Breakage at the GAA_{100} repeat tract will lead to resection, loss of *URA3*, and recombination to restore *ADE2*, and hence growth on 5-FOA -Ade. (D) The *cdc13-1* mutation leads to no effect on fragility of GAA repeats at the semi-permissive temperature. Bars represent 95% confidence intervals; n ≥2. *CAN1*, canavanine resistance gene; CEN V, centromere chromosome V; TEL, telomere; *TRP1*, tryptophan biosynthesis gene; *URA3*, uracil biosynthesis gene.

Rad9-mediated checkpoint activation by single-stranded telomeric overhangs is responsible for increased GAA expansion rate in the *cdc13-1* mutant

The *cdc13-1* mutation is known to induce a potent DDC response due to the presence of uncapped telomeres (Garvik *et al.* 1995). This culminates in Rad53-phosphoryation and, ultimately, in cell cycle arrest (Lydall and Weinert 1995). It is well established that checkpoint activation in the *cdc13-1* mutant occurs during the G2/M transition and is dependent on Rad9 (Weinert and Hartwell 1993). Western blotting using antibodies to Rad53 on protein lysates from α-factor arrested and released cells confirmed the phosphorylation of the Rad53 protein, as visible by its mobility shift during the G2/M transition (120 min post-release) in the *cdc13-1* mutant. This was not observed in the wild-type strain (Figure 4A, top panel). Furthermore, this shift was completely ablated in the *cdc13-1 rad9*Δ double mutant (Figure 4B).

In our experimental setting, we determine repeat expansion rates after multiple cell divisions. Thus, to better correlate the checkpoint activation phenotype with the expansion rates in the *cdc13-1* mutant, we also conducted an experiment in which the *cdc13-1* strain was grown at the semi-permissive temperature for multiple generations (8+) before determining the Rad53 phosphorylation status. In this setting, we also observed the Rad53 phosphorylation only during the G2/M transition (120 min post-release) (Figure 4a, bottom panel). These data confirm that only the G2/M checkpoint is activated in the *cdc13-1* mutant even when it grows for multiple generations at the semi-permissive temperature (Figure 4A).

Rad9 activates the DDC in the *cdc13-1* mutant in response to the presence of long stretches of single-stranded DNA resulting from the uncontrolled end-resection of uncapped telomeres by the Exo1 nuclease (Garvik *et al.* 1995; Zubko *et al.* 2004). We confirm that our *cdc13-1* mutant has longer telomeres even at the permissive temperature (Supplementary Figure S4A, denatured gel; Supplementary Figure S5), as well as long single-stranded telomeric overhangs at the restrictive temperature (Supplementary Figure S4A, native gel; Grandin and Charbonneau 2007). Deleting *RAD9* in the wild-type background had no effect on telomere length or G-tail overhangs (Supplementary Figure S4), while the *cdc13-1 rad9*Δ double mutant showed elongated telomeres, but slightly less single-stranded overhangs (Supplementary Figure S4) (Zubko *et al.* 2004).

Next, we evaluated the effect of *RAD9* deletion on the rate of repeat expansions in the *cdc13-1* mutant. We confirmed that, as previously shown, *RAD9* deletion increases the viability of *cdc13-1* strains (Supplementary Figure S3; Weinert and Hartwell 1993; Zubko *et al.* 2004). While *rad9*Δ single mutants had no effect on GAA expansions, deleting *RAD9* reduced GAA expansions in the *cdc13-1* mutant 3-fold at the semi-permissive temperature (Figure 4C).

Rad9-mediated activation of the checkpoint occurs when large tracts of single-stranded DNA (ssDNA) become exposed due to exonuclease-mediated resection of the 5′-end of the telomere (Lydall and Weinert 1995). One of the major nucleases responsible for telomere end-resection is Exo1 (Maringele and Lydall 2002; Ngo and Lydall 2010). Consistent with the possibility that checkpoint activation due to long tracts of ssDNA at the telomere is responsible for the increased GAA expansions, we observed that deletion of *EXO1* completely rescued GAA expansions in the *cdc13-1* mutant at the semi-permissive temperature, while the *exo1* deletion alone had no effect on instability (Figure 4C).

Mec1 is a PIKK family kinase that senses the RPA-coated single-stranded DNA through its binding partner Ddc2 and phosphorylates the Dpb11 protein, which allows Rad9 recruitment to DNA (Pfander and Diffley 2011). Given that Mec1 is required for both sensing single-stranded DNA and promoting Rad53 phosphorylation for full checkpoint activation, we tested the effect of *MEC1* deletion on GAA repeat expansions. If checkpoint activation is responsible for elevated GAA expansions in the *cdc13-1*

