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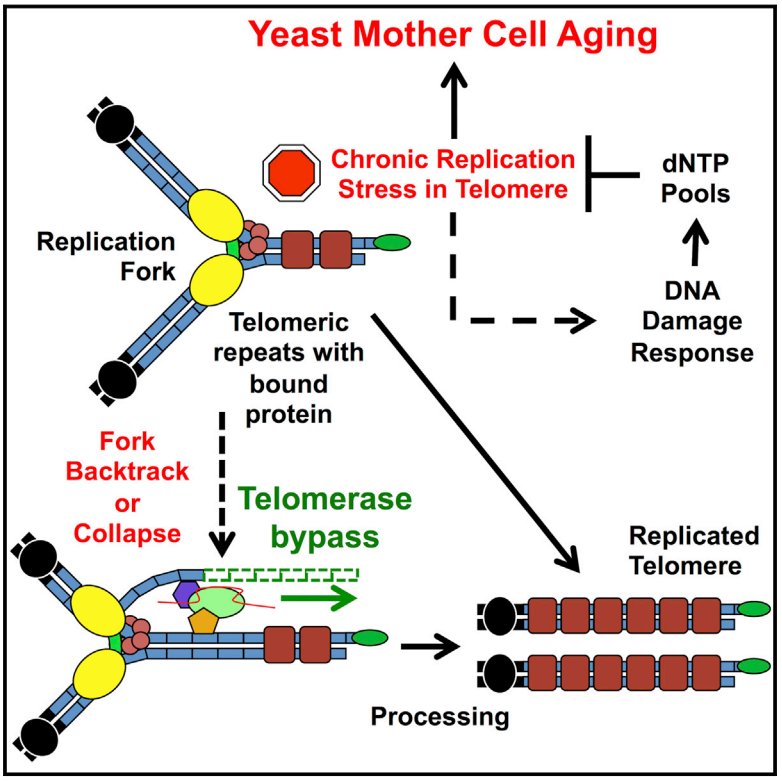
Early Telomerase Inactivation Accelerates Aging Independently of Telomere Length

Graphical Abstract



Highlights

- Early after telomerase inactivation (ETI) mother cell aging is accelerated in yeast
- Accelerated aging occurs before critical telomere shortness-induced senescence
- ETI mother cells show heterogeneous cell cycles that track with lifespan reduction
- ETI acceleration of mother cell aging results from transient DNA damage response

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In Brief

Telomerase is required for telomere maintenance and protection. Here, early telomerase inactivation leads to accelerated aging through a mechanism independent from senescence caused by telomere shortening.

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Early Telomerase Inactivation Accelerates Aging Independently of Telomere Length

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SUMMARY

Telomerase is required for long-term telomere maintenance and protection. Using single budding yeast mother cell analyses we found that, even early after telomerase inactivation (ETI), yeast mother cells show transient DNA damage response (DDR) episodes, stochastically altered cell-cycle dynamics, and accelerated mother cell aging. The acceleration of ETI mother cell aging was not explained by increased reactive oxygen species (ROS), Sir protein perturbation, or deprotected telomeres. ETI phenotypes occurred well before the population senescence caused late after telomerase inactivation (LTI). They were morphologically distinct from LTI senescence, were genetically uncoupled from telomere length, and were rescued by elevating dNTP pools. Our combined genetic and single-cell analyses show that, well before critical telomere shortening, telomerase is continuously required to respond to transient DNA replication stress in mother cells and that a lack of telomerase accelerates otherwise normal aging.

INTRODUCTION

Telomeres, protective DNA-protein complexes at the ends of eukaryotic chromosomes, buffer against the loss of sequence during DNA replication and distinguish normal chromosome ends from potentially dangerous double-strand breaks. Telomeres are composed of sequence-specific DNA binding proteins bound to highly repetitive DNA sequences and are increasingly recognized as genomic regions prone to replication stress (Miller et al., 2006; Sfeir et al., 2009; Drosopoulos et al., 2012). Without the telomeric DNA-elongating enzyme telomerase, progressive telomere shortening eventually causes the collapse of the protective DNA-protein complex (deprotection), but this occurs only after many cell divisions, late after telomerase inactivation (LTI). In LTI cells, telomere deprotection shares many properties with classic DNA damage (Nautiyal et al., 2002; d'Adda di Fagagna et al., 2003) and induces a DNA damage response (DDR) and a permanent G2/M cell-cycle arrest (senescence).

Previously, responses to telomerase deletion have generally been reported only after a significant delay (in *S. cerevisiae*, after ~50–80 divisions). Thus, it was thought that cells sense altered telomere properties that signal senescence only when telomeres become critically short and deprotected. Hence, responses and phenotypes of cells early after telomerase inactivation (ETI) have not been extensively investigated. However, it was previously shown that, in ETI cells, very short telomeres appear at low frequencies that fuse to an induced double-strand break (DSB) ($\sim 10^{-4}$ to 10^{-3}). These rare fusions became molecularly detectable when telomerase was inactivated by either deletion of the telomerase RNA template *TLC1* (*tlc1Δ*) or by replacing the reverse transcriptase subunit, *EST2*, with the mutant *est2-D530A*, which assembles a telomerase ribonucleoprotein enzyme complex lacking telomeric DNA polymerization activity (Chan and Blackburn, 2003; Lingner et al., 1997). These fusogenic telomeres arose in ETI cells well before any signs of bulk population senescence and even if the telomeres had been pre-lengthened. Therefore, even the short-term absence of telomerase activity causes cells to experience a low but detectable genomic instability.

In a process distinct from the permanent bulk population cell-cycle arrest resulting from critically short telomeres in senescent LTI cells, an individual wild-type (WT) yeast mother cell will cease divisions after it has produced ~25 daughter cells. As of yet, there has been very little evidence suggesting interaction between the pathways that regulate these two kinds of aging, hereafter referred to as "LTI senescence" and "mother cell aging/lifespan," respectively. Despite the identification of multiple genes that regulate mother cells lifespans (Bishop and Guarente, 2007; Johnson et al., 1999; Kaeberlein, 2010), the mechanisms causing mother cell aging of even WT yeast remain poorly understood.

Here, we report experiments employing single cell methodologies, supporting a model in which budding yeast mother cells lacking telomerase activity are less able to resolve replication stress inherent to telomeres. These cells show induction of a signaling pathway indicative of transient DNA replication stress, altered cell-cycle dynamics even in young mother cells, and accelerated aging (reduced lifespan), independently of telomere length. Our results demonstrate that this occurs well before the

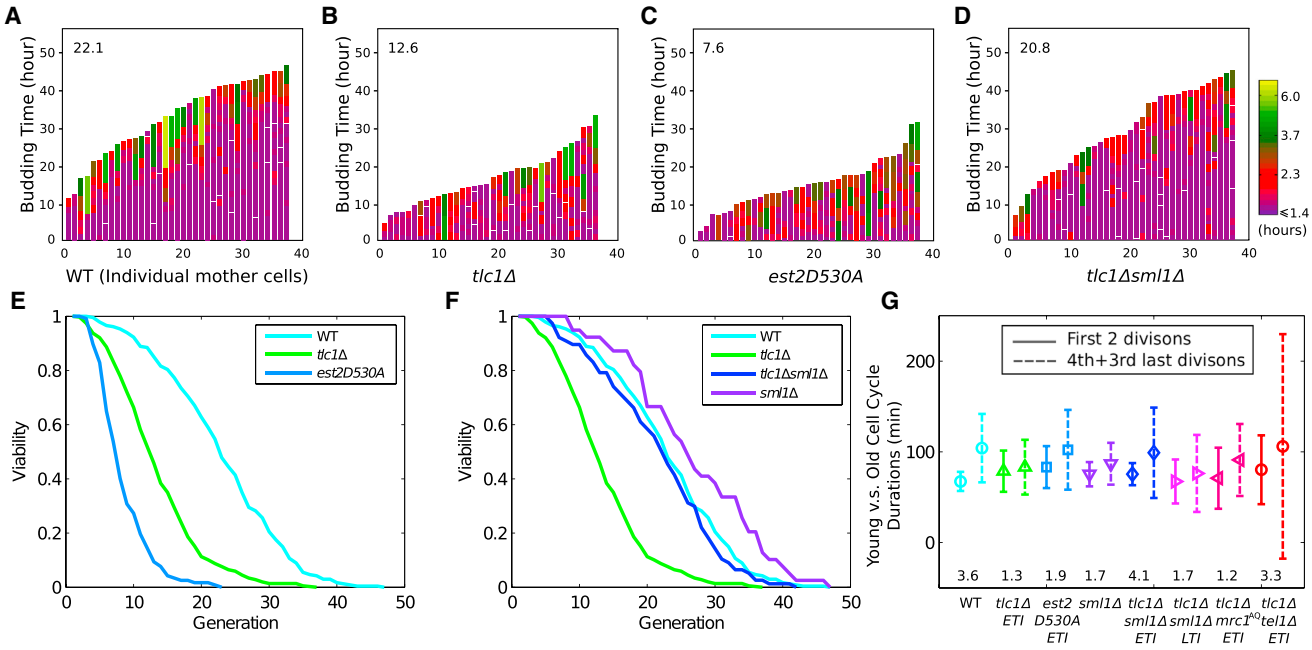

Figure 1. ETI Mother Cells Show a Non-Progressive Cell-Cycle Length Phenotype and Reduced Lifespan that is Rescued by SML1 Deletion

