Suppression of Chromosome Healing and Anticheckpoint Pathways in Yeast Postsenescence Survivors

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ABSTRACT Telomere repeat-like sequences at DNA double-strand breaks (DSBs) inhibit DNA damage signaling and serve as seeds to convert DSBs to new telomeres in mutagenic chromosome healing pathways. We find here that the response to seed-containing DSBs differs fundamentally between budding yeast (*Saccharomyces cerevisiae*) cells that maintain their telomeres via telomerase and so-called postsenescence survivors that use recombination-based alternative lengthening of telomere (ALT) mechanisms. Whereas telomere seeds are efficiently elongated by telomerase, they remain remarkably stable without *de novo* telomerization or extensive end resection in telomerase-deficient (*est2Δ*, *tlc1Δ*) postsenescence survivors. This telomere seed hyper-stability in ALT cells is associated with, but not caused by, prolonged DNA damage checkpoint activity (*RAD9*, *RAD53*) compared to telomerase-positive cells or presenescent telomerase-negative cells. The results indicate that both chromosome healing and anticheckpoint activity of telomere seeds are suppressed in yeast models of ALT pathways.

TELOMERES, the natural chromosome ends, contain a characteristic heterochromatin structure that distinguishes them from DNA double-strand breaks (DSBs) as accidental chromosome ends (de Lange 2009). Telomeric DNA usually consists of arrays of short tandem repeat sequences [(TG₁₋₃)_n in yeast] that serve both as primers for telomere elongation by the reverse transcriptase telomerase and as binding sites for various cap proteins (Wellinger and Zakian 2012). These cap structures maintain genome stability by preventing aberrant recombination between telomeres into dicentric chromosomes or other genome rearrangements (de Lange 2009; Wellinger and Zakian 2012). However, telomerase can sometimes also be a source of genome instability when it acts on internal telomere repeat-like sequences adjacent to DSBs and converts them to *de novo* telomeres in a mutagenic repair process called chromosome healing (Kramer and Haber 1993). To prevent such genome rearrangements, *de novo* telomere addition at DSBs is normally suppressed by DNA damage

checkpoint-signaling pathways (Makovets and Blackburn 2009; Zhang and Durocher 2009). However, checkpoint signals at telomere seed-containing DSBs turn off more quickly than at non-seed-containing DSBs (2–3 hr vs. >10 hr); and, strikingly, a seed-containing end also rapidly turns off the checkpoint signal generated from the other seed-free end of the same DSB, a phenomenon referred to as the anticheckpoint function of telomeres (Michelson *et al.* 2005).

In addition to telomerase, telomere length can also be maintained by a recombination-based mechanism referred to as alternative lengthening of telomeres (ALT). Upon loss of telomerase, cells initially continue to grow normally for several generations until progressive telomere erosion leads to checkpoint-dependent terminal cell cycle arrest. ALT emerges spontaneously via largely unknown mechanisms in a very small subset of senescent telomerase-negative cells undergoing cell cycle crisis (Cesare and Reddel 2010). In budding yeast, cells that have established the ALT mechanism are referred to as postsenescence survivors (Lundblad and Blackburn 1993). As ALT involves rapid telomere elongation using other telomeres or shed extrachromosomal telomeric sequences as templates, in principle, ALT cells should also be able to heal seed-containing DSBs with a new telomere. However, while chromosome healing is very well characterized in telomerase-positive cells (Pennaneach

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et al. 2006), to our knowledge it has not previously been reported to occur in ALT cells. To resolve this knowledge gap, we have here studied chromosome healing in yeast postsenescence survivors. Surprisingly, we found that both *de novo* telomere addition at telomere seeds and the antichkpoint function of telomere seeds appear to be suppressed in ALT cells, which instead repair such breaks after a very long delay (>21 hr) by a mutagenic mechanism resulting in a range of heterogeneous repair products.