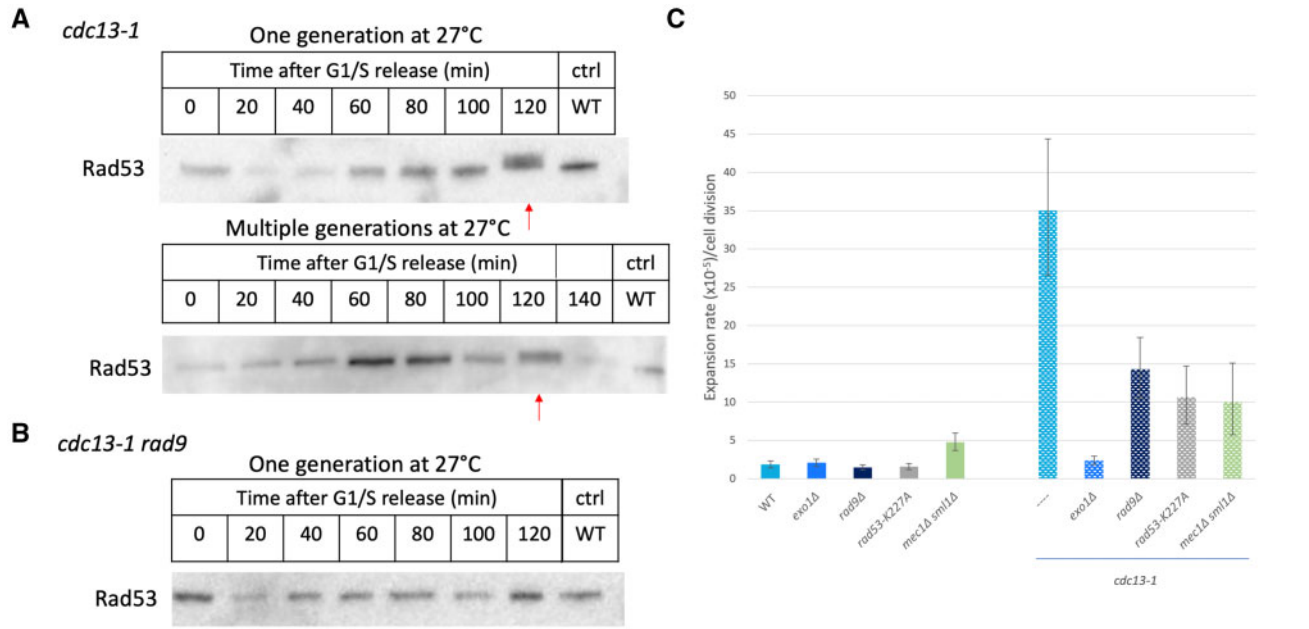

Figure 4 Rad9-mediated Checkpoint activation in G2/M is responsible for increased GAA expansion rate in the *cdc13-1* mutant. (A and B) The *cdc13-1* strain shows Rad9-mediated checkpoint activation in G2/M. Strains were grown in YPD at 23°C or 27°C. To log phase, synchronized in G1/S, and released into the cell cycle at 27°C. Protein extracts for each time-point were probed with anti-Rad53 antibody. Rad53 phosphorylation is observed as band retardation. (C) Checkpoint genes partially rescue the GAA hyper-expansion phenotype in the *cdc13-1* mutant at 27°C. Bars represent 95% confidence intervals; number of experiments (n) ≥2.

mutant, removing the principal sensor kinase, should reverse this effect. Indeed, that is what we saw: deletion of *MEC1*, in the *sml1Δ* background to preserve viability (Zhao *et al.* 1998), decreased the GAA expansion rate 3-fold, similarly to a *RAD9* deletion. Note that in the wild-type background, *MEC1* deletion actually increases repeat expansions 2-fold (Figure 4C). Finally, a kinase-deficient allele of Rad53, *rad53-K227* (Fay *et al.* 1997), also led to a rescue in GAA expansions in the *cdc13-1* mutant at the semi-permissive temperature (Figure 4C).

In sum, we conclude that the elevated rate of GAA repeat expansions observed in the *cdc13-1* mutant primarily, albeit not exclusively, results from G2/M checkpoint activation. Additional support for this conclusion, comes from the fact that we do not see an increase in repeat expansions in the *cdc13-2* (E252K) mutant (Supplementary Figure S2). This mutant impairs telomerase recruitment, but not telomere capping and/or Rad9-mediated G2-M checkpoint activation (Lendvay *et al.* 1996; Nugent *et al.* 1996; Chandra *et al.* 2001; Grandin *et al.* 2005).

The hyper-expansion phenotype in the *cdc13-1* mutant depends on Pol32, the processivity subunit of DNA Polymerase *δ*

Our data indicate that repeat expansions observed in the *cdc13-1* mutant result primarily from the activation of the Rad9-mediated G2/M checkpoint pathway. The DDC in cells with eroded telomeres is known to inhibit mitotic exit (Valerio-Santiago *et al.* 2013) as well as to induce a metaphase to anaphase transition arrest in *S. cerevisiae* (Gardner and Burke 2000). Altogether, this should provide enough time to repair any nicks or gaps left over from replication. We hypothesized, therefore, that repair of nicks or short gaps left within GAA tracts post-replicatively could result in the accumulation of extra expansions upon G2/M checkpoint activation.