(A–D) Mother cell budding profiles for (A) WT, (B) *tlc1Δ*, (C) *est2-D530A*, and (D) *tlc1Δsml1Δ*, showing cell-cycle durations and heterogeneity (see exponential color scale; cell cycles with durations 1.4 hr or less were colored in purple). The x axis displays individual mother cells shown as vertical bars, with budding events indicated as horizontal white divisions. Mean lifespan for each genotype is presented in the upper-left corner of the plot.

(E) ETI mother cells showed reduced replicative lifespans compared to WT cells (number of cells [n]: *tlc1Δ*, 354; *est2-D530A*, 117; WT, 234. p value for difference between *tlc1Δ* and WT < 1e-37).

(F) Deletion of *SML1* restores lifespan of ETI mother cells to WT levels (n: *tlc1Δsml1Δ*, 77; *sml1Δ*, 39).

(G) The heterogeneity of cell-cycle lengths in ETI cells did not progressively worsen relative to WT as mother cells aged. Fold increase in cell-cycle variability from first and second to third and fourth last cell cycles compared for each genotype (shown below each set). The variance of first and second cell cycles of *tlc1Δ* and *est2530A* is significantly greater than that of WT (F test; p < 1e-16 and 1e-13, respectively). Error bars indicate SD.

See also Figures S1 and S2.

onset of LTI senescence and that the accelerated aging of ETI mother cells resembles the normal mother cell aging process.

RESULTS

Mother Cells Lacking Active Telomerase Show Increased Heterogeneity of Cell-Cycle Durations and Reduced Lifespans

We analyzed the properties of individual haploid ETI mother cells, well before any signs of cellular LTI senescence, freshly isolated from sporulation of heterozygous telomerase-competent diploids. Following genotyping, cells were taken from logarithmically growing cell cultures (~25–30 generations after telomerase loss), in which the overwhelming majority of cells were robustly growing newborn or very young mother cells. These cells were placed in a microfluidic device, and the budding cycles and lifespans of individual mother cells were continuously monitored for 2 days by repeated microscopic imaging (Xie et al., 2012; Zhang et al., 2012).

First, even the youngest ETI mother cells (*tlc1Δ* or *est2-D530A*) immediately showed higher frequencies of stochastically longer and more heterogeneous cell-cycle durations than WT (Figures 1A–1C; note especially between times 0 to 5 hr, as marked on Y axes). As the durations of the last two budding cycles were highly heterogeneous in both WT and ETI mothers, they were discarded from all cell-cycle duration analyses discussed here. This cell-cycle heterogeneity was consistent with observations of bulk ETI population budding kinetics, as manifested by cells lingering in the large-budded state (G2/M), enriched for cells with short spindles and unsegregated chromosomes (Figures S1A–S1C). Second, we analyzed the mother cell aging of individual ETI cells and found that the lack of telomerase activity reduced ETI mother cell lifespan. Mean budding lifespan for *tlc1Δ* was 12.6 (7 replicates) and 7.6 generations for *est2-D530A* (3 replicates), compared to 22.1 for WT mother cells (Figures 1A–1C and 1E). Furthermore, the catalytically inactive telomerase *est2-D530A* point mutant showed even longer cell-cycle durations than *tlc1Δ* ETI mother cells, and the lifespan reduction was even more severe. Hence, lack of telomerase enzymatic activity, rather than the absence of an assembled telomerase ribonucleoprotein complex, causes increased cell-cycle heterogeneity and faster mother cell aging.

Heterogeneous Cell Cycles Do Not Progressively Worsen with Shortening Telomeres

If the extended, heterogeneous cell-cycle lengths of ETI mother cells were due solely to telomere shortening, we would have expected the phenotype to worsen progressively with each

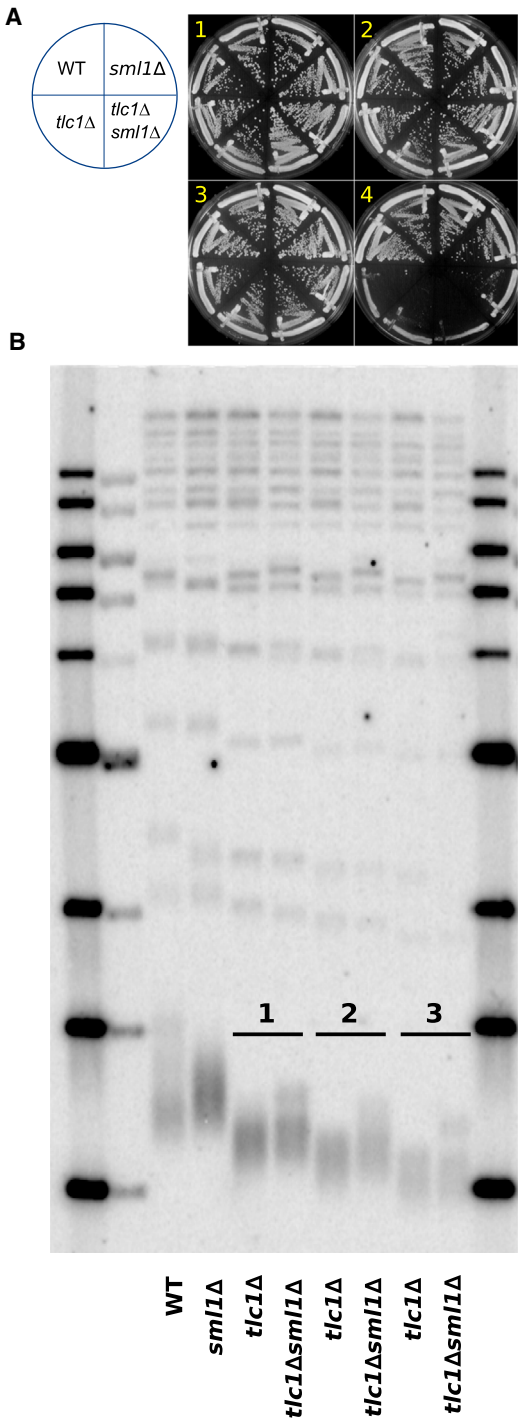

Figure 2. SML1 Deletion Rescues Mother Cell Lifespan of ETI Cells Independently of Telomere Length

(A) *SML1* deletion had no significant effect on the rate of bulk population senescence in ETI cells passaged on solid media to induce LTI-senescence. (B) Southern blot analysis of telomeric DNA restriction fragment lengths of cells taken from serial streaks shown in (A), using $TG_{(1-3)}$ repeat telomeric probe. ETI (*tlc1Δ*) and ETI *sml1Δ* (*tlc1Δsml1Δ*) displayed similar rates of telomere shortening, and the lower end of the telomere length distributions were similar. See also Figure S3.

successive cell division. However, this was not the case. First, as individual mother cells progressed from being very young to old, ETI mother cells did not show any significant progressive increase in mean duration or heterogeneity of mother cell-cycle lengths relative to WT (Figure 1G). Second, during the individual ETI mother cell lineages, a young mother cell whose initial cell cycle was long had no greater probability of having subsequent longer cell cycles or a shorter lifespan than one with an initial short cell cycle (Figure S2), supporting a stochastic and episodic, rather than progressive, nature of the occurrence of longer cell cycles. These highly stochastic episodes of cell-cycle heterogeneity and lack of any progressive worsening of this phenotype as ETI mother cells aged are not the predicted result of progressive telomere shortening.

