Telomere Length Homeostasis Responds to Changes in Intracellular dNTP Pools

Amitabha Gupta,* Sushma Sharma,† Patrick Reichenbach,‡ Lisette Marjavaara,† Anna Karin Nilsson,† Joachim Lingner,‡ Andrei Chabes,†,§ Rodney Rothstein, and Michael Chang**,††,1**

*Department of Cellular, Molecular and Biophysical Studies and **Department of Genetics and Development, Columbia University Medical Center, New York, New York 10032, †Department of Medical Biochemistry and Biophysics and §Laboratory for Molecular Infection Medicine Sweden, Umeå University, SE 901 87 Umeå, Sweden, ‡Swiss Institute for Experimental Cancer Research (ISREC), School of Life Sciences, Frontiers in Genetics National Center of Competence in Research, Ecole Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland, and ††European Research Institute for the Biology of Ageing, University of Groningen, University Medical Center Groningen, 9713 AV Groningen, The Netherlands

ABSTRACT Telomeres, the ends of linear eukaryotic chromosomes, shorten due to incomplete DNA replication and nucleolytic degradation. Cells counteract this shortening by employing a specialized reverse transcriptase called telomerase, which uses deoxyribonucleoside triphosphates (dNTPs) to extend telomeres. Intracellular dNTP levels are tightly regulated, and perturbation of these levels is known to affect DNA synthesis. We examined whether altering the levels of the dNTP pools or changing the relative ratios of the four dNTPs in *Saccharomyces cerevisiae* would affect the length of the telomeres. Lowering dNTP levels leads to a modest shortening of telomeres, while increasing dNTP pools has no significant effect on telomere length. Strikingly, altering the ratio of the four dNTPs dramatically affects telomere length homeostasis, both positively and negatively. Specifically, we find that intracellular deoxyguanosine triphosphate (dGTP) levels positively correlate with both telomere length and telomerase nucleotide addition processivity *in vivo*. Our findings are consistent with *in vitro* data showing dGTP-dependent stimulation of telomerase activity in multiple organisms and suggest that telomerase activity is modulated *in vivo* by dGTP levels.

ALL eukaryotes, as well as some prokaryotes with linear chromosomes, contain repetitive sequences called telomeres at the ends of their DNA. Telomeric DNA is bound by proteins that protect chromosome ends from being recognized as genotoxic DNA double-strand breaks in need of repair (Jain and Cooper 2010). However, telomeres shorten due to incomplete DNA replication and nucleolytic degradation. Left unchecked, this telomere erosion eventually results in very short, unprotected telomeres, leading to cell-cycle arrest and replicative senescence (Lundblad and Szostak 1989; Harley *et al.* 1990; Yu *et al.* 1990).

Telomere shortening is counteracted by a specialized reverse transcriptase called telomerase (Greider and Blackburn 1985), whose core consists of a protein catalytic subunit and an RNA moiety—hTERT and hTR, respectively, in humans (Feng *et al.* 1995; Nakamura *et al.* 1997), and Est2 and TLC1, respectively, in the budding yeast *Saccharomyces cerevisiae* (Singer and Gottschling 1994; Lingner *et al.* 1997). Telomerase extends telomeres by repeated reverse transcription of a short sequence to the 3′ ends of telomeres, using the RNA subunit as a template (Greider and Blackburn 1989; Yu *et al.* 1990; Singer and Gottschling 1994). Although the sequence of the telomeric repeats differs between species, a common feature is that they are all G-rich. In vertebrates, the repeat sequence is TTAGGG (Meyne *et al.* 1989), while in *S. cerevisiae*, the telomeric repeats have a consensus sequence of $(TG)_{0\text{-}6}TGGGTGTG(G)_{0\text{-}1}$ (Forstemann and Lingner 2001).

Deoxyribonucleoside triphosphates (dNTPs) are the building blocks of DNA, and their production needs to be tightly regulated as imbalances in dNTP pools can be mutagenic (Reichard 1988). In *S. cerevisiae*, the sole mode of dNTP production is through *de novo* dNTP synthesis, and the primary enzyme in this process is ribonucleotide reductase (RNR). RNR catalyzes the reduction of adenosine triphosphate (ADP), guanosine diphosphate (GDP), cytidine diphosphate

doi: 10.1534/genetics.112.149120
Manuscript received August 14, 2012; accepted for publication January 11, 2013
Supporting information is available online at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.149120/-/DC1.
[1]Corresponding author: European Research Institute for the Biology of Ageing, University of Groningen, University Medical Center Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands. E-mail: m.chang@umcg.nl

(CDP), and uridine diphosphate (UDP) to their respective deoxy forms, which are then converted to deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and deoxythymidine triphosphate (dTTP) (Reichard 1988). Since RNR is the rate-limiting step in dNTP production, it is tightly regulated to maintain proper dNTP levels. In *S. cerevisiae*, overall pool sizes are controlled by (1) allosteric regulation of RNR activity by dATP (Reichard 1988); (2) transcriptional regulation of the *RNR* genes by Crt1, Ixr1, as well as the MBF complex (Huang *et al.* 1998; de Bruin *et al.* 2006; Tsaponina *et al.* 2011); (3) direct inhibition of the large subunit of RNR, Rnr1, by Sml1 (Zhao *et al.* 1998; Chabes *et al.* 1999), and (4) relocalization of the small subunits of RNR, Rnr2, and Rnr4 to the nucleus by Wtm1 and Dif1 (Lee and Elledge 2006; Lee *et al.* 2008; Wu and Huang 2008). The regulation of the proteins Wtm1, Dif1, Crt1, and Sml1 is dependent on Mec1, the ATR ortholog in yeast, in a manner dependent on Dun1, a CHK2 ortholog (Huang *et al.* 1998; Zhao *et al.* 2001; Lee and Elledge 2006; Lee *et al.* 2008). In addition, an allosteric specificity site on RNR is important for maintaining the proper balance of the four individual dNTPs (Reichard 1988).

Previous studies have implicated dNTP pools in telomere length homeostasis. Both *rnr1*Δ and *dun1*Δ mutants were found to have short telomeres in a genome-wide screen that measured the telomere lengths of strains in the yeast gene deletion library (Gatbonton *et al.* 2006). Given that both Rnr1 and Dun1 are positive regulators of dNTP levels, the shortened telomeres of the *rnr1*Δ and *dun1*Δ mutants may be a product of reduced dNTPs. Indeed, the dNTP pool sizes in a *dun1*Δ strain are reduced twofold compared to wild type (Fasullo *et al.* 2010). Furthermore, *cdc8* and *cdc21* mutants, which express defective thymidylate kinase and thymidylate synthetase, respectively, involved in the production of dTTP, possess shortened telomeres (Adams and Holm 1996). All of these observations suggest a possible link between telomere length homeostasis and regulation of dNTP pools. *In vitro* studies of telomerase from *S. cerevisiae* (Peng *et al.* 2001; Bosoy and Lue 2004), *Euplotes aediculatus* (Hammond and Cech 1997, 1998), *Tetrahymena thermophila* (Hardy *et al.* 2001), and mammals (Morin 1989; Maine *et al.* 1999) also suggest that dGTP levels influence telomerase activity, but the *in vivo* relevance of these observations is unclear.

In this study, we systematically varied dNTP pool levels and observed that reducing the overall pool size does indeed result in shortened telomeres. Interestingly, increasing dNTP levels has no significant effect on telomere length homeostasis. Additionally, we show that Mec1 and Dun1 function in the same pathway to regulate dNTP pools and telomere length homeostasis. However, Mec1 also functions with Tel1, the yeast ATM ortholog, to regulate telomerase in a Dun1-independent manner. Furthermore, by using *rnr1* mutants that perturb the balance of the four dNTPs, we find dramatic effects on telomere length homeostasis, both positive and negative. In particular, intracellular dGTP levels positively correlate with telomere length and telomerase nucleotide addition processivity *in vivo*, consistent with *in vitro* data showing that dGTP levels can stimulate telomerase activity in yeast (Peng *et al.* 2001; Bosoy and Lue 2004), ciliates (Hammond and Cech 1997, 1998; Hardy *et al.* 2001), and mammals (Morin 1989; Maine *et al.* 1999). Thus, our findings reveal an important link between telomere length homeostasis and dNTP pools and show that alterations in intracellular dGTP levels can modulate telomerase activity.

Materials and Methods

Yeast media and strains

Standard yeast media and growth conditions were used (Sherman 1991). Yeast strains used in this study are listed in Supporting Information, Table S1. All the *rnr1* mutants were expressed from the endogenous *RNR1* promoter. For reasons unknown, we were previously unable to generate viable haploid cells carrying the *rnr1-Q288E* allele (Kumar *et al.* 2010), but here we were able to generate the strain without any difficulties with the same methodology that we used to construct the other *rnr1* mutants.

Telomere PCR and sequencing

Yeast genomic DNA was isolated using a Yeast DNA Extraction Kit (Thermo Scientific) or Wizard Genomic DNA Purification Kit (Promega). Y′ telomeres and telomere VI-R were amplified by PCR as previously described (Pardo *et al.* 2006; Chang *et al.* 2007). Telomere VI-R PCR products were cloned using a PCR Cloning Kit (Qiagen). Sequencing was performed by GENEWIZ and BaseClear, and the resulting sequence data were analyzed using Sequencher software (Gene Codes).

Telomere length measurements

Telomeres, whether in a single cell or in a population of cells, exhibit a range of lengths, typically resulting in smears of ~50–100 bp when analyzed after agarose gel electrophoresis. For each sample, the median telomere length (*i.e.*, the most intense part of the smear) is determined. Then data from several isolates of each genotype are combined to determine the average of these median telomere lengths, as well as standard error of the mean values.

Determination of dNTP pools

dNTP levels were measured as previously described (Chabes *et al.* 2003).

Flow cytometry

Flow cytometry was performed as previously described (Sabouri *et al.* 2008).