We have recently demonstrated that nick-repair is responsible for large-scale expansions of GAA repeats in non-dividing yeast cells (Neil *et al.* 2021). This mechanism requires intact, processive DNA polymerase δ (Pol δ) containing the Pol32 subunit. We were wondering, therefore, if a similar mechanism could account for GAA expansions in the G2 phase of the cell cycle in the *cdc13-1* mutant. In order to test the role of Pol δ, we deleted the *POL32* gene, encoding the processivity subunit of Pol δ needed to synthesize long tracts of DNA (Gerik *et al.* 1998; Johansson *et al.* 2004), and thus, required for efficient nick- or gap-repair. Pol32 may also be contributing to the post-replicative repair of gaps or nicks at GAA repeats as an essential subunit of TLS polymerase Pol ζ (Makarova *et al.* 2012), though our preliminary data indicate that this is a less likely scenario in our case. The loss of Pol32 did not have any effect on GAA repeat expansions in the wild-type background, similarly to what we saw earlier for dividing yeast (Neil *et al.* 2018). In contrast, in the *cdc13-1* mutant background, it resulted in a rescue of the hyper-expansion phenotype, which was qualitatively (3-fold) similar to the effect of Rad9 checkpoint gene knockouts (Figure 5A).

We then characterized the telomere structure in the *cdc13-1 pol32Δ* double mutant. We observed an increased, albeit non-significantly, amount of ssDNA at the telomere (Figure 5B), and a partial rescue of the overall telomere length (Supplementary Figure S5) in the *cdc13-1 pol32Δ* double mutant. Hypothetically, the presence of extra ssDNA could lead to additional activation of the Rad9-mediated checkpoint, *i.e.*, more expansions, yet, the lack of Pol32 rescues the hyperexpansion phenotype of the *cdc13-1* mutant (Figure 5A). We conclude, therefore, that Pol32-mediated rescue in GAA expansion rate is not due to the changes in the telomere structure of the *cdc13-1 pol32Δ* mutant.

Pol δ plays a role in several DNA repair pathways. Most notably, its processivity subunit Pol32 was implicated in break-induced replication (BIR) during G2 phase (Kramara *et al.* 2017). The latter process requires strand invasion into the sister chromatid, which depends on either Rad52 or Rad51 proteins. We, thus, tested the GAA_{100} expansion rate in *cdc13-1* mutants lacking homologous-recombination proteins Rad51 and Rad52. It appeared that the lack of these proteins did not have any effect on elevated GAA expansions in the *cdc13-1* mutant (Figure 5A). Besides BIR, we also found that deletion of the *RAD5* gene in the *cdc13-1* mutant did not change its hyper-expansion phenotype (Figure 5A) which effectively rules out the role of post-replicative gap repair via template switching.

We conclude, therefore, that processive DNA polymerase δ synthesis through the repeat, initiated at nicks and/or small gaps, is at the heart of GAA repeat expansions upon G2/M checkpoint activation in the *cdc13-1* mutant (Figure 6).

Discussion

We and others have previously found that GAA repeat expansions in yeast occur primarily during DNA replication (Shishkin *et al.* 2009; Shah *et al.* 2012; Tsutakawa *et al.* 2017; Jedrychowska *et al.* 2019). In this study, we found that expansions can further accumulate upon G2/M checkpoint activation in the *cdc13-1* mutant (Figure 1B). Previously, expanded CAG repeat tracts were shown to cause checkpoint activation and decreased cell viability (Voineagu *et al.* 2009a; Sundararajan and Freudenreich 2011), and CAG repeat expansions preferentially occurred in cells experiencing checkpoint-mediated growth delays (Sundararajan and Freudenreich 2011). Consistent with these results, we show that GAA repeat instability is increased as a result of G2/M checkpoint activation, and that expansions are caused by Pol32-mediated DNA synthesis.

The original observation of the increased repeat expansions in the CST hypomorphs came from an unbiased yeast genetic screen (Zhang *et al.* 2012). It was hypothesized that long tracts of single-stranded DNA at uncapped telomeres, resulting from a deficient CST complex, may titrate the replication stabilizing complex, including the Tof1 protein, additionally complicating fork progression through long GAA repeats. This hypothesis does not seem to agree with our data, as we see no increase (in fact a slight decrease) in the replication fork stalling at GAA repeats in the *cdc13-1* mutant.