Materials and Methods

Yeast strains

All yeast strains used in this study are listed in [Supporting Information, Table S1](#) and were derived from UCC5913 (Diede and Gottschling 1999) provided by Dan Gottschling, except the HO-DSB-repair control strains Y219 (JKM179) and Y496 (TGI354) (Lee *et al.* 1998; Ira *et al.* 2003), which were provided by Jim Haber. All gene disruptions were performed using a PCR-mediated technique (Longtine *et al.* 1998), and *rad53*-K227A was generated by PCR-based allele replacement as described (Erdeniz *et al.* 1997; Tam *et al.* 2008). To generate postsenescence survivors, telomerase-negative presenesence cultures were back-diluted to $\sim 10^5$ cells/ml at 24-hr intervals for 10–15 days until cultures reached growth saturation again; type I or II survivor status was confirmed by Southern blot using Y'-probes as described (Pike and Heierhorst 2007). Presenescent cultures were inoculated from overnight starter cultures of freshly deleted *est2* Δ colonies. Unless stated otherwise, all experiments were carried out in YPR (1% yeast extract, 2% peptone, 2% raffinose) at 30°. For DSB induction, 3% galactose was added for expression of HO endonuclease. *RIF1/2* overexpression plasmids were provided by Maria-Pia Longhese (Viscardi *et al.* 2003; Anbalagan *et al.* 2011).

Protein and DNA blots

DNA for Southern blots was prepared using glass beads and phenol/chloroform extraction and separated in 0.75–1.2% (w/v) agarose gels at 1 V/cm, transferred to nylon membranes, incubated with [α - 32 P]dCTP-labeled probes, and analyzed using GE Healthcare ImageQuant TL software. Protein lysates were prepared using glass beads and urea buffer, separated on 8% acrylamide gels, and transferred to PVDF membranes for immunoblotting.

Results

Impaired chromosome healing in postsenescence survivors

To investigate chromosome healing in ALT cells, we generated *est2* Δ survivors lacking the catalytic protein component of telomerase in a yeast strain containing a galactose-inducible HO endonuclease cleavage site adjacent to an 81-bp telomere seed sequence (in the following also referred to as the TG seed) at an ectopic *ADE2* locus on chromosome VII (Diede and Gottschling 1999) (Figure 1A). This strain also

Figure 1 Absence of *de novo* telomere elongation in postsenescent survivors. (A) Schematic of the assay system. Triangles indicate telomeres, circles indicate centromeres, and short solid bars indicate the location of the probe used for Southern blots. (B–D) Southern blots of *SpeI*-digest DNA of the wild type (WT), four independent *est2* Δ type II survivors (B and C), and a type I survivor (D) at the indicated times after HO induction. PRE, chromosome VII fragment before HO cut; INT, internal control band from chromosome XV; the arrowhead in B points to *de novo* telomeres in the WT.

contains a noncleavable *ade2-101* allele on chromosome XV that can be used as an internal loading control on Southern blots (Figure 1A). As reported previously (Diede and Gottschling 1999; Michelson *et al.* 2005), *de novo* telomere addition to the DSB commenced within ~ 2 hr in wild-type control cells (Figure 1B). In contrast, the TG seed end of the DSB remained remarkably stable without *de novo* telomerization or detectable end resection for at least 21 hr in *est2* Δ survivors (Figure 1B). Similar results were obtained in several independent type II survivors (Figure 1C) as well as in *rad50* Δ *est2* Δ type I survivors (Figure 1D). These results indicate that *de novo* telomere addition is considerably less efficient in postsenescence survivors compared to telomerase-positive cells.

Prolonged checkpoint activation by telomere seed-containing DSBs in postsenescence survivors

The remarkable stability of the TG seed end prompted us to test if survivors are able to perceive the HO-induced DSB as DNA damage by monitoring activation of the Rad53

checkpoint kinase using Western blot mobility shift assays (Pike *et al.* 2003) and a conformation-specific antibody for active Rad53 (Fiorani *et al.* 2008). In the wild-type control, Rad53 was transiently activated for ~2–3 hr after HO break induction. In contrast, prolonged Rad53 activation lasting for 4–6 hr was observed in *est2Δ* survivors (Figure 2A).