Protein blotting

Protein extracts were prepared as previously described (Peter *et al.* 1993). For Rnr2, Rnr3, and Sml1 detection, affinity-purified rabbit polyclonal anti-Rnr2 (AS09 575), anti-Rnr3 (AS09 574), and anti-Sml1 (AS10 847) antibodies

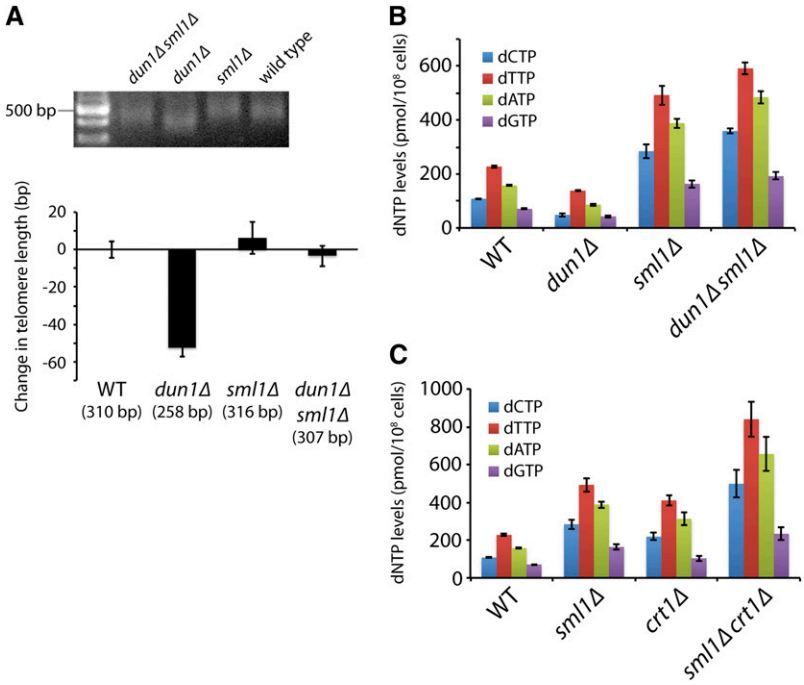

Figure 1 Decreasing dNTP levels results in shorter telomeres. (A) Strains of the indicated genotypes, generated from the sporulation of a *dun1Δ/DUN1 sml1Δ/SML1* diploid, were assayed for telomere length by Y′ telomere PCR after being passaged for at least 100 generations (a representative gel is shown). The change in telomere length, compared to wild-type telomere length, was quantified and plotted. Mean ± SE is shown for four independent isolates of each strain . Similar results were obtained by assaying telomere length by telomere I-L PCR (*i.e.*, PCR amplification of the left telomere of chromosome I) and by denaturing in-gel hybridization (Figure S1). (B) Strains in A were assayed for dNTP levels. Four independent isogenic strains for each genotype were analyzed. Data are represented as mean ± SE. (C) Strains of the indicated genotypes were assayed for dNTP levels, as in B.

(Agrisera AB) were used at 1:500,000, 1:1000, and 1:5000 dilutions, respectively. For detection of both Rnr4 and α-tubulin, YL1/2 rat monoclonal antibody (Sigma) was used at a 1:2500 dilution (Tsaponina *et al.* 2011).

Results

Decreased dNTP pools lead to shortened telomeres

To determine whether altered dNTP pools affect telomere length homeostasis, we isolated meiotic products from the sporulation of a *dun1*Δ/*DUN1 sml1*Δ/*SML1* diploid and examined these haploid progeny for both telomere length and dNTP pool size. Consistent with previous observations (Gatbonton *et al.* 2006), the *dun1*Δ mutant shows slightly shorter telomeres compared to wild-type strains (~50 bp shorter) (Figure 1A and Figure S1). This phenotype is rescued by additional deletion of *SML1* (Figure 1A and Figure S1). Since *dun1*Δ strains have a 2-fold reduction in dNTP pools and *dun1*Δ *sml1*Δ double mutants show a 2.5-fold increase in dNTP pools similar to a *sml1*Δ mutant (Fasullo *et al.* 2010) (Figure 1B), the shortened telomeres in a *dun1*Δ mutant are likely caused by reduced dNTP pools. Interestingly, despite the increased dNTP pools in the *sml1*Δ and *dun1*Δ *sml1*Δ strains, there is no significant change in telomere length in either strain. Since deletion of *CRT1* has previously been shown to increase dNTP pools 2-fold (Tang *et al.* 2009), we constructed *sml1*Δ *crt1*Δ strains hoping to further increase dNTP levels. Indeed, *sml1*Δ *crt1*Δ strains have total dNTP pools ~4-fold above wild-type levels (Figure 1C), but the increase in telomere length is <20 bp (Figure S2A). Similarly, an *rnr1-D57N* mutant (Chabes *et al.* 2003) has dNTP pools almost 3-fold above wild-type levels and an increase in telomere length of ~40 bp (Figure S2B), indicating that an increase in dNTP pools above wild-type levels does not dramatically affect telomere length homeostasis.

Elucidating the role of Mec1 at telomeres

The phosphoinositide-3-kinase-related kinases Tel1 and Mec1, yeast orthologs of human ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related), respectively, are needed for proper regulation of telomerase (Ritchie *et al.* 1999; Arnerić and Lingner 2007). Yeast cells lacking Tel1 have very short telomeres (Greenwell *et al.* 1995), while a *mec1-21* mutant has slightly short telomeres (Ritchie *et al.* 1999). Previous work has shown that *mec1-21*, *dun1*Δ, and *dun1*Δ *mec1-21* mutants all have similar dNTP pool sizes (Fasullo *et al.* 2010), indicating that Mec1 also functions in a pathway with Dun1 to regulate dNTP pools. Therefore, we determined whether Mec1 and Dun1 also function in the same pathway to regulate telomere length homeostasis. We measured telomere length in a *dun1*Δ *mec1-21* double mutant compared to the *dun1*Δ and *mec1-21* single mutants, and all three strains showed the same telomere length (Figure 2). Thus, Mec1 and Dun1 function in the same pathway to regulate both dNTP pools and telomere length. In contrast, we find that Tel1 and Dun1 function in separate pathways to regulate telomere length since the effect of combining *DUN1* and *TEL1* deletions is additive: *dun1*Δ *tel1*Δ double mutants have shorter telomeres than either *dun1*Δ or *tel1*Δ single mutants (Figure 2).

We next examined these three genes for their role in senescence. It was previously shown that *mec1-21 tel1*Δ strains senesce due to a lack of telomerase-mediated telomere extension, a phenotype similar to that of telomerase-negative strains (Ritchie *et al.* 1999; Arnerić and Lingner 2007). Since *MEC1* and *DUN1* act in the same pathway for

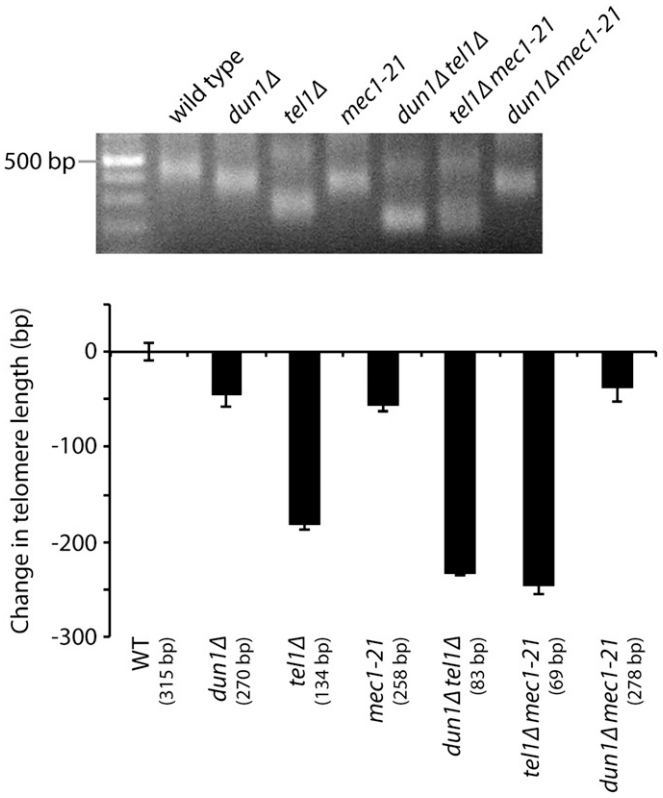

Figure 2 Mec1 and Dun1 function in the same pathway to regulate dNTP pools and telomere length homeostasis. Strains of the indicated genotypes, generated from the sporulation of a *dun1Δ/DUN1 tel1Δ/TEL1 mec1-21/MEC1* diploid, were assayed for telomere length by Y′ telomere PCR after being passaged for ~50 generations (a representative gel is shown). The change in telomere length, compared to wild-type telomere length, was quantified and plotted. Mean ± SE is shown for three independent isolates . Note that the triple mutant is not viable (data not shown).

nucleotide pools and telomere length, we tested whether *dun1*Δ *tel1*Δ double mutants also senesce like *mec1-21 tel1*Δ strains. Interestingly, we find that *dun1*Δ *tel1*Δ cells do not senesce despite repeated subculturings (data not shown). These observations indicate that *MEC1* and *TEL1* function to prevent senescence in a pathway that is not dependent on Dun1.

Altering the ratio of the four dNTPs affects telomere length homeostasis

Having established that limiting dNTP pools leads to shorter telomeres, we explored whether perturbing the balance of the four dNTPs would also affect telomere length homeostasis. We have previously shown that the ratio of the four dNTPs can be severely imbalanced by making mutations in loop 2 of Rnr1 (Kumar *et al.* 2010). We focused on four *rnr1* mutants—*rnr1-Q288E*, *rnr1-Q288A*, *rnr1-R293A*, and *rnr1-Y285A*—with imbalanced dNTP pools (Figure 3A). The *rnr1* mutants were expressed from the endogenous *RNR1* locus. It is important to note that, unlike the mutants previously mentioned (*e.g.*, *dun1*Δ, *sml1*Δ, etc.) and all other mutants where dNTP pools have been measured, to the best of our knowledge, these *rnr1* mutants are the only mutants known to cause an imbalance of the four dNTPs. We find that both the *rnr1-Q288E* and the *rnr1-Q288A* mutants show elongated telomeres compared to wild type while both the *rnr1-R293A* and the *rnr1-Y285A* mutants have dramatically shortened telomeres, almost 200 bp shorter than wild-type telomeres, which is much greater than the ~50-bp decrease seen in the *dun1*Δ mutant (Figures 1A and 3B). These results indicate that disrupting the ratio of the four dNTPs can greatly affect telomere length homeostasis both positively and negatively. The increase in telomere lengths in the *rnr1-Q288E* and *rnr1-Q288A* mutants correlates with increases in total dNTP levels. However, the *rnr1-Y285A* mutant has very short telomeres despite having significantly increased total dNTP levels. Thus, the telomere length changes cannot be easily explained by changes in total dNTP levels (Figure S3A). Neither can growth rate provide an explanation since both the *rnr1-Q288A* and the *rnr1-R293A* mutants are delayed in S phase and grow very poorly (Kumar *et al.* 2010) but exhibit opposite effects on telomere length.

Next, we compared the change in telomere length to the amount of each of the four dNTPs as a percentage of the total dNTP pool size. We found no correlation in length homeostasis in comparison to the dCTP, dTTP, and dATP pools in these strains (Figure S3A). On the other hand, we found a solid association between telomere length and dGTP pools: both the *rnr1-R293A* and the *rnr1-Y285A* mutants, which have short telomeres, show reduced dGTP while the two mutants with long telomeres, *rnr1-Q288E* and *rnr1-Q288A*, have increased dGTP as a percentage of the total dNTP levels (Figure 3C). These results indicate that telomere length is correlated with the percentage of intracellular dGTP more so than the absolute size of the dNTP pools. This correlation remained true when we further analyzed the dNTP pools and telomere length of a fifth *rnr1* mutant, *rnr1-Y285F* (Figure 3C and Figure S3A). Consistent with this idea, the mutants analyzed in Figure 1 do not have altered percentages of dGTP (Figure S3B), resulting in much milder effects on telomere length homeostasis. Interestingly, if we exclude one of the five mutants (*i.e.*, *rnr1-R293A*), it appears that telomere length is inversely correlated with dCTP and dTTP levels and positively correlated with dATP levels. However, *in vitro* data (see below) do not support this view.