In accord with the original study, we observed a strong increase of GAA-mediated chromosome arm-loss in the *cdc13-1* mutant (Figure 3, A and B). However, a different method of measuring fragility, the DDRA assay, showed no effect of the *cdc13-1* mutation on GAA-mediated fragility (Figure 3, C and D). Since the rate of arm loss depends on the efficiency of breakage and the efficiency of telomere addition to the broken chromosome, we conclude that an increase in repeat-mediated chromosome arm loss is likely due to the lack of capping causing profound telomere elongation in the *cdc13-1* mutant and facilitating *de novo* telomere addition. The fact that the *cdc13-1* mutation does not complicate replication fork progression through the repeat (Figure 2, C and D) or cause increased fragility (Figure 3D) strongly argues that additional repeat expansions in this mutant accumulate outside of S-phase. We do not believe, therefore, that Cdc13 directly contributes to the replication of GAA repeats in our system.

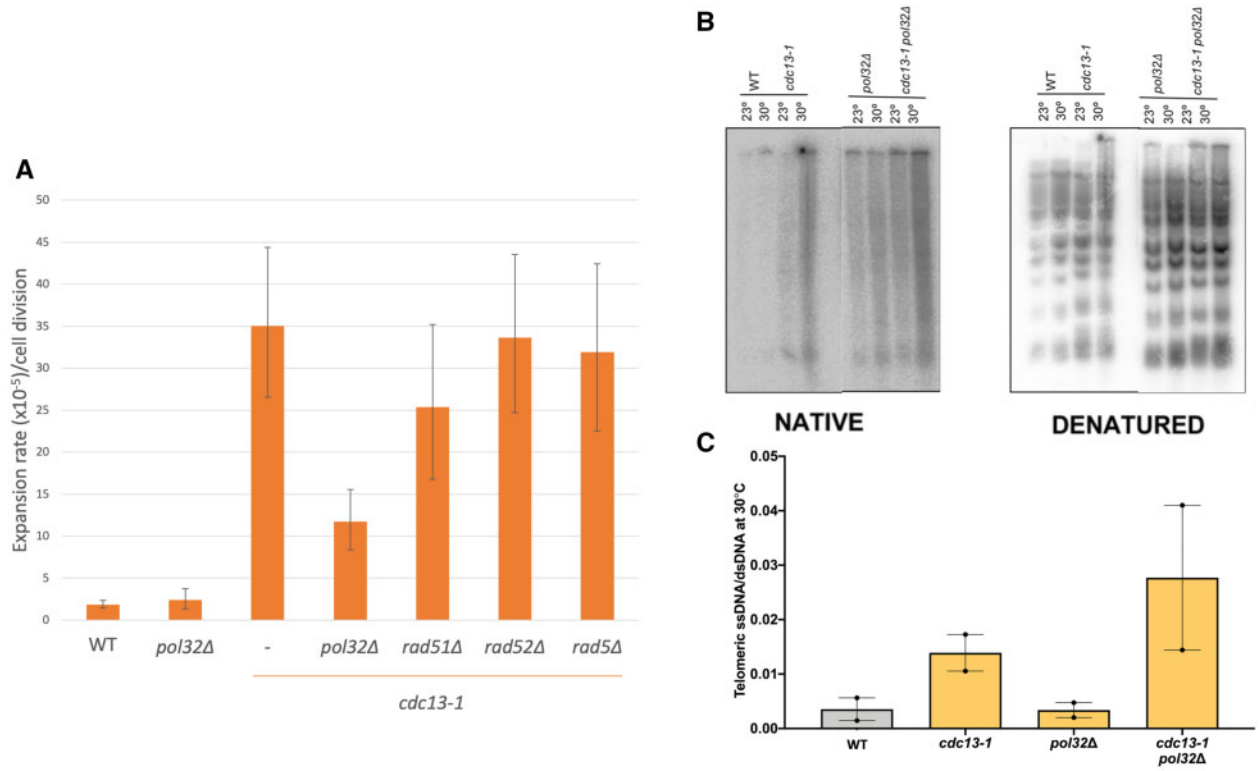

Figure 5 Expansions observed in *cdc13-1* are mediated by loss of Pol32, the processivity subunit of Pol δ, while deletion of *POL32* worsens telomere ssDNA phenotype. (A) Deletion of *POL32* partially rescues GAA hyper-expansion phenotype in the *cdc13-1* mutant at 27°C. Homologous recombination and template-switch-mediated repair do not play a role in GAA expansions in the *cdc13-1* mutant. Bars represent 95% confidence intervals; number of experiments (n) ≥2. (B) Deletion of *POL32* slightly increases the increased single-stranded telomeric G-overhangs at 30°C in the *cdc13-1* mutant. *Native*: The *cdc13-1 pol32Δ* strain shows increased single-stranded telomeric G-tails at 30°C. *Denatured*: The *cdc13-1 pol32Δ* strain shows partial rescue of the *cdc13-1* elongated telomere phenotype. Strains were grown to OD_{600}=1 and then cultured at either 23°C or 30°C for 4 h before DNA isolation. Telomeric G-overhangs were detected as described in Methods using a 5′ dATP γ^{32}p-end-labeled oligo. (C) Quantification of ssDNA relative to dsDNA telomeric DNA in the *cdc13-1 pol32Δ* double mutant shows slightly increased ssDNA compared to the *cdc13-1* single mutant ($n = 2$).