SML1 Deletion Rescues Mother Cell Lifespan of ETI Cells Independently of Telomere Length

Because we observed an extended G2/M phase in bulk population analyses (Figure S1), which is often the result of DDR activation, we determined whether mutations affecting the DDR affected the above ETI phenotypes. Responses to various forms of DNA damage, including that sensed at critically short telomeres in LTI senescence, involve a cascade of phosphorylation events, with early upstream steps occurring at the source of DNA damage through PIKK family member kinases Mec1 (*ATR*) and/or Tel1 (*ATM*). Strains lacking only Mec1 are inviable, but this *mec1Δ* lethality can be rescued by deletion of *SML1* (Zhao et al., 1998). Sml1 inhibits ribonucleotide reductase (RNR), which catalyzes the rate-limiting step in dNTP production (Reichard, 1988). Deletion of Sml1 increases RNR activity and elevates dNTP pools, obviating the need for certain DDR components under healthy growing conditions, and can be protective against some forms of DNA damage (Andreson et al., 2010; Jossen and Bermejo, 2013). Strikingly, deletion of *SML1* in ETI *tlc1Δ* strains efficiently rescued the ETI-induced heterogeneity of budding cycle durations (Figure 1D) as well as the shortening of mother cell lifespan (Figure 1F). However, *SML1* deletion alone produced no change in the rates of bulk telomere shortening in ETI cells, nor in the subsequent onset of LTI senescence (Figures 2 and S3). We also confirmed that the deletion of *SML1* alone caused no significant effect on mother cell lifespans and telomere length compared to WT (Figures 1F and S4B). Hence, the dramatic rescue of ETI cell-cycle heterogeneity and accelerated mother cell aging by *SML1* deletion cannot be explained by increased telomere length or by slower rates of telomere shortening.

ETI Mother Cells Age with Terminal Cellular and Mitochondrial Morphologies Distinct from LTI Senescence but Similar to Those of Normal Mother Cell Aging

We tested further whether budding cessation due to mother cell aging in ETI or WT cells was distinguishable from the G2/M arrest caused by LTI senescence by examining cell and mitochondrial morphology at the end of the lifespans (terminal morphology). Typical WT mother cell aging produces terminal cells that are mostly small budded with minimal or no mitochondrial fluorescence signal from a mitochondrially localized GFP (mtGFP)

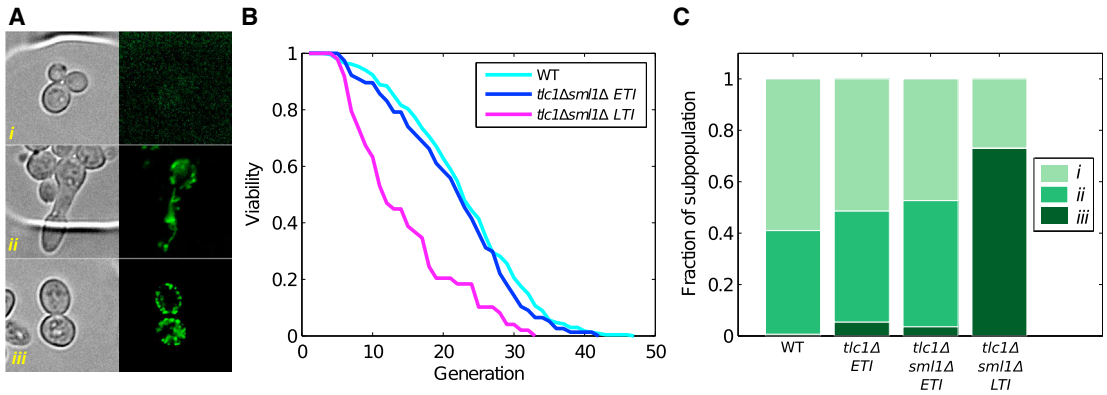

Figure 3. ETI Mother Cells Age with Terminal Cellular and Mitochondrial Morphologies Distinct from LTI Senescence but Similar to Those of Normal Mother Cell Aging

(A) Three possible terminal death morphologies were observed in WT, ETI, and/or LTI (see text for definition) mother cells: small budded (type i), elongated (type ii), and a G2/M large-budded ("dumbbell" shape) (type iii). Mitochondrial volume was measured using mitochondrially localized GFP (mtGFP).

(B) ETI and LTI populations of *tlc1Δsml1Δ* cells were prepared to distinguish cell death from normal mother cell aging and LTI senescence. *tlc1Δsml1Δ* LTI (n = 49) strains senesced and showed reduced lifespan, as expected.

(C) ETI cells terminally arrest in a manner similar to WT mother cells and distinct from LTI senescence. Most of the cells in ETI *tlc1Δ* and ETI *tlc1Δsml1Δ* show type i or type ii death morphologies (> 95%), similar to terminal WT mother cells. In LTI cells, a major fraction (~70%) displayed type iii morphology ($p < 1e\text{-}4$ compared to ETI *tlc1Δsml1Δ* by Fisher's exact test), indicative of senescence induced by critically short telomeres.

(Figure 3Ai) and a smaller population of elongated cells with brighter mitochondrial fluorescence (Figure 3Aii). In contrast, cells terminally arrested due to LTI senescence accumulate with a swollen, large-budded ("dumbbell") morphology and with mitochondrial fluorescence that gradually forms very bright dots (Figure 3Aiii) (Nautiyal et al., 2002). We created and analyzed two populations of *tlc1Δsml1Δ* cells. The first population was isolated as soon as possible after genotyping (ETI) and was enriched for mother cells that would reach their aging limit prior to LTI senescence. The second population was passaged for approximately ten additional generations prior to microfluidics analysis, thus enriching for cells that would undergo LTI senescence (critically short telomeres) before the mother cells reached their aging limit (Figure 3B). Terminally aged ETI *tlc1Δ* and ETI *tlc1Δsml1Δ* mother cells accumulated mostly in two dominant terminal morphologies, which resembled the two dominant WT terminal morphology phenotypes (Xie et al., 2012) and only very rarely in the dumbbell morphology (Figures 3A and 3C). In contrast, in terminal LTI *tlc1Δsml1Δ* mother cells, terminal dumbbell morphologies became the major type observed, indicating that a large proportion of the population had entered LTI senescence (Figures 3Aiii and 3C). These results support terminal cellular and mitochondrial morphology as an accurate distinction between LTI senescence and normal mother cell aging and provide further evidence that ETI mother cells cease divisions as a result of mother cell aging rather than LTI senescence.

Mutation of Specific DDR Components Exacerbates ETI Cell-Cycle and Lifespan Phenotypes

We investigated other proteins previously implicated in yeast telomere maintenance and in the DDR for effects on mother cell aging. Maintenance of yeast telomeres at normal length requires DDR kinases Mec1 and Tel1 (Sabourin and Zakian, 2008; Takata et al., 2004) and the replication stress-specific DDR adaptor protein Mrc1 (Grandin et al., 2005). First, we found that cell-cycle heterogeneity and mother cell lifespan were similar in WT, *tel1Δ*, and *mec1Δsml1Δ* strains (Figures 1A, 4A, 4C, S4C, and S5A). Because *tel1Δ* in haploid cells reduces telomerase action on telomeres, telomeres decline to a short length that is then stably maintained (Greenwell et al., 1995; Lustig and Petes, 1986). The *tel1Δ* cells used here were isolated immediately after sporulation of heterozygous parent diploids and analyzed when telomeres were still shortening from near-WT lengths. Therefore, having telomeres that are shortening but eventually stably maintained is not alone sufficient to alter cell-cycle duration and lifespan.

Next, we examined how mutations of Mec1 and Tel1 affect the ETI phenotypes. Because *sml1Δ*, as shown above, efficiently rescues the accelerated aging of ETI mother cells, it is difficult to determine whether Mec1 has a role in this process, due to the necessity of deleting *SML1* for viability in *mec1Δ* strains. However, ETI *tlc1Δtel1Δ* double-mutant mother cells had even greater cell-cycle heterogeneity and shorter budding lifespan (mean 9.8 generations, 2 replicates) than control ETI *tlc1Δ* single-mutant mother cells (Figures 1B, 4B, and 4D). As shown previously, freshly isolated ETI haploid cells that are also mutated for Tel1 or Mec1 (*tlc1Δmec1Δsml1Δ* or *tlc1Δtel1Δ*) have a rate of initial telomere shortening and progression to LTI population senescence similar to *tlc1Δ* single mutants (Chan and Blackburn, 2003) (Figures 4E and 4F). Hence, the exacerbation of the ETI cell-cycle heterogeneity and lifespan reduction phenotypes caused by lack of Tel1 is not explained by faster telomere shortening or accelerated population senescence.