To determine whether telomere length changes in the *rnr1-Q288E*, *rnr1-Q288A*, *rnr1-R293A*, and *rnr1-Y285A* mutants are dependent on telomerase, we made double mutants of the *rnr1* point mutants with *tlc1*Δ by isolating haploid meiotic progeny after the sporulation of *rnr1/RNR1 tlc1*Δ*/TLC1* diploids. We were unable to carefully measure the telomere length of *rnr1-R293A tlc1*Δ double mutants as isolates of this strain senesced before we were able to extract DNA. In the one double-mutant strain that grew (of a possible 15), the telomeres were extremely short as measured by sequencing (see below). Thus, this synergistic

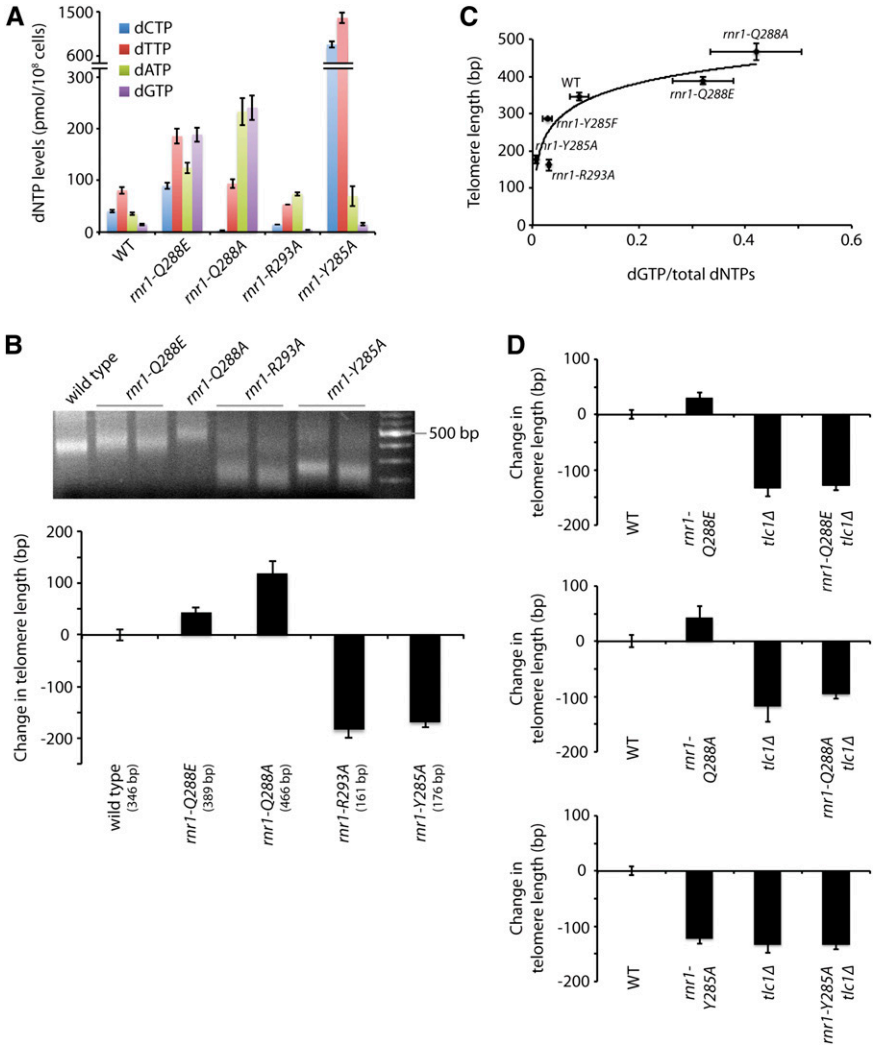

Figure 3 Telomere length positively correlates with percentage of intracellular dGTP. (A) dNTP concentrations in a wild-type strain and the *rnr1* mutants were measured. Mean ± SE is shown for each strain. Data for the wild type, *rnr1-Q288A*, *rnr1-R293A*, and *rnr1-Y285A* strains were previous reported (Kumar *et al.* 2010). (B) Strains of the indicated genotypes were assayed for telomere length by telomere VI-R PCR after being passaged for at least 100 generations (a representative gel is shown). The change in telomere length, compared to wild-type telomere length, was quantified and plotted. Mean ± SE is shown for at least three independent isolates. (C) Strains of the indicated genotype were plotted for telomere length *vs.* dGTP as a fraction of total dNTP levels. Each point indicates the mean for each of these values, and error bars indicate the standard error. (D) Strains of the indicated genotypes, generated from the sporulation of *rnr1/RNR1 tlc1Δ/TLC1* diploids, were assayed for telomere length by Y′ telomere PCR after being passaged for ∼30 generations. The change in telomere length, compared to wild-type telomere length, was quantified and plotted. Mean ± SE is shown for three independent isolates.

interaction suggests that an additional factor(s) contributes to the shortened telomeres of the *rnr1-293A* mutant. In contrast, the other double mutants, *rnr1-Q288E tlc1*Δ, *rnr1-Q288A tlc1*Δ, and *rnr1-Y285A tlc1*Δ, all exhibit telomere lengths that are similar to *tlc1*Δ single mutants (Figure 3D), indicating that the telomere length changes associated with the *rnr1-Q288E*, *rnr1-Q288A*, and *rnr1-Y285A* mutants are all telomerase-dependent.

Altering dGTP affects telomerase nucleotide addition processivity

Since the telomere length changes were telomerase-dependent in the three *rnr1* mutants that we could test, we examined the effect of imbalanced dNTP pools on the efficiency with which telomerase adds dNTPs to the 3′ terminus of telomeres (*i.e.*, the nucleotide addition processivity of telomerase). Although the *S. cerevisiae* telomerase RNA subunit, TLC1, is predicted to specify the synthesis of the sequence 5′-TGTGTGGGTGTGGTG-3′ if reverse transcription of each repeat is completely processive, yeast telomerase adds imperfect, degenerate repeats with a consensus sequence of 5′-$(TG)_{0\text{-}6}$TGGGTGTG$(G)_{0\text{-}1}$-3′ (Forstemann and Lingner 2001). Thus, telomerase nucleotide addition processivity can be assessed by analyzing the frequency of sequence elements within this consensus. Since almost all telomeric repeats contain the -TGGGTGT- sequence motif, we can analyze reverse transcription of the 5′ portion of the TLC1 template region after the synthesis of this core motif, as well as reverse transcription of the 3′ portion of the template region leading up to the core motif. To study the telomeric sequences, we amplified, cloned, and sequenced telomere VI-R from wild type and the four different *rnr1* strains. The distal region of the telomeres has sequences that are divergent because telomerase adds imperfect, degenerate repeats (Forstemann *et al.* 2000). To ensure that the sequences analyzed were due to telomerase-mediated extension events, only sequences that diverged from bulk telomere sequences were examined.

To assay reverse transcription of the 5′ portion of the TLC1 template region, we analyzed how often the core -TGGGTGT- sequence is followed by a GG dinucleotide, as predicted from the template region of TLC1, to produce

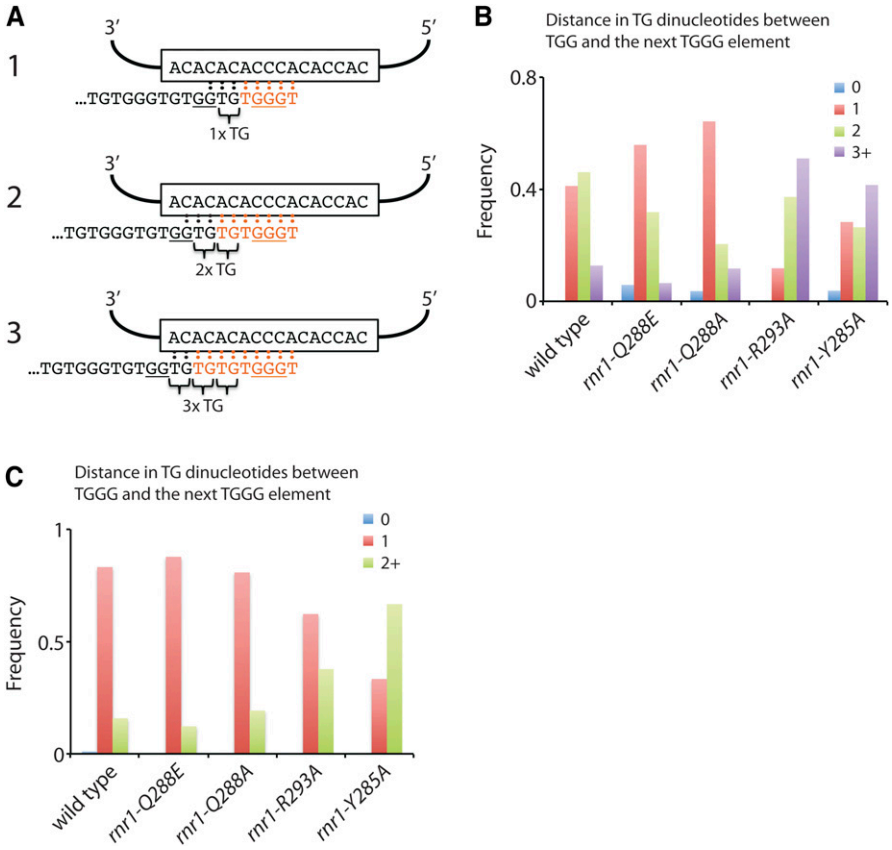

Figure 4 Telomerase nucleotide addition processivity is affected by dGTP levels. (A) Schematic illustrating three possible alignments for a telomere ending in -TGGTG with the template region of TLC1. Following reverse transcription and extension of the telomere (with added nucleotides shown in orange), the number of TG dinucleotides between the TGG motif and the following TGGG motif will vary. (B and C) For strains of the indicated genotypes, telomere VI-R was amplified by PCR, cloned, and sequenced. (B) The frequency of having 0, 1, 2, or 3 and higher TG dinucleotides between a TGG and the following TGGG was plotted for each strain. (C) The frequency of having 0, 1, or 2 and higher TG dinucleotides between a TGGG and the following TGGG was plotted for each strain.

either -TGGGTGTGGT- or -TGGG(TG)$_n$TGGGT- repeats (Figure S4A). In a wild-type strain, the core -TGGGTGT- sequence is followed in 52% of all cases by a GG dinucleotide (Figure S4A), similar to what has been previously reported (Forstemann and Lingner 2001). We find that the fraction of repeats that contain the GG dinucleotide in the *rnr1-Q288E* and *rnr1-Q288A* mutants is increased to 59% and 61%, respectively, indicating that telomerase processivity for the 5′ region is enhanced in these two mutants ($P = 0.018$ for *rnr1-Q288E* and $P = 0.003$ for *rnr1-Q288A*, as determined by chi-square tests; Figure S4B). This observation may provide an explanation for the elongated telomeres seen in the *rnr1-Q288E* and *rnr1-Q288A* strains. However, no statistically significant difference was observed for the other two *rnr1* mutants.