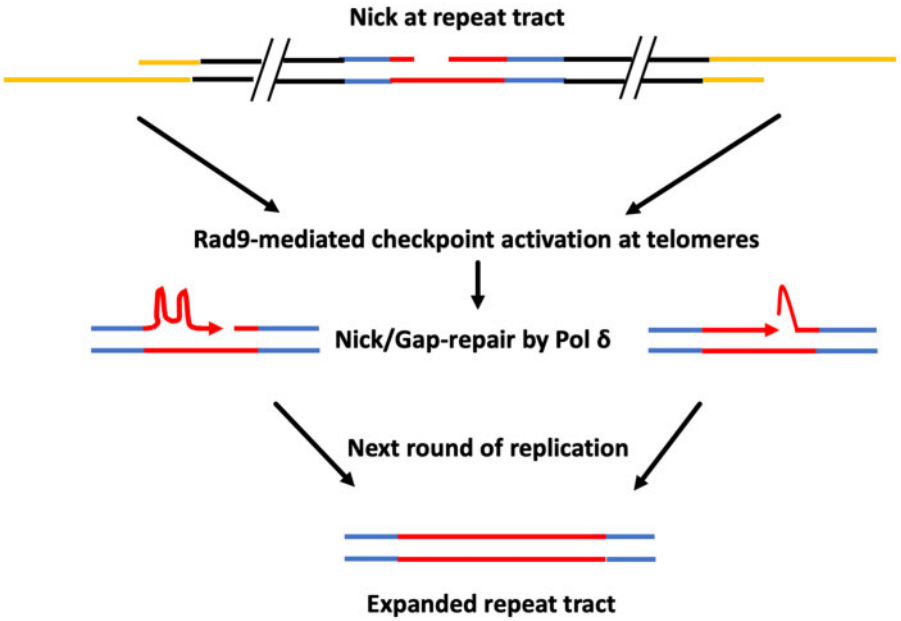

Figure 6 Model for GAA repeat expansions in the *cdc13-1* mutant following Rad9-mediated checkpoint activation. Nicks or gaps left over from GAA replication may result in repair by "slippage-prone" Pol δ following proper flap removal, which adds extra GAA repeats and results in an expansion event visible in the next cell-division. Alternatively, expansions may result from flap equilibration and ligation if repetitive flaps are not properly removed. GAA repeats in red; *URA3* gene in blue; and telomeric ends in orange.

In agreement with earlier observations by others (Weinert and Hartwell 1993; Lydall and Weinert 1995), we demonstrated that the G2/M checkpoint is activated in the *cdc13-1* mutant after a single cell-cycle at the semi-permissive temperature (Figure 4A, top panel). It was foreseeable, however, that following just one cell cycle, we could have missed the intra-S checkpoint activation caused by persistent DNA damage in *the cdc13-1* mutant. Following Rad53 phosphorylation after multiple generations at the semi-permissive temperature, we found recurrent activation of the G2/M checkpoint and still no activation of the intra-S checkpoint (Figure 4a, bottom panel). These results cumulatively indicate that only the G2/M chekpoint is on in the *cdc13-1* mutant. In addition, deleting genes involved in the G2/M checkpoint, including *EXO1*, *RAD9*, *MEC1*, and *RAD53* in the *cdc13-1* genetic background rescued the increased GAA expansion rate in the *cdc13-1* mutant (Figure 4C). We conclude that the majority of extra GAA expansions in the *cdc13-1* mutant originate during the G2 phase of the cell cycle upon G2/M checkpoint activation.

What could be the mechanisms of post-replicative repeat expansions? We showed that they do not occur via post-replicative template-switching, or any type of homologous recombination. At the same time, they depend on Pol32 (Figure 5A). All these data combined suggest that repair of nicks and/or small gaps involving Pol32-mediated DNA synthesis is at the heart of GAA repeat expansions upon G2/M checkpoint activation in the *cdc13-1* mutant (Figure 6). This idea is additionally supported by the data that deletion of Exo1 nuclease led to strongest rescue of the hyper-expansion phenotype caused by the *cdc13-1* mutation (Figure 4C), which could be attributed to its role in both chekpoint activation and postreplicative gap repair (García-Rodríguez *et al.* 2018). Expansions can then arise by two processes. First, DNA polymerase δ is notoriously slippery (Stith *et al.* 2008; Kim *et al.* 2017; Kramara *et al.* 2017; Neil *et al.* 2021), which may result in the accumulation of slip-outs in the repaired DNA strand (Figure 6, left pathway). Second, Pol δ-mediated strand displacement can produce 5′ flaps (Stith *et al.* 2008), and the product of the repair synthesis can be ligated to the unprocessed repetitive flap, resulting in expansions (Liu *et al.* 2004) (Figure 6, right pathway). Expansions could also result from the combination of both scenarios.