Because *sml1Δ* rescues the cell-cycle and lifespan phenotypes of *tlc1Δ* mother cells and is known to facilitate DNA replication by increasing nucleotide levels (Chabes et al., 2003), we suspected that ETI cells may be more vulnerable to telomeric

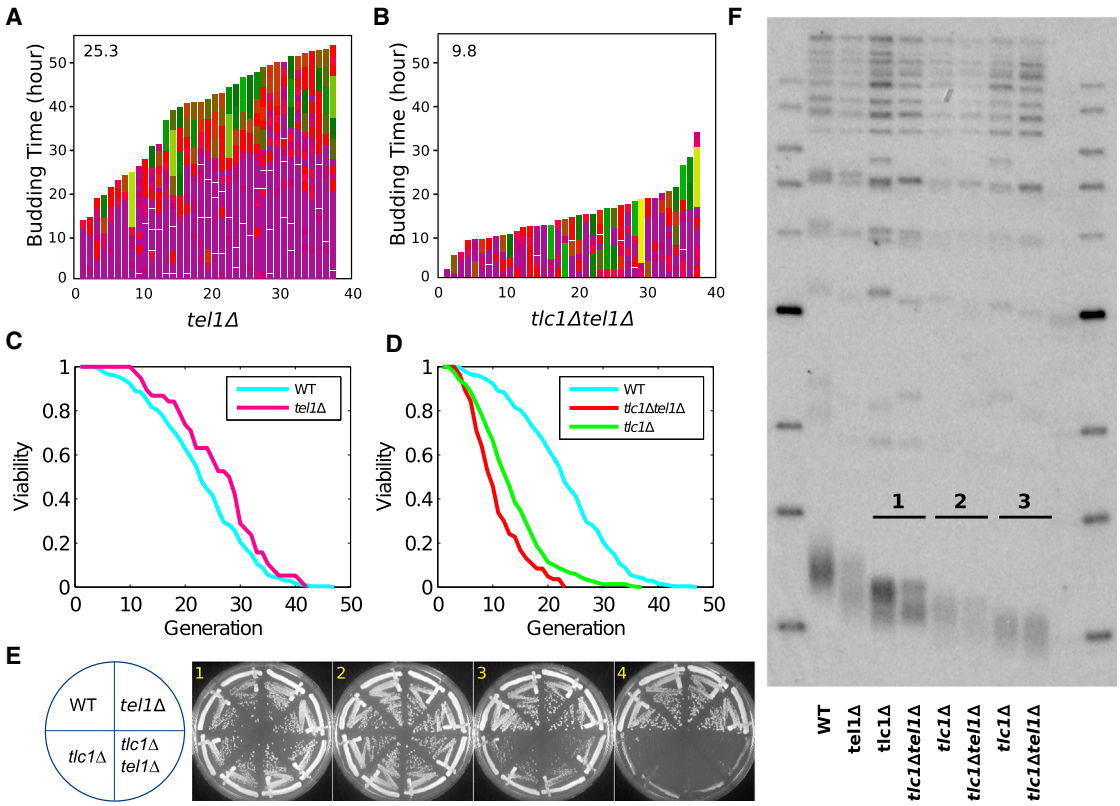

Figure 4. TEL1 Deletion Exacerbates ETI Cell Cycle and Lifespan Phenotypes, but Not Senescence or Telomere-Shortening Rates

(A and B) Mother cell budding profiles for *tel1Δ* (A) and *tlc1Δ tel1Δ* (B).

(C) *tel1Δ* (n = 38) strain lifespan does not differ from WT.

(D) *tlc1Δtel1Δ* (n = 83) mutation worsens the lifespan reduction caused by ETI mutations in mother cells ($p < 1e\text{-}4$, compared with *tlc1Δ* alone).

(E) ETI and ETI *tel1Δ* mutants displayed similar rates of senescence when passaged on solid media.

(F) Southern blot analysis of telomeric DNA restriction fragment lengths of cells taken from plates after serial streaks shown in (E).

See also Figures S4 and S5.

DNA replication stress. Therefore, we mutated the DDR adaptor protein Mrc1, which is required specifically for the DNA replication stress checkpoint (Alcasabas et al., 2001; Osborn and Elledge, 2003) and has a minor role in telomere length maintenance (Tsolou and Lydall, 2007). Mutation of 17 potential PIKK family kinase consensus phosphorylation sites on Mrc1 (*mrc1^{AQ}*) allows full cell viability but disables the DNA replication stress response (Osborn and Elledge, 2003). Despite the lack of any mother cell lifespan or cell-cycle effect of *mrc1^{AQ}* alone (Figures 5A and 5C), *tlc1Δmrc1^{AQ}* double-mutant ETI mother cells showed even greater cell-cycle length heterogeneity than the *tlc1Δ* single-mutant ETI cells (Figure 5B). Consistent results were also seen in the G2/M durations in bulk populations (Figure S1D), and mean lifespan was markedly reduced to 8.8 generations (2 replicates), compared with 12.6 generations for the control *tlc1Δ* ETI strains (Figures 1B and 5D). These effects were not explainable by reduced telomere length or accelerated senescence, as the *mrc1^{AQ}* mutant allele produced stable telomeres only slightly shorter than WT and had no effect on the kinetics of telomere shortening or bulk population senescence (Figures 5E and 5F). We also tested the epistasis relationship of *tel1Δ* and *mrc1^{AQ}* in the ETI context. ETI triple-mutant *tlc1Δ tel1Δ mrc1^{AQ}* cells showed the same lifespan shortening as the double ETI mutants (Figure S5B). We conclude that *Tel1* and *Mrc1* checkpoint functions act in the same pathway and that lack of either one acts synthetically with the ETI mother cell phenotypes.

In the DDR cascade, downstream of Tel1 or Mec1, the DDR adaptor protein Rad9 can act semi-redundantly with the adaptor protein Mrc1. Mrc1 is specifically involved in the replication stress response while Rad9 is mostly important for responding to DNA breaks and other DNA damage. In contrast to *tlc1Δmrc1^{AQ}* ETI cells, *tlc1Δrad9Δ* ETI mother cell-cycle durations and lifespans were not significantly different from *tlc1Δ* ETI cells, consistent with bulk population analyses (Figure S1 and data not shown). The ETI *tlc1Δ rad9Δ* mother cells had a mean lifespan of 16.5 generations (2 replicates), while the control *tlc1Δ* strain had a mean lifespan of 13.7 generations (Figure S5C). Thus, *rad9Δ* did not significantly affect the accelerated aging phenotypes of ETI mother cells. These results confirmed the specificity of the Mrc1 checkpoint function in the ETI mother cell phenotypes and indicate the involvement of a DNA replication stress response, rather than a response to other forms of DNA damage, which requires Rad9. In summary, disrupting the DDR via *tel1Δ* or *mrc1^{AQ}* mutations, but not by *mec1Δsml1Δ*