To assess the processivity of the reverse transcription of the 3′ portion of TLC1 template region, we first considered all repeats containing the GG dinucleotide (*i.e.*, -TGGGTGTGGT- repeats). Since the 3′ portion of the template consists of a stretch of CA dinucleotides, multiple alignments are possible for a telomere ending in -TGGT or -TGGTG (Figure 4A). This variable alignment gives rise to the variable number of TG dinucleotides between a GG dinucleotide and the following TGGG motif (Figure 4, A and B). In wild-type cells, we find that there are typically one or two TG dinucleotides between a TGG and the subsequent TGGG motif (Figure 4B), consistent with previous observations (Forstemann and Lingner 2001). In the *rnr1-Q288E* and the *rnr1-Q288A* mutants, both of which have increases in the percentage of dGTP and elongated telomeres, there is a shift toward having fewer TG dinucleotides ($P = 1 \times 10^{-6}$ for *rnr1-Q288E* and $P = 4 \times 10^{-10}$ for *rnr1-Q288A*, as determined by chi-square tests; Figure 4B). In contrast, the *rnr1-R293A* and the *rnr1-Y285A* mutants, which both have decreased percentages of dGTP and shortened telomeres, show an increase in the number TG dinucleotides between a TGG and the following TGGG motif ($P = 6 \times 10^{-16}$ for *rnr1-R293A* and $P = 2 \times 10^{-9}$ for *rnr1-Y285A*, as determined by chi-square tests; Figure 4B). If nucleotide addition processivity is low, telomerase would dissociate before reverse transcription proceeds to the next TGGG motif. A cycle of stalling—where telomerase dissociates prematurely, realigns, and reattempts reverse transcription—would increase the number of TG dinucleotides between a TGG and the next TGGG motif. Thus, telomerase in the *rnr1-R293A* and *rnr1-Y285A* mutants exhibits reduced nucleotide addition processivity likely due to the low relative levels of dGTP in these mutants.

Similarly, we can consider all repeats that do not have a GG dinucleotide (*i.e.*, -TGGG(TG)$_n$TGGGT- repeats) and measure the number of TG dinucleotides between a TGGG and the following TGGG. In wild-type cells, one TG dinucleotide usually separates a TGGG and the next TGGG (Figure 4C). Similar to the scenario above, if telomerase nucleotide addition processivity is low, the number of TG dinucleotides between the TGGG motifs would be increased. The *rnr1-R293A* and *rnr1-Y285A* mutants, both of which have

a reduced percentage of dGTP and shortened telomeres, show a striking increase in the number of TG dinucleotides ($P = 1 \times 10^{-5}$ for *rnr1-R293A* and $P = 2 \times 10^{-23}$ for *rnr1-Y285A*, as determined by chi-square tests; Figure 4C), indicating that telomerase processivity is reduced in these strains.

It is formally possible that a change in dGTP levels causes telomerase to preferentially adopt different alignments with the telomere 3′ overhang without altering telomerase processivity. For example, lower dGTP levels may cause telomerase to adopt alignment #3 instead of alignment #1 depicted in Figure 4A, resulting in an increase in TG dinucleotides between TGG and TGGG motifs. However, we do not favor this model because, for both the *rnr1-293A* and *rnr1-Y285A* mutants, there is a dramatic increase in the frequency of four or more TG dinucleotides between a TGG motif and the next TGGG motif (Figure S5). Four or more TG dinucleotides can occur only if telomerase dissociates before reverse transcription proceeds to the next TGGG motif because three TG dinucleotides is the maximum possible from a processive telomerase enzyme from the TLC1 sequence (Figure 4A, alignment #3).

It is also possible that these sequence changes are not telomerase-dependent and are instead a consequence of mutations inserted by DNA polymerases. This is not the case as sequencing the *rnr1 tlc1*Δ double mutants described earlier showed that sequence divergence was mostly eliminated in the *rnr1 tlc1*Δ double mutants (Figure S6A) to levels similar to what has been previously observed in telomerase-negative strains (Teixeira *et al.* 2004; Chang *et al.* 2011). Furthermore, almost all telomeres analyzed in this study, regardless of the strain, have an identical internal region of 60 bp (Figure S6B), meaning that the sequence changes in the *rnr1 TLC1* mutants recorded in Figure 4 were confined to the distal end of the telomeres, where telomerase acts. If replication-induced mutations were responsible for the sequence changes in the *rnr1 TLC1* mutants, these changes would still be observed in the *rnr1 tlc1*Δ mutants, and the changes would also be observed within the internal 60-bp region. Thus, the telomere sequence changes in all four *rnr1* mutants are telomerase-dependent.

In this section, we show that all four *rnr1* mutants have altered telomerase processivity, even the *rnr1-R293A* mutant, which shows synergistic effects in the absence of telomerase. We find that telomerase nucleotide addition processivity is influenced by intracellular dGTP levels, with reverse transcription of the 3′ portion of the TLC1 template region being more dramatically affected than the 5′ portion. Altogether, we show that the percentage of dGTP positively correlates both with telomerase processivity and with telomere length.

*Characterization of the rnr1*Δ *deletion*

Given our results with the *rnr1* point mutants, we decided to examine the effect of an *rnr1*Δ deletion. Interestingly, while *RNR1* is essential in the YNN402 (Elledge and Davis 1990) and W303 backgrounds (I. Sunjevaric and R. Rothstein, unpublished data), an *rnr1*Δ strain is present in the nonessential gene deletion collection (Giaever *et al.* 2002), which is in the BY4741 strain background, a derivative of the S288C background. This *rnr1*Δ strain has been reported to have really short telomeres, >200 bp shorter than those in wild-type strains (Gatbonton *et al.* 2006), but considering the discrepancy in the reported viability of *rnr1*Δ mutants, we decided to validate the reported phenotypes ourselves. We first confirmed that the *rnr1*Δ mutant from the deletion collection has really short telomeres (data not shown). In this strain, the *RNR1* gene has been replaced with the *kanMX4* cassette, which provides resistance to the drug geneticin (also known as G418). We backcrossed the *rnr1*Δ strain twice to a BY4741 wild-type strain and found that the short telomere phenotype always cosegregated with resistance to G418 and slow growth (Figure 5, A and B). We then verified the location of the *kanMX4* cassette by PCR amplification of the junctions between the cassette and locations upstream and downstream of *RNR1* (data not shown).

Flow cytometric analysis revealed defects in cell-cycle progression in the *rnr1*Δ mutant, with many cells delayed in S phase (Figure 5C). We also find that there is upregulation of Rnr2, Rnr4, and most significantly, Rnr3 (Figure 5D). Rnr3 is a minor isoform of the large subunit of RNR that is expressed only following DNA damage or replication blocks in response to an increased need for dNTPs (Elledge and Davis 1990). Furthermore, Sml1 is degraded in *rnr1*Δ strains (Figure 5D), which normally occurs during S phase or in response to DNA damage (Zhao *et al.* 2001). We suspect that the changes in RNR and Sml1 levels are likely responsible for keeping the cells viable. However, we are still uncertain why a deletion of *RNR1* is viable in the BY4741 background, but lethal in the YNN402 and W303 backgrounds. Interestingly, we find that the difference in viability is due to one genetic locus, which is still unknown (see File S1, *Supporting information regarding the viability of rnr1*Δ).

We next examined the levels of the four dNTPs in the *rnr1*Δ mutant. While the levels of dATP appear similar between wild-type cells and *rnr1*Δ mutants, the levels of the other three dNTPs are substantially reduced (Figure 5E). In particular, the percentage of dGTP is reduced from 16% in wild-type cells to 6.6% in the *rnr1*Δ strain (Figure 5E). This reduction in dGTP, combined with the overall reduction in dNTPs, likely contributes significantly to the drastic shortening of telomeres seen in *rnr1*Δ mutants (Figure 5A).

Human telomerase activity is dramatically affected by changes in dGTP concentration

Since our results suggest that telomerase and telomere length are extremely sensitive to dGTP levels, we decided to test whether our findings in yeast are evolutionarily conserved. Previous *in vitro* studies of ciliate (Hammond and Cech 1997, 1998; Hardy *et al.* 2001) and mammalian (Morin 1989; Maine *et al.* 1999) telomerase suggested that

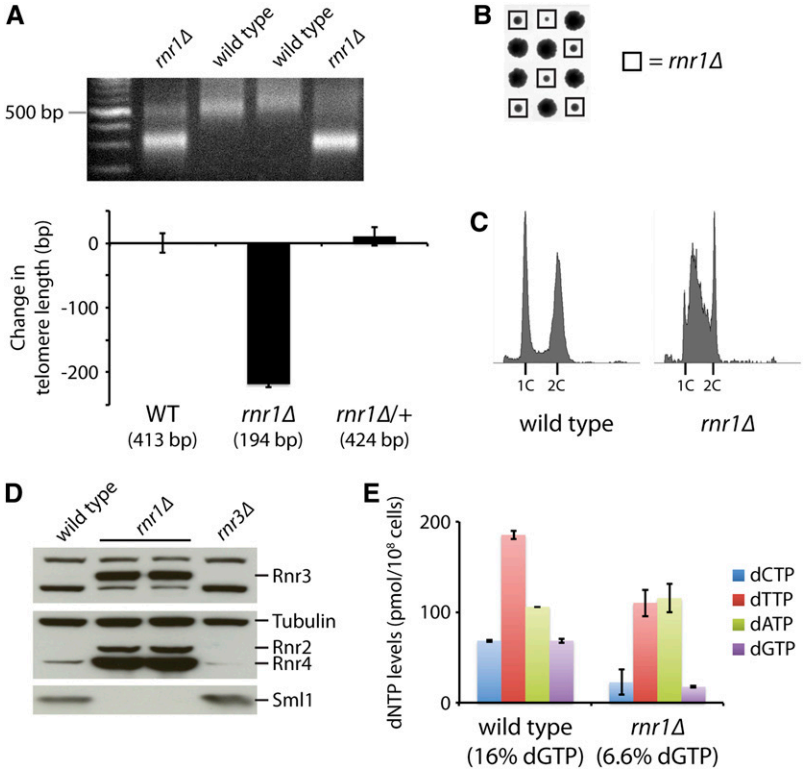

Figure 5 *rnr1*Δ mutants have shortened telomeres due to reduced dGTP levels. (A) An *rnr1*Δ mutant from the yeast gene deletion collection was backcrossed to a wild-type strain (BY4741) twice. The resulting wild-type and *rnr1*Δ progeny strains, along with a heterozygous *rnr1*Δ/*RNR1* diploid, were assayed for telomere length by Y′ telomere PCR after being passaged for at least 100 generations (a representative gel is shown). The change in telomere length, compared to wild-type telomere length, was quantified and plotted. Mean ± SE is plotted for at least four independent isolates (two for the *rnr1*Δ/*RNR1* diploid). (B) Tetrad analysis reveals that an *rnr1*Δ strain exhibits slow growth. Each column of four colonies is a single tetrad derived from the sporulation of an *rnr1*Δ/*RNR1* diploid followed by the separation of the four haploid spores by micromanipulation. (C) Flow cytometry histograms for the indicated strains derived from B. (D) Wild-type, *rnr1*Δ, and *rnr3*Δ strains were assayed for Rnr3, Rnr2, Rnr4, and Sml1 protein levels by protein blot analysis. Tubulin levels were also assayed as a loading control. (E) dNTP pools in the wild-type and *rnr1*Δ strains were measured. Data are represented as mean ± SE. dGTP levels, as a percentage of total dNTPs, are indicated for each strain.

telomerase activity is stimulated by dGTP. However, these studies did not address the effect of separately changing the concentration of all four dNTPs. In light of our yeast *in vivo* observations, we decided to measure human telomerase activity, using the Telospot assay (Cristofari and Lingner 2006), while systematically titrating all four dNTPs. In this assay, both the telomerase RNA subunit, hTR, and the protein catalytic subunit, hTERT, are strongly overexpressed after transient transfection, yielding a situation referred to as "super-telomerase." Crude super-telomerase extract is incubated with a telomeric $(TTAGGG)_3$ primer with varying concentrations of dNTPs. A small fraction of the reaction is directly spotted onto a nylon membrane, which is then probed with a randomly radiolabeled telomeric probe. Since mammalian and *S. cerevisiae* dNTP pools are similar in concentration and in terms of the ratio of the four dNTPs (Traut 1994; Sabouri *et al.* 2008; Nick McElhinny *et al.* 2010), we used the yeast dNTP concentrations determined from the wild-type strain used in Figure 3A as the starting point for our assays (Figure S7A). We maintained three of the dNTPs at these "physiological concentrations" while varying the fourth. Titration of dCTP, dATP, or dTTP does not dramatically affect telomerase activity (Figure S7, A and B). However, consistent with previous results (Maine *et al.* 1999), alteration of dGTP had a striking effect on telomerase activity (Figure S7, A and B). Human telomerase activity is markedly reduced when dGTP concentrations are lowered, while activity is greatly increased even with modest increases in dGTP concentration.