As the checkpoint was activated despite lack of notable processing of the seed-containing DSB end, we also monitored the stability of the distal, non-TG-seed-containing DSB end by Southern blot using a *LYS2* probe (Figure S1). Relative to the 6-kbp internal loading control signal corresponding to the *lys2-801* allele (on chromosome II), the signal from the *LYS2* gene adjacent to the DSB disappeared by 2 hr after HO induction, indicating that end resection had progressed past the *SpeI* site ~2.3 kbp distal from the HO site (Figure S1). Thus, this result indicates that DSB end resection as such is unimpaired in our survivors and that the stability of the TG-containing end must be due to the telomere seed sequence itself.

While single-stranded DNA generated from processing of the distal DSB end could explain checkpoint activation, the prolonged Rad53 activity in survivors was surprising as it had previously been reported that an 81-bp TG tract at a DSB acts as a telomerase-independent antichkpoint that restricts checkpoint activation by the non-TG-seeded end in *trans* to 2–3 hr (Michelson *et al.* 2005). As this previous result was obtained by deleting the catalytic RNA component of telomerase, *TLC1*, we repeated our analyses in *tlc1Δ* survivors. Again, compared to the telomerase-positive control, Rad53 activation was considerably prolonged in *tlc1Δ* survivors (Figure 2B) to a similar extent as in *est2Δ* survivors (Figure 2A). We therefore tested if checkpoint activation differed between cells that had freshly lost telomerase but still had long-enough telomeres to proliferate normally and postsenescent survivors that were maintaining their telomeres by the ALT pathway. In contrast to prolonged Rad53 activation in postsenescent *est2Δ* and *tlc1Δ* survivors, checkpoint activation in presenescent telomerase-deficient *est2Δ* cells was as short-lived as in wild-type cells (Figure 2C). Thus, these data indicate that it was the establishment of the ALT pathway, rather than just the loss of telomerase, that was responsible for prolonged checkpoint activation in our survivors.

Abolition of the checkpoint response does not restore *de novo* telomere addition in survivors

As indicated above, DNA damage checkpoints inhibit *de novo* telomere addition at DSBs by telomerase to prevent genome instability (Makovets and Blackburn 2009; Zhang and Durocher 2009). We thus investigated if prolonged checkpoint activation also contributes to impaired *de novo* telomere addition in postsenescent survivors. For this purpose, we initially monitored repair of the HO-induced DSB in *rad53-K227A* kinase-deficient *est2Δ* survivors. As the *in vivo* site-directed mutagenesis procedure involves the transient disruption of the essential *RAD53* gene (Erdeniz *et al.* 1997; Tam *et al.*

Figure 2 Persistent checkpoint activation in type II postsenescent survivors. (A–C) Western blot analysis of extracts of indicated cells with activation-state-specific (Fiorani *et al.* 2008) and general Rad53 antibodies (Pike *et al.* 2003) and actin as loading control at the indicated times after galactose addition. Note that, in C, identical time points with and without galactose addition were collected to control for DSB-independent checkpoint activation due to replicative telomere shortening in presenescent cells (bottom); wild-type and *est2Δ* survivor cultures (top) were treated at the same time with presenescent cells for comparison.

2008), these experiments were performed in the presence of the *sml1Δ* suppressor of *rad53Δ* lethality (Zhao *et al.* 1998). *sml1Δ* by itself affected neither prolonged checkpoint activation nor the hyper-stability of the TG seed end of the DSB in *est2Δ* survivors (Figure 3A). However, impaired Rad53 checkpoint function in *rad53-K227A* cells did not interfere with the stability of the TG-seed-containing DSB end relative to the internal control band in survivors (Figure 3A). We also performed similar analyses in the absence of the Rad9 mediator that is required for DSB-induced Rad53 phosphorylation and activation by Mec1. Again, abolition of Rad53 activation in *rad9Δ est2Δ* survivors did not significantly affect the stability of the TG-seed-containing DSB end (Figure 3B). Thus, the prolonged checkpoint response does not seem to be responsible for impaired *de novo* telomere addition in postsenescent survivors.