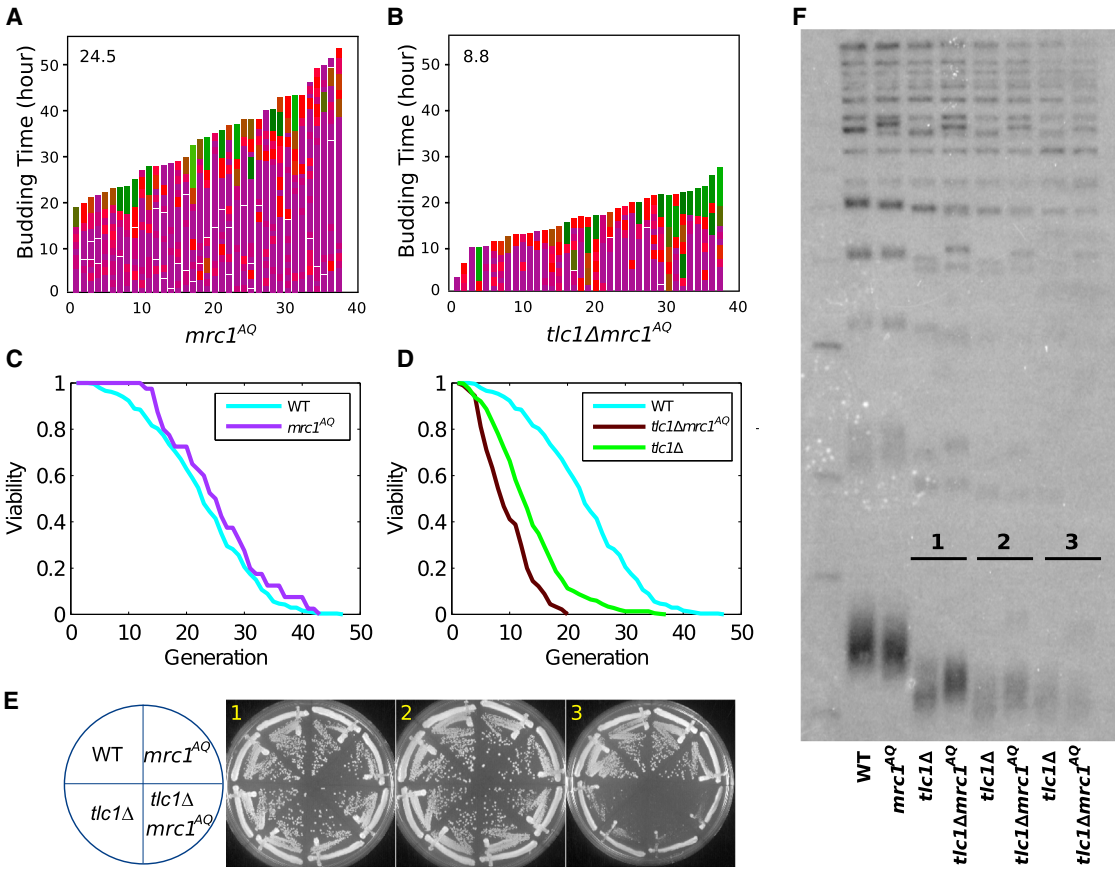

Figure 5. *MRC1* Mutation Exacerbates ETI Cell Cycle and Lifespan Phenotypes, but not Senescence or Telomere-Shortening Rates

(A and B) Mother cell budding profiles for *mrc1*^{AQ}(A) and *tlc1Δ mrc1*^{AQ} (B).

(C) *mrc1*^{AQ} (n = 40) strain lifespan does not differ from WT.

(D) *tlc1Δmrc1*^{AQ} (n = 90) mutation worsens the lifespan reduction caused by ETI mutations in mother cells ($p < 2e\text{-}7$, compared with *tlc1Δ*).

(E) ETI and ETI *mrc1*^{AQ} mutants displayed similar rates of senescence when passaged on solid media.

(F) Southern blot analysis of telomeric DNA restriction fragment lengths of cells taken from plates after serial streaks shown in (E).

See also Figures S4 and S5.

or *rad9Δ*, strongly exacerbated the cell-cycle abnormalities and acceleration of mother cell aging in ETI cells, independently of telomere length and without accelerating LTI senescence.

ETI Mother Cell Phenotypes Are Not Caused by Deprotected Telomeres

Previous results showed that short, fusogenic telomeres occur spontaneously at very low frequencies in ETI cells (Chan and Blackburn, 2003). These fusogenic telomeres derive from rare individual deprotected telomeres and can be detected by PCR assays upon their fusion to an induced DNA double-stranded break. We tested whether the amount of such fusogenic telomeres correlated with the severity of our ETI mother cell phenotypes using the same system (Chan and Blackburn, 2003) for semiquantitative PCR analyses. In agreement with the published work, we found that single-mutant ETI (*tlc1Δ*) and *tel1Δ* strains each showed detectable but low amounts of fusions resulting from a deprotected telomere fusing to an induced DSB and that *tlc1Δtel1Δ* strains showed a synergistic increase (Figures 6A and 6B). However, in *tlc1Δmrc1*^{AQ} ETI cells (Figure 6A), the *mrc1*^{AQ} mutation produced no further significant increase over a *tlc1Δ* single mutant. Furthermore, *sml1Δ* did not reduce (and possibly increased) the number of fusogenic telomeres detected (Figures 6A and 6B). This complete non-concordance in these various ETI genotypes with the phenotypes we have observed here in ETI mother cells argues strongly against deprotected telomeres as a cause for the exacerbated cell-cycle heterogeneity and accelerated mother cell aging.

Further evidence that ETI phenotypes are not caused by deprotected telomeres, which induce a robust DDR (Nautiyal et al., 2002; d'Adda di Fagagna et al., 2003), came from comparing the genetic dependencies of ETI cell phenotypes versus DNA damage sensitivity. As previously reported, *mec1Δsml1Δ* and *rad9Δ* mutations made cells highly sensitive to treatment with various classic DNA damaging agents (HU, UV, phleomycin, or MMS) (Figure S4D). This is in dramatic contrast to the experiments described above, in which *mec1Δsml1Δ* and *rad9Δ* did not exacerbate the ETI phenotypes.

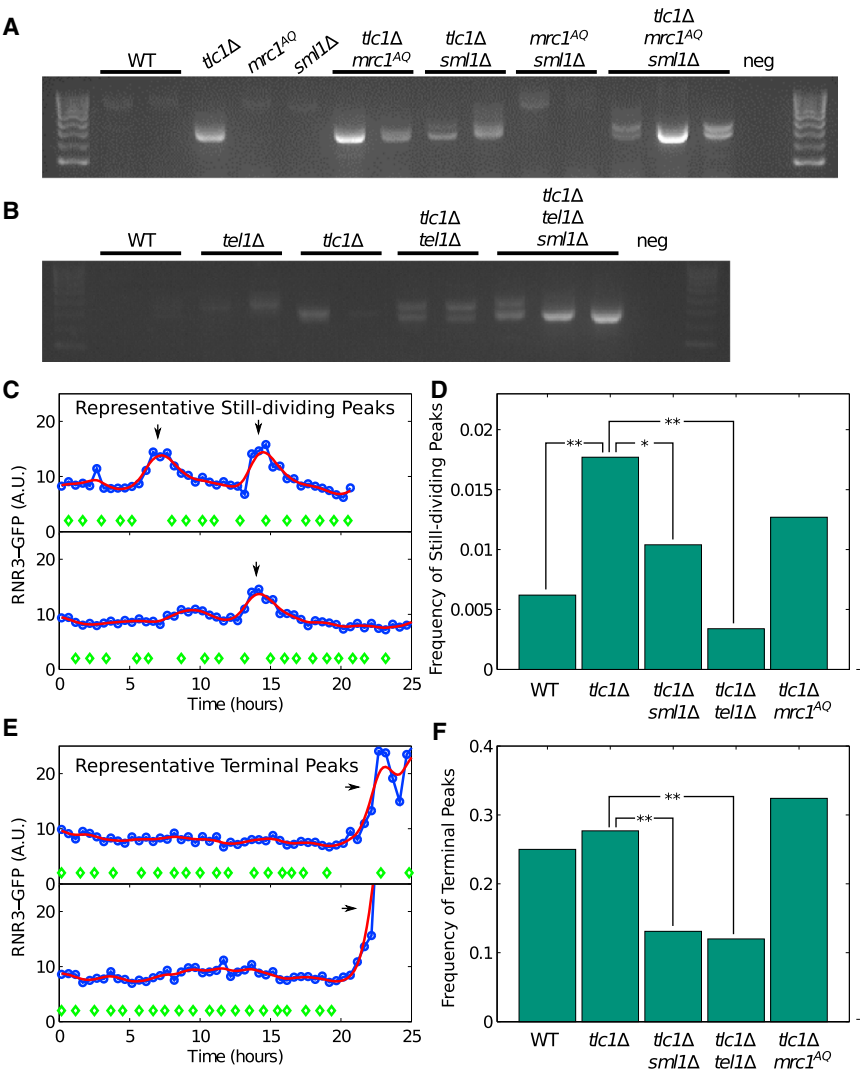

Figure 6. Genotype Dependence of Telomere Fusions and Transient DNA Damage Response Episodes in Mother Cells

(A) Semiquantitative PCR of DNA species resulting from the fusion of a deprotected telomere with an induced double-strand break in genetic backgrounds containing *ETI* and *mrc1^{AQ}* mutant combinations.