An increase in telomerase activity in the Telospot assay is likely due to processive addition of telomere repeats to the primers to yield long extended products, but it could also result from many primers extended only a short distance. To differentiate between these two scenarios, we repeated the reactions where dGTP concentration was varied using a 5′-biotinylated $(TTAGGG)_3$ primer and resolved the products on a polyacrylamide gel after purification with streptavidin-coated beads. The DNA was transferred onto a nylon membrane and probed as in a standard Telospot assay (Figure S7C). Consistent with previous observations (Maine *et al.* 1999), processive telomerase activity, as measured by the increase in size of the fragments, positively correlates with dGTP concentration. These results indicate that the specific effect of dGTP levels on telomerase activity is an evolutionarily conserved feature.

Discussion

In this study, we examined the effect of changing intracellular dNTP levels, including unbalancing the four dNTPs, on telomere length homeostasis in *S. cerevisiae*. When the ratio of the four dNTPs is maintained, we find that telomere length decreases modestly when total dNTP levels are reduced, while there is no significant change in telomere length upon increasing total dNTP levels. However, we demonstrate that altering the ratio of the dNTPs has a much more pronounced effect on telomere length homeostasis. Specifically, we show that both telomerase nucleotide addition processivity and telomere length positively correlate with dGTP levels *in vivo*.

Reduction of dNTPs leads to shorter telomeres but increase of dNTPs does not significantly affect telomere length

Although previous studies hinted at a connection between dNTP pools and telomere length homeostasis, our work is the first to document their precise relationship. We find that changing the total levels of dNTPs, without altering the ratio of the dNTPs, leads to a modest change in telomere length. Deletion of *DUN1* leads to a twofold reduction in dNTPs and an ~50-bp reduction in telomere length (Figure 1A and Figure S1). However, a *sml1*Δ *crt1*Δ double mutant has a fourfold increase in dNTP levels but an increase in telomere length of <20 bp (Figure 1C and Figure S2A). Thus, an excess of dNTPs results in a rather minimal increase in telomere length. We were unable to probe the telomere length effects of a reduction of dNTP levels greater than twofold without altering the ratio of the four dNTPs because a mutant with such low levels has not been reported. Presumably, such a mutant would be inviable due to insufficient levels of dNTPs required for DNA synthesis.

Mec1 mediates telomere length homeostasis by regulating dNTP levels

Previous work has shown that *mec1-21* mutants have shortened telomeres that can be rescued by deletion of *SML1* (Ritchie *et al.* 1999). Given that *mec1-21*, *dun1*Δ, and *dun1*Δ *mec1-21* mutants all have similar dNTP levels (Fasullo *et al.* 2010), we asked whether the shortened telomeres in the *mec1-21* mutant are due to reduced activation of Dun1. By epistasis analysis, we show this to be the case, with a *dun1*Δ *mec1-21* double mutant having the same telomere length as each of the single mutants (Figure 2). However, Mec1 has functions at the telomere that are Dun1-independent. While *mec1-21 tel1*Δ double mutants senesce, similar to a telomerase-negative strain (Ritchie *et al.* 1999), *dun1*Δ *tel1*Δ mutants fail to senesce, despite repeated subculturings (data not shown). Thus, while Mec1 affects telomere length homeostasis through Dun1-mediated regulation of dNTP pools, Mec1 also has Dun1-independent functions at the telomere.

Intracellular dGTP levels affect telomere length homeostasis by altering telomerase nucleotide addition processivity

The most remarkable finding from our work is the strong dependence of telomere length homeostasis and telomerase activity on the levels of dGTP in the cell. Specifically, we find that the length of yeast telomeres and nucleotide addition processivity of telomerase positively correlate with intracellular dGTP levels (Figures 3 and 4).

Consistent with our observations, yeast telomerase mutants that alter nucleotide addition processivity, as measured *in vitro*, positively correlate with the *in vivo* length of the telomeres (Peng *et al.* 2001), and this processivity is enhanced by increasing dGTP concentrations (Bosoy and Lue 2004). However, these *in vitro* telomerase assays examined only the processivity of nucleotide addition using the 5′ portion of the TLC1 template region, whereas we were able to examine *in vivo* both the 3′ and 5′ portions and have found that reverse transcription of the 3′ portion is more dramatically affected by changes in dGTP levels.

Telomerase enzymes can also be characterized by their ability to add multiple repeats before dissociating (*i.e.*, their repeat addition processivity). Yeast telomerase is generally nonprocessive at adding repeats, both *in vitro* (Cohn and Blackburn 1995) and *in vivo* (Chang *et al.* 2007). However, yeast telomerase has the ability to processively elongate critically short telomeres *in vivo* (Chang *et al.* 2007), and limited repeat addition processivity can be observed *in vitro* by increasing dGTP concentration (Bosoy and Lue 2004).

In agreement with these findings in budding yeast, previous studies have also observed dGTP-dependent stimulation of human telomerase activity *in vitro* (Morin 1989; Maine *et al.* 1999), and by careful titration of all four dNTPs, we show that this effect is specific to dGTP (Figure S7C). Similar *in vitro* studies of endogenous *Euplotes* telomerase and recombinant *Tetrahymena* telomerase have also revealed that it is the binding of dGTP to telomerase that stimulates its repeat addition processivity (Hammond and Cech 1997, 1998; Hardy *et al.* 2001). Interestingly, while we find that the percentage of total dNTPs that is dGTP is important *in vivo* in yeast (Figure 4), it is the absolute concentration of dGTP, independent of the levels of the other three dNTPs, which is important for *in vitro* human telomerase activity (Figure S7). It is noteworthy that the biochemical characteristics of yeast, ciliate, and human telomerases are quite different. For example, they show differences in processivity and associate with different complements of proteins, and their RNA templates differ vastly (Mason *et al.* 2011). Thus, it is quite remarkable that the importance of dGTP levels on telomerase activity is highly conserved, even if the precise mechanism may differ in different species.

Our results indicate that intracellular dGTP levels are rate-limiting for both yeast and human telomerase activity. dNTP levels are most likely optimized for the DNA synthesis machinery. Low levels of dNTPs cause DNA replication fork stalling (Desany *et al.* 1998) while high levels of dNTPs result in an increase in mutation rate (Reichard 1988; Chabes *et al.* 2003). It has also been shown that even mild dNTP pool imbalances are mutagenic (Kumar *et al.* 2010). However, our work suggests that telomere length homeostasis may also impose selective pressure on optimal intracellular dGTP levels. Indeed, of the four dNTPs, dGTP levels are kept the lowest in both yeast (Chabes *et al.* 2003) and mammalian cells (Traut 1994), perhaps reflecting an evolutionarily conserved mechanism to regulate telomerase activity and telomere length homeostasis.

Finally, our findings may provide new strategies to regulate telomerase activity therapeutically. For example, telomerase is repressed in most human somatic cells but is expressed in ~85% of cancers (Shay and Bacchetti 1997), and telomerase has been viewed as an ideal target to inhibit the growth of

a wide range of tumors. Identifying agents that reduce intracellular dGTP levels may be effective in limiting the proliferation of cancer cells. Furthermore, several human diseases are associated with shortened telomeres. Individuals born with reduced telomerase activity have short telomeres, leading to telomere dysfunction in highly proliferative cells (Armanios 2009). Indeed, many of these individuals are haploinsufficient for telomerase and have shortened life spans, suggesting that full telomerase activity is important in preventing these diseases. Perhaps elevating the levels of dGTP to increase telomerase activity will be effective in treating these individuals. Thus, it will be of significant interest to find ways to modulate intracellular dGTP levels as a mechanism to regulate telomerase activity.

Acknowledgments

We thank Peter Thorpe for constructive comments on the manuscript; Stephen Elledge for providing strains; Dinesh Kumar for help in constructing yeast strains; Ivana Sunjevaric for allowing us to cite her unpublished data; and Gaël Cristofari for advice on the Telospot assays. S.S. was supported by a stipend from the Wenner-Gren Foundations. M.C. was supported by a Terry Fox Foundation Fellowship Award. This work was supported by funds from the Swiss National Science Foundation and a European Research Council advanced investigator grant (to J.L.); the Swedish Foundation for Strategic Research, the Swedish Research Council, and the Swedish Cancer Society (A.C.); and National Institutes of Health grants GM50237 and GM67055 (to R.R.).

Literature Cited

Adams, A. K., and C. Holm, 1996 Specific DNA replication mutations affect telomere length in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 16: 4614–4620.

Armanios, M., 2009 Syndromes of telomere shortening. Annu. Rev. Genomics Hum. Genet. 10: 45–61.

Arnerić, M., and J. Lingner, 2007 Tel1 kinase and subtelomere-bound Tbf1 mediate preferential elongation of short telomeres by telomerase in yeast. EMBO Rep. 8: 1080–1085.

Bosoy, D., and N. F. Lue, 2004 Yeast telomerase is capable of limited repeat addition processivity. Nucleic Acids Res. 32: 93–101.

Chabes, A., V. Domkin, and L. Thelander, 1999 Yeast Sml1, a protein inhibitor of ribonucleotide reductase. J. Biol. Chem. 274: 36679–36683.

Chabes, A., B. Georgieva, V. Domkin, X. Zhao, R. Rothstein *et al.*, 2003 Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. Cell 112: 391–401.

Chang, M., M. Arneric, and J. Lingner, 2007 Telomerase repeat addition processivity is increased at critically short telomeres in a Tel1-dependent manner in *Saccharomyces cerevisiae*. Genes Dev. 21: 2485–2494.

Chang, M., J. C. Dittmar, and R. Rothstein, 2011 Long telomeres are preferentially extended during recombination-mediated telomere maintenance. Nat. Struct. Mol. Biol. 18: 451–456.

Cohn, M., and E. H. Blackburn, 1995 Telomerase in yeast. Science 269: 396–400.

Cristofari, G., and J. Lingner, 2006 Telomere length homeostasis requires that telomerase levels are limiting. EMBO J. 25: 565–574.

de Bruin, R. A., T. I. Kalashnikova, C. Chahwan, W. H. McDonald, J. Wohlschlegel *et al.*, 2006 Constraining G1-specific transcription to late G1 phase: the MBF-associated corepressor Nrm1 acts via negative feedback. Mol. Cell 23: 483–496.