Efficient long-term repair of seed-containing DSBs with heterogeneous repair products in postsenescent survivors

To test if survivors were able to eventually repair the TG end of the DSB or convert it to a *de novo* telomere at a time beyond 21 hr, we plated 10-fold serial dilutions of *est2Δ* survivors on galactose-containing plates and monitored

Figure 3 Abolition of the checkpoint response does not restore *de novo* telomere elongation in type II postsenescent survivors. (A and B) Analysis of the indicated strains using Western blots as in Figure 1 and Southern blots as in Figure 2.

colony formation as a marker of viability after 3–4 days. Under these conditions, *est2Δ* survivors formed colonies with similar efficiency to the wild-type control, whereas a DSB repair-deficient negative control strain formed >100-fold fewer colonies than on plates lacking galactose (where no break is induced; Figure 4A). Analysis of all six tested independent wild-type control colonies from galactose plates by Southern blot with the *ADE2* probe revealed a characteristic smear at the expected size indicative of *de novo* telomere addition to the seed-containing DSB end (Figure 4B). In contrast, the survivor colonies exhibited a diverse range of heterogeneous repair products (Figure 4B). In all cases except the fifth sample (from the left), these repair products differed in size from the internal *ade2-101* control band, indicating that a repair pathway other than homologous recombination with the seemingly ideal repair template *ade2-101* had been used (Figure 4B). However, *est2Δ* survivors were able to repair another HO-induced DSB not associated with a TG seed by gene conversion with the corresponding homologous repair template on another chromosome with similar efficiency to wild-type cells (Figure S2). Thus, these results indicate that TG seeds may also impair homologous recombination repair of DSBs in postsenescent survivors.

Discussion

Here we have found that the processing of telomere seed-containing DSBs differs in several fundamental aspects between telomerase-positive cells and telomerase-negative postsenescent survivors in that both telomere healing and the antieckpoint function of seed-containing breaks seem to be suppressed in postsenescent survivors. The finding that the seed-containing DSB end is initially remarkably stable (in contrast to rapid degradation of the nonseeded end) suggests that the 81-bp seed may be sufficient to serve as a temporary telomere cap until it gradually erodes due to inevitable replicative telomere shortening after a few S

phases. Interestingly, once the seed is sufficiently uncapped to allow access to the DNA repair machinery, for an unknown reason the residual TG repeats appear to be able to suppress recombination of adjacent sequences with the most preferred homologous repair templates (Figure 4). Although we have not determined the sequence of the repair products that eventually form, we presume that they are the products of homeologous recombination with diverse repair templates. It is unlikely that these products were generated by other previously described rare telomerase- and recombination-independent chromosome end-capping pathways, as the latter act only on initially extremely long telomeres (Grandin and Charbonneau 2009; Lebel *et al.* 2009) or depend on the absence of *Exo1* (Maringele and Lydall 2004) (which seems functional in our survivors based on efficient resection of the non-seed-containing DSB end; Figure S1), respectively.