(B) Same as in (A) but with genetic backgrounds containing *ETI* and *tel1Δ* mutations.

(C) Two representative profiles of RNR3-GFP peaks occurring in still-dividing individual mother cells. Cell divisions (green diamonds) and RNR3-GFP reporter levels (blue circles) were plotted throughout an individual mother cell's lifespan. Spline fitting is shown as red lines.

(D) Frequencies of RNR3-GFP induction peaks in still-dividing cells such as those shown in (C). *p < 0.01 and **p < 0.001 by Fisher's exact test are indicated.

(E) Two representative mother cell profiles shown, as in (C), with cells displaying terminal RNR3-GFP induction peaks.

(F) Frequencies of RNR3-GFP induction peaks in terminal mother cells, such as those shown in (E). See also Figure S6.

Hence, the genotype dependencies of ETI mother cell phenotypes are quite distinct from the dependencies of responses to classic DNA-damaging agents.

Altered Recombination Levels Are Not Responsible for ETI Mother Cell Phenotypes

Recombination is another process that has been implicated in maintaining yeast telomeres and occurs when telomeres lose protection, such as in LTI cells (McEachern and Blackburn, 1996; Basenko et al., 2011). Following the onset of LTI senescence, Rad52-dependent recombination at telomeres allows a small fraction (~10^{-4}) of senescing LTI yeast cells to survive and continue dividing (Lundblad and Blackburn, 1993). Also, DNA replication stress can be relieved by mechanisms involving recombination. We therefore asked whether recombination plays any role in the ETI accelerated mother cell-cycle kinetics and aging response. Deletion of *RAD52* alone causes no changes in telomere length maintenance, and telomeres in *tlc1Δrad52Δ* strains shorten no faster than with *tlc1Δ* alone (Lundblad and Blackburn, 1993). However, *rad52Δ* alone caused increased mother cell-cycle duration heterogeneity (data not shown) and an acceleration of mother cell budding aging (Park et al., 1999). Notably, these *rad52Δ* phenotypes were not substantially rescued by *SML1* deletion (mean lifespan, *rad52Δ*: 9.4, n = 130 versus *rad52Δsml1Δ*: 13.2, n = 70) (Figure S6A and data not shown). Furthermore, the mean lifespan of ETI *tlc1Δrad52Δsml1Δ* mother cells was even lower than *rad52Δsml1Δ*: 8.2 versus 13.2 (Figure S6A). Hence, lack of Rad52 function appears to act additively to the effect of *TLC1* deletion. This epistasis relationship indicates that absence of telomerase activity and of Rad52 each causes acceleration of mother cell aging but by two distinct mechanisms.

ETI Phenotypes Are Not Caused By Relocalization of Sir Proteins

Another pathway previously implicated in yeast mother cell aging involves changes in Sir protein concentration and localization. For example, Sir2 overexpression has been shown to increase mother cell lifespan (Kaeberlein et al., 1999). However, several lines of evidence argue that Sir2 sequestration in ETI cells does not explain their accelerated aging. First, all of our ETI strains mated normally, implying that the mating type loci were still silenced and arguing against a large relocalization of Sir proteins. Second, localized puncta of Sir3-GFP, indicative of telomere-bound Sir complex proteins (Martin et al., 1999), were not significantly different between ETI and WT mother cells

(Figure S6B). Third, although a single induced unrepairable DNA break has been reported to cause Rad9-dependent delocalization of Sir2 from telomeres (Martin et al., 1999; Mills et al., 1999), as described above, *rad9Δ* neither exacerbated nor significantly rescued the accelerated aging in ETI mother cells. Together, these findings indicate that altered sequestration of the Sir complex is not the mechanism causing the accelerated aging of ETI cells.

Lifespan Reduction of ETI Mother Cells Is Not Caused by Increased Reactive Oxygen Species

We re-examined the previously described transcriptional profile data sets (Nautiyal et al., 2002; Table S2, passage 1) of ETI *tlc1Δ* cells. In an unbiased approach, we compared the large available number of yeast gene expression profiles, measured under different environmental conditions and genetic backgrounds (Edgar et al., 2002), to that of ETI cells (Tables S3, S4, S5). The top hit (Pearson correlation = 0.495, $p < 1e\text{-}254$) was treatment with diamide, a thiol-oxidizing agent that causes oxidative stress. Because intracellular ROS have long been theorized to play a role in aging and ROS levels can be elevated as a result of DNA damage (Rowe et al., 2008; Salmon et al., 2004), we tested whether oxidative stress caused the ETI mother cell phenotypes.

We assessed oxidative stress in ETI cells by quantifying ROS levels in our strains. If the accelerated aging of ETI cells is caused by higher intracellular ROS levels, it would be predicted that ETI *tlc1Δ* mother cells would have higher ROS than WT and that ETI *tlc1Δ sml1Δ* cells would have lower ROS levels than ETI single *tlc1Δ* mutants. However, ETI cells did not have significantly higher levels of ROS than WT (Figure S6C). Furthermore, *sml1Δ* and *tlc1Δsml1Δ* strains showed even higher levels of ROS, the opposite effect from that predicted if ETI causes faster aging of cells via higher intracellular ROS level. We also tested the effects of anti-oxidants by treatment with N-Acetyl-L-Cysteine (NAC). However, NAC equally and only modestly lengthened mother cell lifespans of both ETI and WT mother cells (Figure S6D). Hence, we conclude that, even though the transcriptional profile changes in ETI cells include features of an oxidative stress response, increased ROS levels and oxidative stress are not a primary cause of accelerated mother cell aging elicited by ETI.

ETI Cells Show Transient RNR3 Upregulation during Mother Cell Divisions

Given the connections between DNA damage and cell-cycle regulation, we turned to a more detailed analysis to establish whether DDR signaling was induced in ETI mother cells. Notably, phosphorylation of DDR components such as Rad53 and Mrc1 is only detectable in LTI cells and not in ETI cells (Grandin et al., 2005) (Figure S4A). Therefore, we employed a more sensitive single-cell monitoring method to detect evidence for DDR signaling. We examined DDR activation during mother cell aging using a GFP-tagged allele of *RNR3*, a gene that is strongly induced as a downstream component of the DDR. We monitored GFP intensity during mother cell lifespan assays and quantified RNR3-GFP peaks (at least 1.3-fold above background) using strains containing *RNR3-GFP* and all relevant combinations of *tlc1Δ*, *sml1Δ*, *mrc1^{AQ}*, and *tel1Δ* mutations. Peaks were classified as occurring before the last two cell divisions or during/after the last two divisions, referred to here as "still-dividing" and "terminal" peaks, respectively (Figures 6C and 6E). Terminal peaks were scored as peaks per mother cell, and still-dividing peaks were scored as peaks per cell cycle as mother cells underwent a different average number of cycles depending on genotype (Figures 6D and 6F). In WT mother cells (two replicates), a transient RNR3-GFP peak appeared at a low frequency during the cell cycles of still-dividing mother cells (0.0062, 95% confidence limits 0.0031–0.0111; n = 11 events in 1,766 cell cycles) and terminal peaks occurred in 25.0% of mother cells (24/96 cells, 95% confidence limits 0.1736–0.3456). In contrast, in *tlc1Δ* ETI mother cell lineages (three replicates), RNR3-GFP peaks occurred at significantly greater frequency in still-dividing mother cells (0.0177, 95% confidence limits 0.0123–0.0252; n = 30 events out of 1,696 cell cycles, $p < 0.0025$ compared with WT), and in 27.7% of terminal mother cells (44/159 cells, 95% confidence limits 0.213–0.351). Hence, in still-dividing mother cells, ETI elicits an increased number of transient episodes of DDR signaling.

Notably, relative to *tlc1Δ* single mutants, RNR3-GFP peaks in *tlc1Δsml1Δ* ETI mother cells (three replicates) were significantly diminished in frequency in the still-dividing mother cells (0.0104, 95% confidence limits 0.0071–0.0152; n = 27 events in 2,587 cell cycles, $p < 0.05$ compared with *tlc1Δ*), and terminal peaks occurred in only 13.1% of mother cells (22/168 cells, 95% confidence limits 0.087–0.191, $p < 0.01$ compared with *tlc1Δ*). This result is explainable, as *sml1Δ* raises nucleotide pools, making replication fork stalling less likely to occur (Andreson et al., 2010; Jossen and Bermejo, 2013) and hence reducing the possibility of eliciting a DNA replication stress response.