Desany, B. A., A. A. Alcasabas, J. B. Bachant, and S. J. Elledge, 1998 Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev. 12: 2956–2970.

Elledge, S. J., and R. W. Davis, 1990 Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase. Genes Dev. 4: 740–751.

Fasullo, M., O. Tsaponina, M. Sun, and A. Chabes, 2010 Elevated dNTP levels suppress hyper-recombination in *Saccharomyces cerevisiae* S-phase checkpoint mutants. Nucleic Acids Res. 38: 1195–1203.

Feng, J., W. D. Funk, S. S. Wang, S. L. Weinrich, A. A. Avilion *et al.*, 1995 The RNA component of human telomerase. Science 269: 1236–1241.

Forstemann, K., and J. Lingner, 2001 Molecular basis for telomere repeat divergence in budding yeast. Mol. Cell. Biol. 21: 7277–7286.

Forstemann, K., M. Hoss, and J. Lingner, 2000 Telomerase-dependent repeat divergence at the 3′ ends of yeast telomeres. Nucleic Acids Res. 28: 2690–2694.

Gatbonton, T., M. Imbesi, M. Nelson, J. M. Akey, D. M. Ruderfer *et al.*, 2006 Telomere length as a quantitative trait: genome-wide survey and genetic mapping of telomere length-control genes in yeast. PLoS Genet. 2: e35.

Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles *et al.*, 2002 Functional profiling of the *Saccharomyces cerevisiae* genome. Nature 418: 387–391.

Greenwell, P. W., S. L. Kronmal, S. E. Porter, J. Gassenhuber, B. Obermaier *et al.*, 1995 *TEL1*, a gene involved in controlling telomere length in *S. cerevisiae*, is homologous to the human ataxia telangiectasia gene. Cell 82: 823–829.

Greider, C. W., and E. H. Blackburn, 1985 Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell 43: 405–413.

Greider, C. W., and E. H. Blackburn, 1989 A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. Nature 337: 331–337.

Hammond, P. W., and T. R. Cech, 1997 dGTP-dependent processivity and possible template switching of *Euplotes* telomerase. Nucleic Acids Res. 25: 3698–3704.

Hammond, P. W., and T. R. Cech, 1998 *Euplotes* telomerase: evidence for limited base-pairing during primer elongation and dGTP as an effector of translocation. Biochemistry 37: 5162–5172.

Hardy, C. D., C. S. Schultz, and K. Collins, 2001 Requirements for the dGTP-dependent repeat addition processivity of recombinant *Tetrahymena* telomerase. J. Biol. Chem. 276: 4863–4871.

Harley, C. B., A. B. Futcher, and C. W. Greider, 1990 Telomeres shorten during ageing of human fibroblasts. Nature 345: 458–460.

Huang, M., Z. Zhou, and S. J. Elledge, 1998 The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell 94: 595–605.

Jain, D., and J. P. Cooper, 2010 Telomeric strategies: means to an end. Annu. Rev. Genet. 44: 243–269.

Kumar, D., J. Viberg, A. K. Nilsson, and A. Chabes, 2010 Highly mutagenic and severely imbalanced dNTP pools can escape

detection by the S-phase checkpoint. Nucleic Acids Res. 38: 3975–3983.
Lee, Y. D., and S. J. Elledge, 2006 Control of ribonucleotide reductase localization through an anchoring mechanism involving Wtm1. Genes Dev. 20: 334–344.
Lee, Y. D., J. Wang, J. Stubbe, and S. J. Elledge, 2008 Dif1 is a DNA-damage-regulated facilitator of nuclear import for ribonucleotide reductase. Mol. Cell 32: 70–80.
Lingner, J., T. R. Hughes, A. Shevchenko, M. Mann, V. Lundblad *et al.*, 1997 Reverse transcriptase motifs in the catalytic subunit of telomerase. Science 276: 561–567.
Lundblad, V., and J. W. Szostak, 1989 A mutant with a defect in telomere elongation leads to senescence in yeast. Cell 57: 633–643.
Maine, I. P., S. F. Chen, and B. Windle, 1999 Effect of dGTP concentration on human and CHO telomerase. Biochemistry 38: 15325–15332.
Mason, M., A. Schuller, and E. Skordalakes, 2011 Telomerase structure function. Curr. Opin. Struct. Biol. 21: 92–100.
Meyne, J., R. L. Ratliff, and R. K. Moyzis, 1989 Conservation of the human telomere sequence (TTAGGG)n among vertebrates. Proc. Natl. Acad. Sci. USA 86: 7049–7053.
Morin, G. B., 1989 The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 59: 521–529.
Nakamura, T. M., G. B. Morin, K. B. Chapman, S. L. Weinrich, W. H. Andrews *et al.*, 1997 Telomerase catalytic subunit homologs from fission yeast and human. Science 277: 955–959.
Nick McElhinny, S. A., B. E. Watts, D. Kumar, D. L. Watt, E. B. Lundstrom *et al.*, 2010 Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. Proc. Natl. Acad. Sci. USA 107: 4949–4954.
Pardo, B., E. Ma, and S. Marcand, 2006 Mismatch tolerance by DNA polymerase Pol4 in the course of nonhomologous end joining in *Saccharomyces cerevisiae*. Genetics 172: 2689–2694.
Peng, Y., I. S. Mian, and N. F. Lue, 2001 Analysis of telomerase processivity: mechanistic similarity to HIV-1 reverse transcriptase and role in telomere maintenance. Mol. Cell 7: 1201–1211.
Peter, M., A. Gartner, J. Horecka, G. Ammerer, and I. Herskowitz, 1993 *FAR1* links the signal transduction pathway to the cell cycle machinery in yeast. Cell 73: 747–760.
Reichard, P., 1988 Interactions between deoxyribonucleotide and DNA synthesis. Annu. Rev. Biochem. 57: 349–374.
Ritchie, K. B., J. C. Mallory, and T. D. Petes, 1999 Interactions of *TLC1* (which encodes the RNA subunit of telomerase), *TEL1*, and *MEC1* in regulating telomere length in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. 19: 6065–6075.
Sabouri, N., J. Viberg, D. K. Goyal, E. Johansson, and A. Chabes, 2008 Evidence for lesion bypass by yeast replicative DNA polymerases during DNA damage. Nucleic Acids Res. 36: 5660–5667.
Shay, J. W., and S. Bacchetti, 1997 A survey of telomerase activity in human cancer. Eur. J. Cancer 33: 787–791.
Sherman, F., 1991 Getting started with yeast. Methods Enzymol. 194: 3–21.
Singer, M. S., and D. E. Gottschling, 1994 *TLC1*: template RNA component of *Saccharomyces cerevisiae* telomerase. Science 266: 404–409.
Tang, H. M., K. L. Siu, C. M. Wong, and D. Y. Jin, 2009 Loss of yeast peroxiredoxin Tsa1p induces genome instability through activation of the DNA damage checkpoint and elevation of dNTP levels. PLoS Genet. 5: e1000697.
Teixeira, M. T., M. Arneric, P. Sperisen, and J. Lingner, 2004 Telomere length homeostasis is achieved via a switch between telomerase-extendible and -nonextendible states. Cell 117: 323–335.
Traut, T. W., 1994 Physiological concentrations of purines and pyrimidines. Mol. Cell. Biochem. 140: 1–22.
Tsaponina, O., E. Barsoum, S. U. Astrom, and A. Chabes, 2011 Ixr1 is required for the expression of the ribonucleotide reductase Rnr1 and maintenance of dNTP pools. PLoS Genet. 7: e1002061.
Wu, X., and M. Huang, 2008 Dif1 controls subcellular localization of ribonucleotide reductase by mediating nuclear import of the R2 subunit. Mol. Cell. Biol. 28: 7156–7167.
Yu, G. L., J. D. Bradley, L. D. Attardi, and E. H. Blackburn, 1990 *In vivo* alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. Nature 344: 126–132.
Zhao, X., E. G. Muller, and R. Rothstein, 1998 A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol. Cell 2: 329–340.
Zhao, X., A. Chabes, V. Domkin, L. Thelander, and R. Rothstein, 2001 The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. EMBO J. 20: 3544–3553.

Communicating editor: N. M. Hollingsworth

GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.149120/-/DC1

Telomere Length Homeostasis Responds to Changes in Intracellular dNTP Pools

Amitabha Gupta, Sushma Sharma, Patrick Reichenbach, Lisette Marjavaara, Anna Karin Nilsson, Joachim Lingner, Andrei Chabes, Rodney Rothstein, and Michael Chang

DOI: 10.1534/genetics.112.149120

File S1

SUPPORTING MATERIAL AND METHODS

DNA isolation and denaturing in-gel hybridization: Yeast genomic DNA was isolated using a Yeast DNA Extraction Kit (Thermo Scientific). The DNA was digested with XhoI restriction endonuclease before running on a 0.8% agarose gel. Denaturing in-gel hybridization using a telomeric CA oligonucleotide radio- labeled probe was performed as described (DIONNE and WELLINGER 1996)

Telospot: Telospot assays were performed as previously described (CRISTOFARI *et al.* 2007), except that the membrane was not denatured with NaOH. For Figure 6C, Telospot reactions were performed with 35 nM 5′-biotinylated $(TTAGGG)_3$ primer, which were then purified using 10 µl of streptavidin-coated M-280 Dynabeads (Invitrogen) according to the manufacturer's protocol. Samples were heated to 98°C for 5 min in 98% formamide-10 mM EDTA, resolved on a 12% polyacrylamide-urea gel, and transferred onto a positively charged Nylon membrane (GE Healthcare) using a semi-dry electrophoretic transfer cell (Transblot SD, BIO-RAD). After UV-crosslinking, the membrane was probed as in a standard Telospot assay.

SUPPORTING INFORMATION REGARDING THE VIABILITY OF *rnr1Δ*

To further examine the viability of an *rnr1Δ* deletion mutation in the BY4741 background, we backcrossed MCY602 (*rnr1Δ::kanMX* in the BY4741 background) to W9100-12C (a wild type strain of the W303 background). Half of the *rnr1Δ* progeny were dead (22 of 42 possible spores from 21 tetrad dissections), suggesting that one genetic locus was responsible for the difference in viability in the two genetic backgrounds.

Since Rnr3 protein levels are increased in *rnr1Δ* mutants (Figure 5), we wanted to determine if this single genetic locus was linked to *RNR3*. To do so, we crossed MCY605 (*rnr3Δ::natMX*; BY4742 background) and MCY606 (*yil067cΔ::natMX*; BY4742 background) to MCY602. *YIL067C* is adjacent to *RNR3* on chromosome IX. We were able to obtain viable *rnr1Δ yil067cΔ* double mutants, but not *rnr1Δ rnr3Δ* double mutants, indicating that Rnr3 is required for viability in the absence of Rnr1. We then crossed MCY607 (*rnr1Δ::kanMX yil067cΔ::natMX*; BY4741 background) to W9100-12C. As expected, half of the *rnr1Δ* progeny were dead (13 of 24 possible spores from 12 tetrad dissections). If the locus in question is linked to *RNR3*, then all the viable *rnr1Δ* strains should also contain *yil067cΔ::natMX* (of the 11 viable *rnr1Δ* colonies, only 8 were big enough to be genotyped by replica plating). This was not the case: only half did (5 of the 8). Thus, while a single genetic locus is responsible for the viability of *rnr1Δ* in the BY4741 background, the locus is not linked to *RNR3*.