This study shows that trinucleotide instability is exacerbated in cells with uncapped telomeres that undergo checkpoint activation. It may serve as a further proof that instability at trinucleotide repeats is exacerbated during conditions of arrested replication, perhaps as a corollary to other studies that show that profound instability effects can accrue in non-dividing cells (Neil *et al.* 2021).

Data availability

All data, yeast strains and plasmids underlying this article will be shared on request to the corresponding author. Table S1 contains a list of the strains used. Table S2 contains a list of the primers used. Supplemental Material provided at figshare: https://doi.org/10.25386/genetics.15062436.

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Conflicts of interest

The authors declare that there is no conflict of interest.

Literature cited

Bertuch AA, Lundblad V. 2003. The Ku heterodimer performs separable activities at double-strand breaks and chromosome termini. Mol Cell Biol. 23:8202–8215.

Bourn RL, De Biase I, Pinto RM, Sandi C, Al-Mahdawi S, *et al.* 2012. Pms2 suppresses large expansions of the (GAA·TTC)n sequence in neuronal tissues. PLoS One. 7:e47085.

Campuzano V, Montermini L, Moltò MD, Pianese L, Cossée M, *et al.* 1996. Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. Science. 271: 1423–1427.

Chandra A, Hughes TR, Nugent CI, Lundblad V. 2001. Cdc13 both positively and negatively regulates telomere replication. Genes Dev. 15:404–414.

Chastain M, Zhou Q, Shiva O, Fadri-Moskwik M, Whitmore L, *et al.* 2016. Human CST facilitates genome-wide RAD51 recruitment to GC-rich repetitive sequences in response to replication stress. Cell Rep. 16:2048.

Fay DS, Sun Z, Stern DF. 1997. Mutations in SPK1/RAD53 that specifically abolish checkpoint but not growth-related functions. Curr Genet. 31:97–105.

Foiani M, Marini F, Gamba D, Lucchini G, Plevani P. 1994. The B subunit of the DNA polymerase alpha-primase complex in *Saccharomyces cerevisiae* executes an essential function at the initial stage of DNA replication. Mol Cell Biol. 14:923–933.

Foster PL. 2006. Methods for determining spontaneous mutation rates. Methods Enzymol. 409:195–213.

Freudenreich CH, Kantrow SM, Zakian VA. 1998. Expansion and length-dependent fragility of CTG repeats in yeast. Science. 279: 853–856.

Gacy AM, Goellner GM, Spiro C, Chen X, Gupta G, *et al.* 1998. GAA instability in Friedreich's Ataxia shares a common, DNA-directed and intraallelic mechanism with other trinucleotide diseases. Mol Cell. 1:583–593.

Gadgil R, Barthelemy J, Lewis T, Leffak M. 2017. Replication stalling and DNA microsatellite instability. Biophys Chem. 225:38–48.

García-Rodríguez N, Morawska M, Wong RP, Daigaku Y, Ulrich HD. 2018. Spatial separation between replisome- and template-induced replication stress signaling. EMBO J. 37:e98369.

Gardner RD, Burke DJ. 2000. The spindle checkpoint: two transitions, two pathways. Trends Cell Biol. 10:154–158.

Garvik B, Carson M, Hartwell L. 1995. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol Cell Biol. 15:6128–6138.

Gellon L, Kaushal S, Cebrián J, Lahiri M, Mirkin SM, *et al.* 2019. Mrc1 and Tof1 prevent fragility and instability at long CAG repeats by their fork stabilizing function. Nucleic Acids Res. 47:794–805.

Gerik KJ, Li X, Pautz A, Burgers PM. 1998. Characterization of the two small subunits of Saccharomyces cerevisiae DNA polymerase delta. J Biol Chem. 273:19747–19755.

Gietz RD, Woods RA. 2006. Yeast transformation by the LiAc/SS Carrier DNA/PEG method. Methods Mol Biol. 313:107–120.

Grandin N, Bailly A, Charbonneau M. 2005. Activation of Mrc1, a mediator of the replication checkpoint, by telomere erosion. Biol Cell. 97:799–814.

Grandin N, Charbonneau M. 2007. Mrc1, a non-essential DNA replication protein, is required for telomere end protection following loss of capping by Cdc13, Yku or telomerase. Mol Genet Genomics. 277:685–699.

Grandin N, Damon C, Charbonneau M. 2001. Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13. EMBO J. 20:1173–1183.

Jedrychowska M, Denkiewicz-Kruk M, Alabrudzinska M, Skoneczna A, Jonczyk P, *et al.* 2019. Defects in the GINS complex increase the instability of repetitive sequences via a recombination-dependent mechanism. PLoS Genet. 15:e1008494.

Johansson E, Garg P, Burgers PM. 2004. The Pol32 subunit of DNA polymerase delta contains separable domains for processive replication and proliferating cell nuclear antigen (PCNA) binding. J Biol Chem. 279:1907–1915.