ETI *tlc1Δtel1Δ* mother cells (two replicates) showed fewer RNR3 peaks than WT (or *tlc1Δ* ETI) in still-dividing cells (0.006, 95% confidence limits 0.0034–0.0124; n = 10 events in 1,505 cell cycles, $p < 0.006$ compared with *tlc1Δ*), and peaks occurred in 12.0% of terminal mother cells (15/125 cells, 95% confidence limits 0.0730–0.1897, $p < 0.009$ compared with *tlc1Δ*). This finding indicates that abrogating Tel1 greatly exacerbated the ETI mother cell aging phenotypes (Figures 4B and 4D) while reducing RNR3 induction events. We propose that the optimal response to the replication stress in *tlc1Δ* ETI cells requires Tel1 checkpoint function to activate DDR signaling, monitored here as downstream RNR3 induction.

Interestingly, the *tlc1Δmrc1^{AQ}* ETI mother cells showed significantly more RNR3 peaks than WT cells in both still-dividing (0.0193, 95% confidence limits 0.0127–0.0289; n = 23 events in 1,192 cell cycles) and terminal mothers (32.4%, 35/108 cells, 95% confidence limits 0.243–0.417) and also trends to more RNR3 peaks than *tlc1Δ* (three replicates). Because the *mrc1^{AQ}* mutation exacerbates the ETI cell-cycle duration and lifespan reduction phenotypes, this finding indicates that telomeric replication stress requires Mrc1 checkpoint function in order to elicit an appropriate response in the absence of telomerase, but not for induction of the RNR3 reporter.

As the Rad9 adaptor protein is semi-redundant with Mrc1 in the DDR cascade, we investigated whether Rad9 is required for induction of RNR3 in the absence of Mrc1 checkpoint function. However, when combined with the *tlc1Δ* mutation, the *mrc1^{AQ} rad9Δ* double mutation induced rapid lethality of the

bulk ETI cell population, precluding mother cell analyses. Remarkably, this loss of viability in *tlc1Δmrc1^{AQ}rad9Δ* cells was also completely rescued by *sml1Δ* (data not shown). We conclude that, if ETI mother cells lack either Tel1 or Mrc1 checkpoint function, the response to, or repair of, telomeric DNA replication stress-induced damage is compromised.

The Degree of Heterogeneity of Cell-Cycle Durations and Mitochondrial Changes in Young ETI Mother Cells Each Quantitatively Predict Lifespan

Strikingly, for each mother cell genotype described above, the frequency and degree of lengthened cell cycles in young mother cells predicted the degree of reduction in mean lifespan (Figures S7A and S7B). Furthermore, for both WT and ETI mother cells, the extent of the mitochondrial fluorescence quantified at a given early time point in the budding lineage (4 hr) predicted the lifespan of that particular mother cell (Figures S7C and S7D). The finding that these relationships held across multiple genotypes suggests that responses that occur in even the youngest mother cells are caused by the same problem that eventually regulates the lifespan of the cell.

DISCUSSION

Here, we have shown that lack of active telomerase affects yeast mother cells much earlier than expected, well before any effect on cells that can be attributed to critical telomere shortness. Notably, early telomerase inactivation in yeast mother cells caused increased heterogeneity of the cell cycle and accelerated aging. These phenotypes were rescued by increasing nucleotide pool levels and were sensitive to inactivation of specific DDR components. By several criteria, the ETI mother cell aging phenotype is consistent with an acceleration of normal mother cell aging processes and not senescence caused by loss of telomere protective function. These criteria included terminal cell and mitochondrial morphologies that were characteristic of aging WT mother cells and distinct from those in senescent cells.

Previously, it was thought that telomeres had to become critically short in order to elicit a cellular DDR. In contrast, our results suggest that, independent of critical telomere shortness, ETI cells initiate signaling that accelerates an otherwise normal mother cell aging pathway. In the ETI setting, across multiple genotypes, the premature onset of mother cell aging is anticipated by the frequency and severity of stochastically slower cell-cycling events occurring in even young mother cells. In addition, we have shown that it is a lack of telomerase activity, rather than the lack of an assembled telomerase complex, that is the proximate cause of the response. Therefore, the action of telomerase on telomeres appears to be the most likely molecular property whose alteration causes these effects in ETI cells.

Our combined findings provide evidence for the model shown in Figure 7. In this model, during mother cell divisions in ETI cells, lack of telomerase activity may eliminate a potential bypass mechanism for replication stress in the telomere. This causes transient episodes of a much milder DDR than the robust and sustained DDR elicited by critically short telomeres (Nautiyal et al., 2002; d'Adda di Fagagna et al., 2003). We propose that deletion of Sml1, via its known phenotype of increasing dNTP levels, alleviates this replication stress at an upstream level, preventing DNA damage signaling. This explains why Sml1 deletion suppressed both the transient DDR signaling and the accelerated aging in ETI mother cells. We propose that such replication stress arises often in telomeres and is sensed by Tel1, which is required to cause the observed transient rises in RNR3 levels in ETI cells. This response promotes either repair or tolerance of the replication stress that allows ETI cells to bypass it and enter the next cell cycle. In ETI cells lacking the checkpoint functions of Tel1 or Mrc1 (*tlc1Δtel1Δ* or *tlc1Δ mrc1^{AQ}*), cells cannot activate the appropriate response to this replication stress, thus exacerbating the ETI phenotypes. Without Mrc1 checkpoint function, compensation by the semi-redundant adaptor Rad9 occurs to some degree, but the telomeric replication stress is not fully resolved and further damage can ensue. However, in the absence of telomerase, even a fully functional DDR is not sufficient to fully alleviate telomeric replication stress and prevent accelerated mother cell aging.

We determined that neither ROS, recombination, deprotected (fusogenic) telomeres, redistribution of SIR protein complexes, nor a DDR similar to that in response to classic DNA damaging agents can account for the accelerated mother cell aging of ETI cells. Deletion of *SML1* rescued the cell cycle, lifespan, and DDR (RNR3) induction phenotypes in dividing ETI mother cells and is known to suppress replication fork stalling (Andreson et al., 2010; Jossen and Bermejo, 2013). The majority of mutant phenotypes known to be suppressed by *sml1Δ* are related to DNA replication, including replication fork progression. This suggests that suppression by *sml1Δ* is very specific and occurs through elevated nucleotide pools via the release of inhibition of the RNR complex. Furthermore, the transcriptional profile of ETI *tlc1Δ* cells indicates that they upregulate *RNR2*, *3*, and *4* gene expression (Nautiyal et al., 2002) (Table S2). Taken together with previous findings, our results suggest that the higher nucleotide pools in *sml1Δ* cells prevent telomeric replication stress from occurring, thus suppressing the ETI phenotype by preventing any need for DDR activation or telomerase intervention.

Telomerase is predicted to be recruited to backtracked replication forks resulting from stalling, which has been proposed to occur at measurable frequencies in telomeric DNA (Miller et al., 2006; Drosopoulos et al., 2012). Such backtracked forks will expose single-stranded leading-strand $TG_{(1–3)}$ repeat sequence DNA, which is the substrate for telomerase elongation. Also, telomerase could aid in the repair of a broken telomeric fork generated when a stalled replisome collapses (Chang et al., 2009). The resulting shortened telomere is a known preferred telomerase substrate (Miller et al., 2006). Other known interactions between DNA polymerase and telomerase actively engaged on telomeres may normally be required to optimize fork movement or fork restarting in telomeres. For example, the telomere-binding Cdc13-Stn1-Ten1 complex interacts via its Cdc13 subunit with a subunit of the telomerase complex (Est1) and also interacts (via Stn1) with a subunit of DNA polymerase alpha (Grossi et al., 2004).