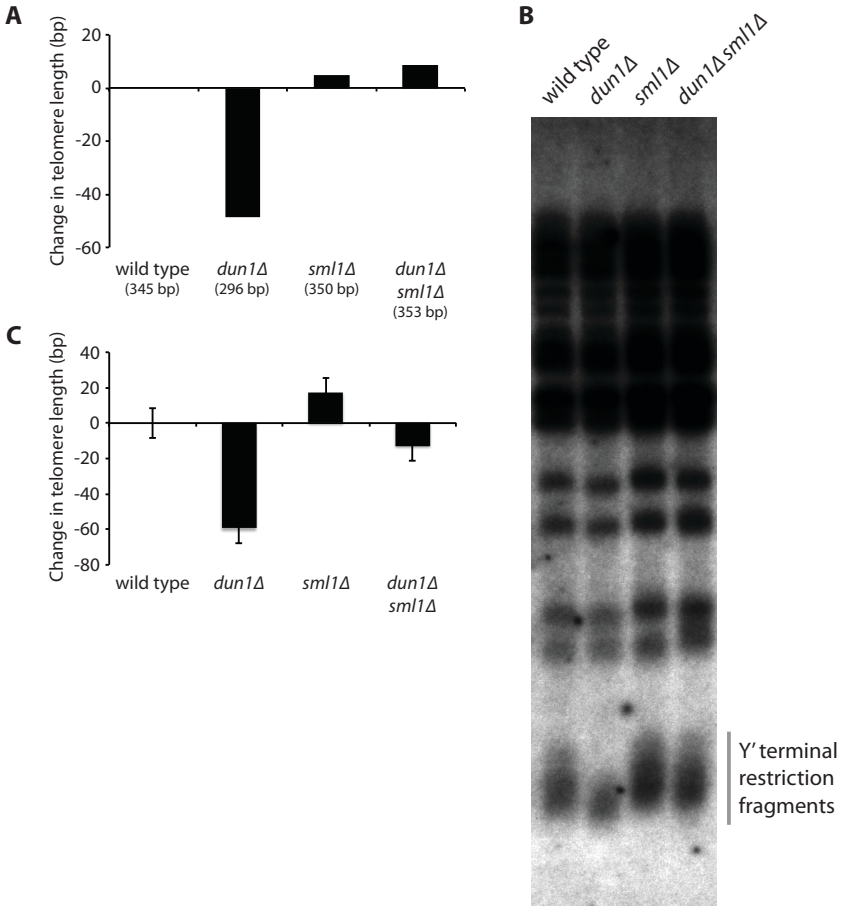

Figure S1. Telomeres are shortened in cells lacking Dun1. (A) Strains of the indicated genotypes were assayed for telomere length by telomere I-L PCR after being passaged for at least 100 generations. The change in telomere length, compared to wild type telomere length, was quantified and plotted. (B) Strains in A were assayed for telomere length by denaturing in-gel hybridization (see Supplementary Materials and Methods; a representative gel is shown). The vertical bar indicates the position of the terminal restriction fragments of Y′ telomeres, which represent more than half of yeast telomeres. Larger bands represent non-Y′-containing telomeres. (C) The change in telomere length, compared to wild type telomere length, of each strain indicated in B was quantified and plotted. Mean ± standard error for four independent isolates for each genotype are shown.

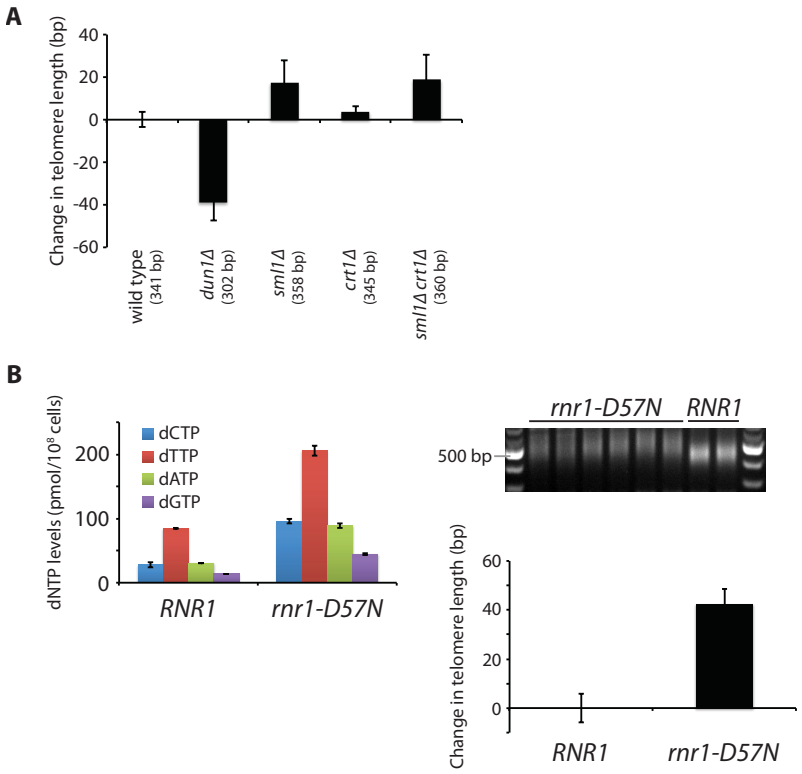

Figure S2. (A) The change in telomere length, compared to wild type telomere length, of strains of the indicated genotypes was quantified and plotted. Telomere length was assayed by Y′ telomere PCR. Mean ± standard error for at least three independent isolates for each genotype are shown. (B) Wild type (*RNR1*) and *rnr1-D57N* strains were assayed for dNTP levels. Data are represented as mean ± standard error. Multiple isolates of these strains were assayed for telomere length by Y′ telomere PCR (a representative gel is shown) and plotted as in A.

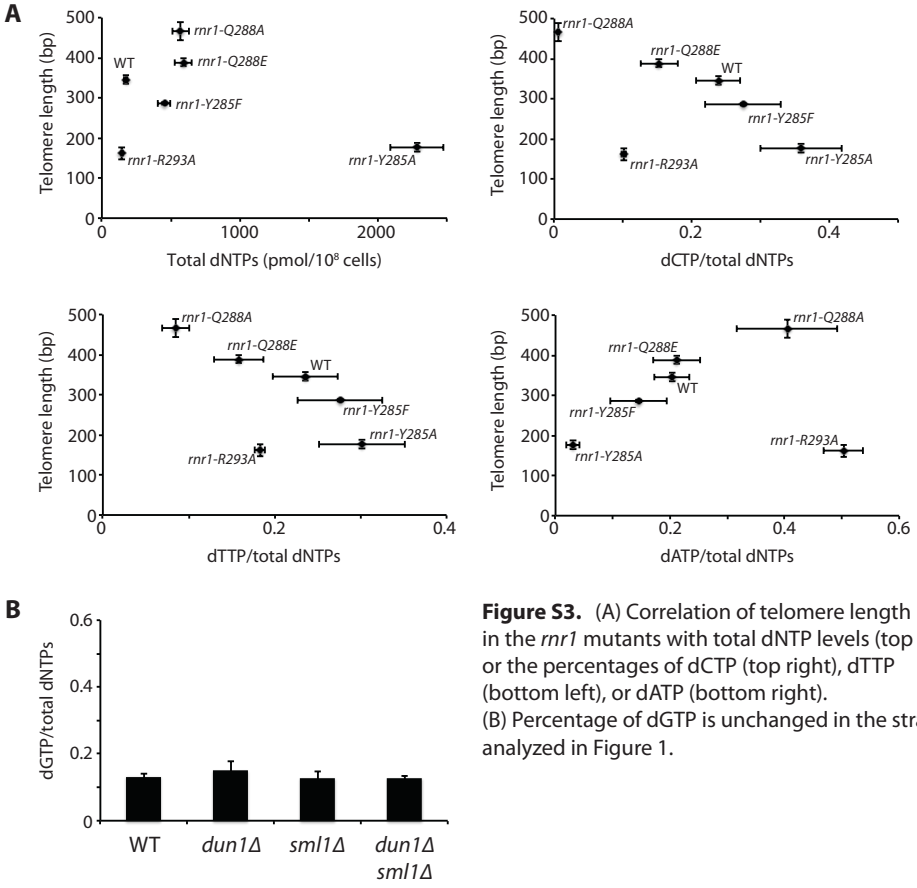

Figure S3. (A) Correlation of telomere length in the *rnr1* mutants with total dNTP levels (top left), or the percentages of dCTP (top right), dTTP (bottom left), or dATP (bottom right).
(B) Percentage of dGTP is unchanged in the strains analyzed in Figure 1.

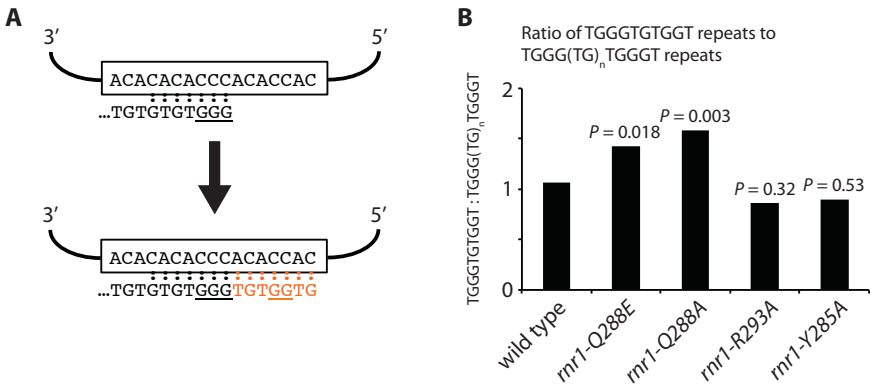

Figure S4. The processivity of reverse transcription of the 5′ portion of the TLC1 template region is increased in the *rnr1-Q288E* and *rnr1-Q288A* mutants. (A) Schematic illustrating the reverse transcription of the 5′ portion of the TLC1 template region, which is shown in the boxed area. Almost all telomeric repeats contain a GGG trinucleotide, but only about 50% of these repeats also contain the GG dinucleotide specified by the 5′ portion of the template region. (B) The ratio of GG-containing repeats (i.e. TGGGTGTGGT) to non-GG-containing repeats (i.e. TGGG(TG)$_n$TGGGT) is shown for a wild type strain and the four *rnr1* mutants. The *rnr1-Q288E* and *rnr1-Q288A* mutants, which have elongated telomeres, exhibit an increase in the presence of GG-containing repeats, indicating that telomerase repeat addition processivity for the 5′ portion of the TLC1 template region is increased. *P* values were determined using a chi-squared test to look whether the ratio in the mutant was significantly different from the wild type ratio.