Kerrest A, Anand RP, Sundararajan R, Bermejo R, Liberi G, *et al.* 2009. SRS2 and SGS1 prevent chromosomal breaks and stabilize triplet repeats by restraining recombination. Nat Struct Mol Biol. 16: 159–167.

Khristich AN, Armenia JF, Matera RM, Kolchinski AA, Mirkin SM. 2020. Large-scale contractions of Friedreich's ataxia GAA repeats in yeast occur during DNA replication due to their triplex-forming ability. Proc Natl Acad Sci USA. 117:1628–1637.

Khristich AN, Mirkin SM. 2020. On the wrong DNA track: molecular mechanisms of repeat-mediated genome instability. J Biol Chem. 295:4134–4170.

Kim JC, Harris ST, Dinter T, Shah KA, Mirkin SM. 2017. The role of break-induced replication in large-scale expansions of (CAG)(n)/(CTG)(n) repeats. Nat Struct Mol Biol. 24:55–60.

Kramara J, Osia B, Malkova A. 2017. Break-induced replication: an unhealthy choice for stress relief? Nat Struct Mol Biol. 24:11–12.

Krasilnikova MM, Mirkin SM. 2004a. Analysis of triplet repeat replication by two-dimensional gel electrophoresis. Methods Mol Biol. 277:19–28.

Krasilnikova MM, Mirkin SM. 2004b. Replication stalling at Friedreich's ataxia (GAA)n repeats *in vivo*. Mol Cell Biol. 24:2286–2295.

Lendvay TS, Morris DK, Sah J, Balasubramanian B, Lundblad V. 1996. Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional EST genes. Genetics. 144:1399–1412.

Lin JJ, Zakian VA. 1996. The Saccharomyces CDC13 protein is a single-strand TG1-3 telomeric DNA-binding protein in vitro that affects telomere behavior in vivo. Proc Natl Acad Sci USA. 93: 13760–13765.

Liu CC, Gopalakrishnan V, Poon LF, Yan T, Li S. 2014. Cdk1 regulates the temporal recruitment of telomerase and Cdc13-Stn1-Ten1 complex for telomere replication. Mol Cell Biol. 34:57–70.

Liu Y, Kao HI, Bambara RA. 2004. Flap endonuclease 1: a central component of DNA metabolism. Annu Rev Biochem. 73:589–615.

Lue NF, Chan J, Wright WE, Hurwitz J. 2014. The CDC13-STN1-TEN1 complex stimulates Pol α activity by promoting RNA priming and primase-to-polymerase switch. Nat Commun. 5:5762.

Lydall D, Weinert T. 1995. Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. Science. 270: 1488–1491.

Majka J, Binz SK, Wold MS, Burgers PM. 2006. Replication protein A directs loading of the DNA damage checkpoint clamp to 5'-DNA junctions. J Biol Chem. 281:27855–27861.

Makarova AV, Stodola JL, Burgers PM. 2012. A four-subunit DNA polymerase ζ complex containing Pol δ accessory subunits is essential for PCNA-mediated mutagenesis. Nucleic Acids Res. 40: 11618–11626.

Maringele L, Lydall D. 2002. EXO1-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast yku70Delta mutants. Genes Dev. 16:1919–1933.

Mason M, Wanat JJ, Harper S, Schultz DC, Speicher DW, *et al.* 2013. Cdc13 OB2 dimerization required for productive Stn1 binding and efficient telomere maintenance. Structure. 21:109–120.

Matellán L, Monje-Casas F. 2020. Regulation of Mitotic Exit by Cell Cycle Checkpoints: Lessons From Saccharomyces cerevisiae. Genes. 11:195 10.3390/genes11020195.

McGinty RJ, Puleo F, Aksenova AY, Hisey JA, Shishkin AA, *et al.* 2017. A defective mRNA cleavage and polyadenylation complex facilitates expansions of transcribed (GAA)(n) repeats associated with Friedreich's ataxia. Cell Rep. 20:2490–2500.

Mersaoui SY, Bonnell E, Wellinger RJ. 2018. Nuclear import of Cdc13 limits chromosomal capping. Nucleic Acids Res. 46:2975–2989.

Mirkin SM. 2006. DNA structures, repeat expansions and human hereditary disorders. Curr Opin Struct Biol. 16:351–358.

Mirkin SM. 2007. Expandable DNA repeats and human disease. Nature. 447:932–940.

Miyake Y, Nakamura M, Nabetani A, Shimamura S, Tamura M, *et al.* 2009. RPA-like mammalian Ctc1-Stn1-Ten1 complex binds to single-stranded DNA and protects telomeres independently of the Pot1 pathway. Mol Cell. 36:193–206.

Navas TA, Sanchez Y, Elledge SJ. 1996. RAD9 and DNA polymerase epsilon form parallel sensory branches for transducing the DNA damage checkpoint signal in *Saccharomyces cerevisiae*. Genes Dev. 10:2632–2643.