The causal mechanism underlying mother cell aging remains unknown even for WT yeast despite extensive identification of genetic and environmental modifiers of this process. Our findings indicate that telomerase functionality is required throughout

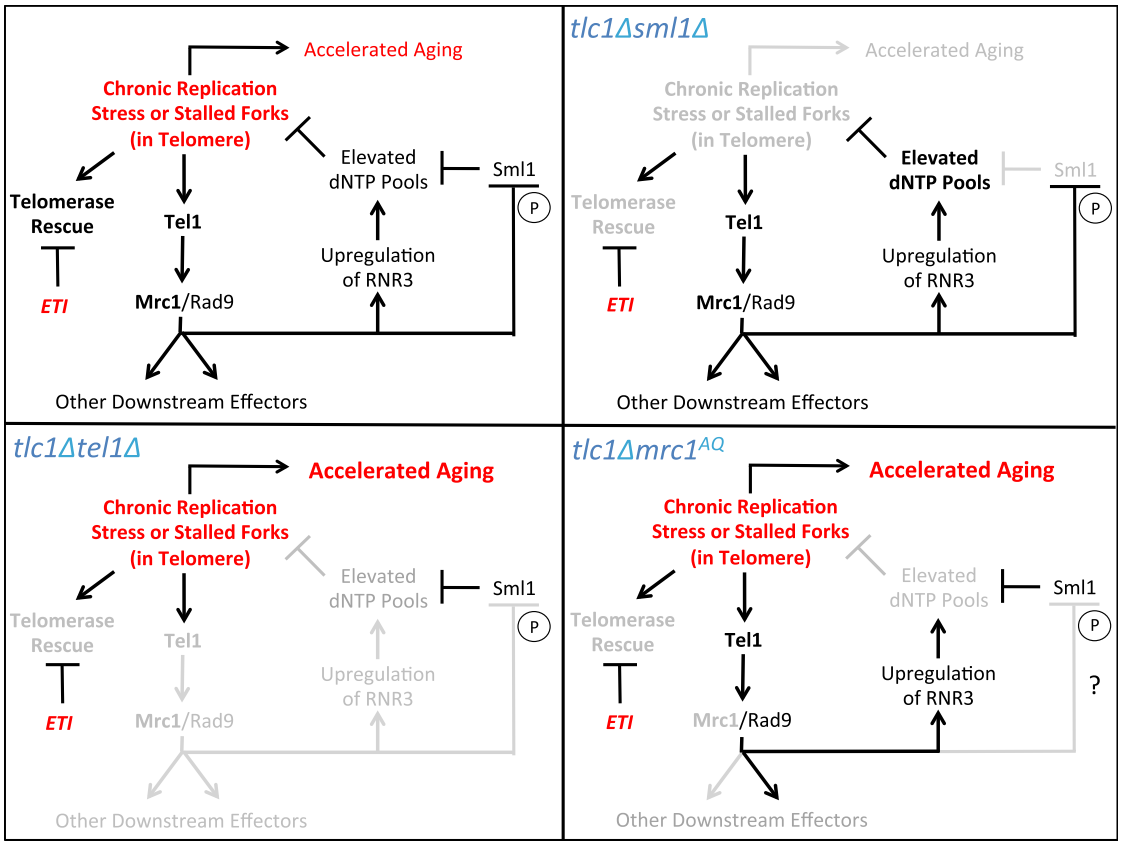

Figure 7. Proposed Model

(Upper-left) Proposed signaling interactions that regulate aging in response to telomeric DNA stress. Deleterious effects shown in red. (Upper-right) In ETI cells lacking Sml1 (*tlc1Δsml1Δ*), telomerase cannot alleviate replication stress. However, due to elevated dNTP levels, replication stress is prevented and aging is not accelerated. Eliminated or reduced signaling is shown in gray. (Lower-left) In ETI cells lacking Tel1 (*tlc1Δtel1Δ*), as well as lacking telomerase rescue, the DDR response is unavailable to alleviate replication stress, as indicated by the elimination of RNR3 signaling, and ETI-accelerated aging is exacerbated. (Lower-right) In ETI cells lacking Mrc1 function (*tlc1Δmrc1^{AQ}*), telomerase rescue is unavailable and the DDR response to telomeric replication stress is partially hindered. Rad9 is able to partially compensate and induce RNR3 induction, but other downstream DDR targets cannot be induced, thus exacerbating the ETI-accelerated aging. See also Figure S7.

the divisions of yeast mother cells in a more continuous mode than previously thought. The findings reported here indicate that telomerase activity is required to alleviate normal telomeric replication stress and allow mother cell aging to occur with wild-type kinetics. In addition, mutations known to inhibit telomerase activity or telomere maintenance have been implicated in the premature onset of diseases of aging and reduced lifespan in humans and mice (Codd et al., 2013, Armanios and Blackburn, 2012), and replication stress has been shown to induce aging in mouse cells (Flach et al., 2014). Therefore, this early requirement for active telomerase in preventing premature mother cell aging in yeast suggests a new possibility: that loss of telomerase activity may have telomere-length-independent consequences that accelerate aging and cause aging-related diseases in other eukaryotes.

EXPERIMENTAL PROCEDURES

See Extended Experimental Procedures for supplemental experiments: bulk population budding and chromosome segregation kinetics, NAC treatment, and ROS straining.

Yeast Strain Construction

All strains used in this study are listed in Table S1. Plasmid and oligo sequences are available upon request. Complete disruption of ORFs was carried out using PCR-mediated gene disruption (Rose et al., 1990). *mrc1^{AQ}* mutant strains were made either via a loop-in, loop-out of a plasmid containing the mutant, followed by PCR verification, or via plasmid transformation of the mutant into an *mrc1Δ* strain.

Growth of Mutants for Monitoring Early Loss of Telomerase

ETI cells were produced by two methods: by sporulation of diploid heterozygote strains (*tlc1Δ/TLC1, est2-D530A/EST2*) or by loss of a covering plasmid in a haploid telomerase-deficient background strain struck on solid media. Colonies underwent 2 days of growth at 30°C, were genotyped, and were grown overnight (five to ten generations) in YPD prior to experimentation.

Microfluidics Technique Analyses of Mother Cells

Mother cells were monitored for 2 days by repeated microscopic imaging as described (Xie et al., 2012; Zhang et al., 2012). Microposts contained within the microfluidic device were used to clamp mother cells in place while daughter cells were washed away by hydrodynamically controlled flow of the surrounding liquid medium. Cell-cycle durations analyzed here excluded the first cycle observed and the terminal two divisions.

Southern Blotting Analysis of Telomere Length

Genomic DNA was prepared from cells from serial streaks on solid media after the indicated number of passages. Genomic DNA was then digested with XhoI and run on 0.8% agarose gels. DNA was transferred from the gels to Hybond N+ membranes and probed with γ^{32}P end-labeled WT telomeric repeat oligonucleotide (TGTGGTGTGTGGGTGTGGTGT) as described previously (Rose et al., 1990; Sambrook and Russel, 2001) and visualized using a phosphoimager.

Mitochondrial and SIR3 Quantification

Cells containing mito-tagged (mt)GFP were placed on a microfluidics chip as described above. Images were taken every 2 hr, and mtGFP intensity was measured relative to WT to determine volume of mitochondria. For Sir3-GFP foci, 11 images were taken for the Z stack and projected to a single image using the maximum value of the column, and the fluorescence intensity of the foci was measured using customized software Cellseg 5.4.

Statistical Analysis

Lifespans were compared using the Wilcoxon rank-sum test. Significance for the variation of cell-cycle length was determined by F tests. We used Fisher's exact test to determine the significance of frequency of RNR3 peaks in different genotypes.

Quantification of RNR3 Peaks

Fluorescence was measured every 30 min during lifespan tracking. Frequency of RNR3 peaks (at least 1.3-fold over background) for "still-dividing" mother cells was calculated as the number of peaks divided by all cell cycles occurring within that genotype and, for "terminal" mother cells, as the percentage of mother cell lifespans that contained a peak during or after the last two divisions.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five tables, and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.02.002.

AUTHOR CONTRIBUTIONS

Z.X. performed the microfluidic lifespan assays, cell counting and scoring, image segmentation, and fluorescence quantification. K.A.J. performed strain constructions, sample genotyping, bulk population studies, telomere length (Southern blot) experiments, and PCR-based telomere deprotection assays. D.L.S. performed strain constructions, cell-cycle and budding analyses, and DNA damage assays. E.H.B., Z.X., and H. L. analyzed the statistics. Y.Z. performed the Rad52 mutant lifespan assays. Z.L. synthesized the mtGFP plasmids. J.Z. and H.L. performed the bioinformatics analyses. R.T. aided in strain preparation. E.H.B., H.L., K.A.J., and Z.X. conceived the plans, interpreted and analyzed the data, and wrote the paper.

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