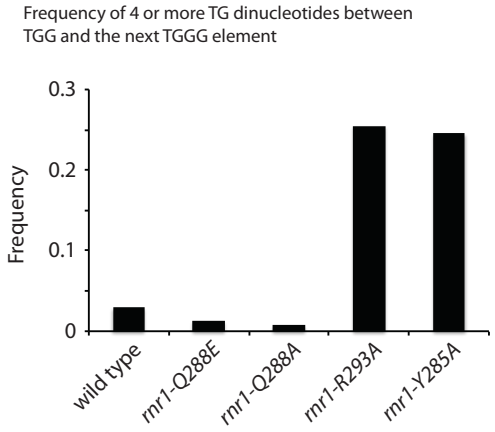

Figure S5. The frequency of having 4 or higher TG dinucleotides between a TGG and the following TGGG was plotted for the indicated strains.

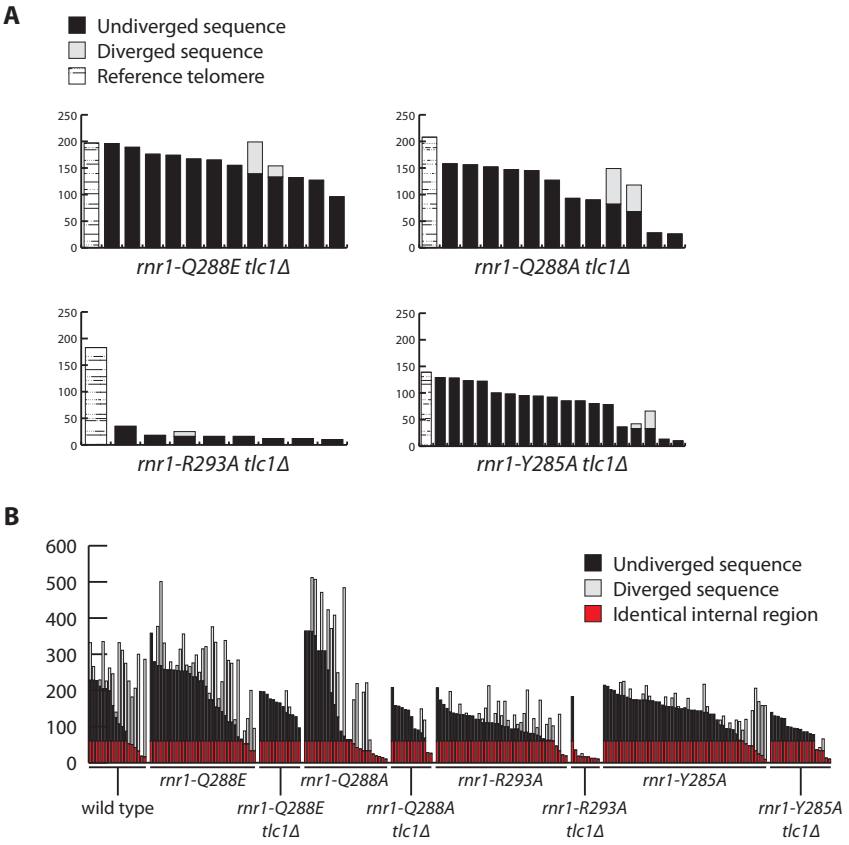

Figure S6. The telomere sequence changes in the *rnr1* mutants recorded in Figure 4 are telomerase-dependent. (A) Analysis of sequenced VI-R telomeres after ~30 generations of clonal expansion. Each bar represents an individual VI-R telomere and bars are sorted by the length of the undiverged sequence. The black portion of each bar represents the undiverged region of the telomere. The light gray portion represents the diverged region of the telomere. For each strain, the longest telomere without divergent sequence (hashed bar) is used as a reference telomere to which all other telomeres are compared to determine whether divergence has occurred. (B) All telomeres from all strains analyzed in this study share an identical internal region (as indicated by the red portion of each bars).

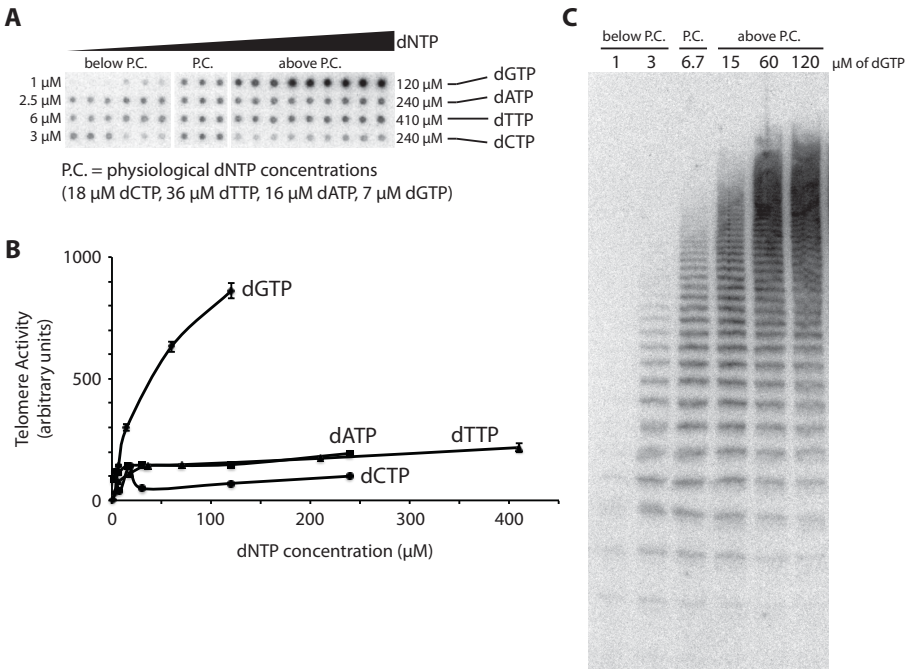

Figure S7. Human telomerase activity positively correlates with dGTP levels. (A) Telospot assay was performed by incubating crude "super-telomerase" extracts with a telomeric $(TTAGGG)_3$ primer and varying concentrations of dNTPs. "Physiological dNTP concentrations" used are derived from concentrations in yeast, although concentrations in mammalian cells are in the same range, as explained in the text. A small fraction of the reaction was directly spotted onto a nylon membrane, which is then probed with a randomly radiolabeled telomeric probe. Each reaction was spotted in triplicate. Each row of spots varies one of the four dNTPs, as indicated, from the lowest concentration shown on the left side to the highest concentration shown on the right. The reaction performed using physiological concentrations of all four dNTPs were spotted in triplicate, and then copy and pasted into each row for clarity. (B) Activity in each spot from A was quantified, and the mean for each reaction was plotted as a function of the concentration of the indicated dNTP. Error bars indicate the standard error. (C) The Telospot reactions with varying dGTP concentrations were repeated using a 5′-biotinylated $(TTAGGG)_3$ primer, purified with streptavidin-coated beads, resolved on a polyacrylamide gel, and transferred onto a nylon membrane. The membrane was then probed as in A. The concentration of dGTP used for each reaction is indicated above each lane.

TABLE S1 Yeast strains used in this study. All strains are derivatives of W1588-4C, except for BY4742, W9882 and the MCY strains. W9882 and the MCY strains are derivatives of BY4742.

Strain	Genotype	Reference
W1588-4C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5*	(ZHAO *et al.* 1998)
W9557	*MAT***a**/α *dun1Δ::TRP1*/+ *sml1Δ::HIS3*/+	This study
W9875	*MAT***a**/α *dun1Δ::TRP1*/+ *sml1Δ::HIS3*/+ *crt1Δ::LEU2*/+	This study
W9877	*MAT***a**/α *dun1Δ::TRP1*/+ *tel1Δ::URA3*/+ *mec1-21*/+	This study
DK2A	*MAT*α *rnr1::[rnr1-R293A-URA3-pGAL-RNR1]*	(KUMAR *et al.* 2010)
DK2D	*MAT***a** *rnr1::[rnr1-R293A-URA3-pGAL-RNR1]*	(KUMAR *et al.* 2010)
DK8A	*MAT***a** *rnr1::[rnr1-Y285A-URA3-pGAL-RNR1]*	(KUMAR *et al.* 2010)
DK8E	*MAT*α *rnr1::[rnr1-Y285A-URA3-pGAL-RNR1]*	(KUMAR *et al.* 2010)
DK10E	*MAT***a** *rnr1::[rnr1-Q288A-URA3-pGAL-RNR1]*	(KUMAR *et al.* 2010)
JAK11-5B	*MAT*α *rnr1::[rnr1-Q288E-URA3-pGAL-RNR1]*	This study
JAK11-5C	*MAT***a** *rnr1::[rnr1-Q288E-URA3-pGAL-RNR1]*	This study
W9878	*MAT***a**/α *rnr1::[rnr1-Q288E-URA3-pGAL-RNR1]*/+ *tlc1Δ::HIS3*/+	This study
W9879	*MAT***a**/α *rnr1::[rnr1-Q288A-URA3-pGAL-RNR1]*/+ *tlc1Δ::HIS3*/+	This study
W9880	*MAT***a**/α *rnr1::[rnr1-R293A-URA3-pGAL-RNR1]*/+ *tlc1Δ::HIS3*/+	This study
W9881	*MAT***a**/α *rnr1::[rnr1-Y285A-URA3-pGAL RNR1]*/+ *tlc1Δ::HIS3*/+	This study
DK3A	*MAT***a** *rnr1::[rnr1-Y285F-URA3-pGAL RNR1]*	(KUMAR *et al.* 2010)
DK3C	*MAT***a** *rnr1::[rnr1-Y285F-URA3-pGAL RNR1]*	(KUMAR *et al.* 2010)
W4069-4C	*MAT***a** *RNR1 CAN1*	(CHABES *et al.* 2003)
W4069-8C	*MAT***a** *rnr1-D57N CAN1*	(CHABES *et al.* 2003)
W9100-12C	*MAT*α *ADE2*	This study
BY4742	*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(BRACHMANN *et al.* 1998)
W9882	*MAT***a**/α *rnr1Δ::kanMX*/+	This study
MCY602	*MAT***a** *rnr1Δ::kanMX LYS2*	This study
MCY603	*MAT*α *rnr1Δ::kanMX*	This study
MCY605	*MAT*α *rnr3Δ::natMX*	This study
MCY606	*MAT*α *yil067cΔ::natMX*	This study
MCY607	*MAT*α *rnr1Δ::kanMX yil067cΔ::natMX*	This study

SUPPORTING REFERENCES

BRACHMANN, C. B., A. DAVIES, G. J. COST, E. CAPUTO, J. LI *et al.*, 1998 Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:** 115-132.

CHABES, A., B. GEORGIEVA, V. DOMKIN, X. ZHAO, R. ROTHSTEIN *et al.*, 2003 Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. Cell **112:** 391-401.

CRISTOFARI, G., P. REICHENBACH, P. O. REGAMEY, D. BANFI, M. CHAMBON *et al.*, 2007 Low- to high-throughput analysis of telomerase modulators with Telospot. Nat Methods **4:** 851-853.

DIONNE, I., and R. J. WELLINGER, 1996 Cell cycle-regulated generation of single-stranded G-rich DNA in the absence of telomerase. Proc Natl Acad Sci USA **93:** 13902-13907.

KUMAR, D., J. VIBERG, A. K. NILSSON and A. CHABES, 2010 Highly mutagenic and severely imbalanced dNTP pools can escape detection by the S-phase checkpoint. Nucleic Acids Res **38:** 3975-3983.

ZHAO, X., E. G. MULLER and R. ROTHSTEIN, 1998 A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol Cell **2:** 329-340.