Neil AJ, Liang MU, Khristich AN, Shah KA, Mirkin SM. 2018. RNA-DNA hybrids promote the expansion of Friedreich's ataxia (GAA)n repeats via break-induced replication. Nucleic Acids Res. 46:3487–3497.

Neil AJH, JA, IQuasem, RJMcGinty, M Hitczenko, ANKhristich, *et al.* 2021. Replication-independent instability of Friedreich's ataxia GAA repeats during chronological aging. Proc Natl Acad Sci USA. 118:e2013080118.

Ngo HP, Lydall D. 2010. Survival and growth of yeast without telomere capping by Cdc13 in the absence of Sgs1, Exo1, and Rad9. PLoS Genet. 6:e1001072.

Nugent CI, Hughes TR, Lue NF, Lundblad V. 1996. Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. Science. 274:249–252.

Pelletier R, Krasilnikova MM, Samadashwily GM, Lahue R, Mirkin SM. 2003. Replication and expansion of trinucleotide repeats in yeast. Mol Cell Biol. 23:1349–1357.

Pfander B, Diffley JF. 2011. Dpb11 coordinates Mec1 kinase activation with cell cycle-regulated Rad9 recruitment. EMBO J. 30:4897–4907.

Polleys EJ, Freudenreich CH. 2020. Genetic assays to study repeat fragility in *Saccharomyces cerevisiae*. Methods Mol Biol. 2056:83–101.

Radchenko EA, McGinty RJ, Aksenova AY, Neil AJ, Mirkin SM. 2018. Quantitative analysis of the rates for repeat-mediated genome instability in a yeast experimental system. Methods Mol Biol. 1672:421–438.

Shah KA, Shishkin AA, Voineagu I, Pavlov YI, Shcherbakova PV, *et al.* 2012. Role of DNA polymerases in repeat-mediated genome instability. Cell Rep. 2:1088–1095.

Shishkin AA, Voineagu I, Matera R, Cherng N, Chernet BT, *et al.* 2009. Large-scale expansions of Friedreich's ataxia GAA repeats in yeast. Mol Cell. 35:82–92.

Stith CM, Sterling J, Resnick MA, Gordenin DA, Burgers PM. 2008. Flexibility of eukaryotic Okazaki fragment maturation through regulated strand displacement synthesis. J Biol Chem. 283:34129–34140.

Sundararajan R, Freudenreich CH. 2011. Expanded CAG/CTG repeat DNA induces a checkpoint response that impacts cell proliferation in *Saccharomyces cerevisiae*. PLoS Genet. 7:e1001339.

Taggart AK, Teng SC, Zakian VA. 2002. Est1p as a cell cycle-regulated activator of telomere-bound telomerase. Science. 297:1023–1026.

Tsutakawa SE, Thompson MJ, Arvai AS, Neil AJ, Shaw SJ, *et al.* 2017. Phosphate steering by Flap Endonuclease 1 promotes 5'-flap specificity and incision to prevent genome instability. Nat Commun. 8:15855.

Valerio-Santiago M, de Los Santos-Velázquez AI, Monje-Casas F. 2013. Inhibition of the mitotic exit network in response to damaged telomeres. PLoS Genet. 9:e1003859.

Voineagu I, Freudenreich CH, Mirkin SM. 2009a. Checkpoint responses to unusual structures formed by DNA repeats. Mol Carcinog. 48: 309–318.

Voineagu I, Surka CF, Shishkin AA, Krasilnikova MM, Mirkin SM. 2009b. Replisome stalling and stabilization at CGG repeats, which are responsible for chromosomal fragility. Nat Struct Mol Biol. 16: 226–228.

Webb CJ, Wu Y, Zakian VA. 2013. DNA repair at telomeres: keeping the ends intact. Cold Spring Harb Perspect Biol. 5:a012666.

Weinert TA, Hartwell LH. 1993. Cell cycle arrest of cdc mutants and specificity of the RAD9 checkpoint. Genetics. 134:63–80.

Yu X, Gabriel A. 1999. Patching broken chromosomes with extranuclear cellular DNA. Mol Cell. 4:873–881.

Zhang Y, Shishkin AA, Nishida Y, Marcinkowski-Desmond D, Saini N, *et al.* 2012. Genome-wide screen identifies pathways that govern GAA/TTC repeat fragility and expansions in dividing and nondividing yeast cells. Mol Cell. 48:254–265.

Zhao X, Muller EG, Rothstein R. 1998. A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol Cell. 2:329–340.

Zou L, Elledge SJ. 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science. 300:1542–1548.

Zubko MK, Guillard S, Lydall D. 2004. Exo1 and Rad24 differentially regulate generation of ssDNA at telomeres of Saccharomyces cerevisiae cdc13-1 mutants. Genetics. 168:103–115.

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