Higher affinity of telomerase to telomere seeds compared to the recombination machinery, and its unique ability to elongate very short but still capped telomeres, may explain why telomere seeds are rapidly and quantitatively elongated in wild-type cells (Figures 1 and 4) but not in survivors. Interestingly, it has recently been shown that—in contrast to the preference of telomerase for short telomeres—the recombination machinery preferentially elongates longer telomeres during the establishment of type II survivors (Chang *et al.* 2011). As the cells used here already had an established type II survivor mechanism with very long telomeres before induction of the HO break, a possible explanation for our observations could be that ongoing recombination of very long telomeres in these survivors might hijack the recombination machinery away from other DSBs with shorter homology sequences. However, this is unlikely to be the case because survivors—despite a preference for elongating long telomeres—are still able to also elongate short telomeres (Chang *et al.* 2011). Moreover, other DSBs that were not adjacent to telomere seeds were still

Figure 4 Heterogeneous repair products of the telomere seed-containing DSB end in survivors. (A) Drop tests of 10-fold serial dilutions of the indicated strains on raffinose plates (top) and raffinose+galactose plates (bottom) incubated for 3–4 days. The repair-deficient strain (Y219) lacks *HML* and *HMR* to repair the HO-induced break at the endogenous mating-type locus. (B) Southern blot of individual colonies isolated from galactose plates similar to A and probed with the *ADE2* probe as indicated in Figure 1.

efficiently recombined in our type II survivors (Figure S2).

A striking feature of telomere seeds is that they also quickly turn off the checkpoint signal emanating from the other, seed-free end of the same DSB and that this is independent of telomerase (Michelson *et al.* 2005). We have here confirmed that this antieckpoint function as such is indeed telomerase-independent, but only in presenescent cells, whereas it is compromised once these cells have switched to the ALT pathway (Figure 2). However, even in survivors the antieckpoint may still be partially active, as *Rad53* activation in response to the seed DSB was turned off after ~6 hr, which is considerably earlier than checkpoint adaptation to an irreparable break after ~10–12 hr (Pellicioli *et al.* 2001). A possible explanation for the suppression of the antieckpoint at seed-containing DSBs could be that capping factors required for checkpoint attenuation might be sequestered at excessively long survivor telomeres and might therefore be limiting for local action at the DSB. Interestingly, the telomere cap component *Rif1* has recently been identified as a telomeric antieckpoint factor that may antagonize *Rad53* activation even independently of its *Rap1*-dependent telomere-capping function (Xue *et al.* 2011; Ribeyre and Shore 2012). However, overexpression of neither *Rif1* nor *Rif2* restored the telomere seed antieckpoint function in our survivors (Figure S3), indicating that its suppression is not due to limiting *Rif1/2* protein levels.

Chromosome healing is an evolutionarily conserved source of genome instability that has also been observed in telomerase-positive human cancer cells (Pennaneach *et al.* 2006). While telomerase-independent chromosome healing has been reported in murine embryonic stem cells, these cells did not have an established ALT mechanism (Gao *et al.* 2008), and it is thus unclear how mammalian ALT cells respond to telomere seed-containing DSBs. About 15% of human cancers utilize an

ALT pathway resembling yeast type II survivors for proliferation (Cesare and Reddel 2010). Given the importance of checkpoint signaling and genome instability in cancer biology, it would thus be interesting to investigate how human ALT cancers respond to DSBs near endogenous telomere-like seed sequences.

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Supporting Information

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Figure S1 Efficient degradation of the distal, non-seed-containing DSB end in post-senescence survivors. **(A)** Diagram of the assay system similar to Fig. 1A. **(B)** The same blot as shown in Fig. 1B was reprobed with a *LYS2* probe. Note the disappearance of the *LYS2* (chr. VII) signal compared to the *lys2-801* signal (chr. II).

Figure S2 Normal gene conversion at a non-telomere-seed-containing DSB. **(A)** Schematic diagram of the assay system, where an HO break at an ectopic mating type locus on chromosome V is repaired with the endogenous, but HO-resistant, mating type locus repair template on chromosome III. **(B)** Southern blot of the wild type control (WT) and an isogenic *est2Δ* survivor treated with galactose for the indicated times.

Figure S3 Rif1/2 overexpression does not restore the anti-checkpoint function of telomere seeds in post-senescence survivors. **(A)** *In lieu* of available Rif1/2 antibodies, rapid telomere shortening as a consequence of Rif1/2 overexpression (WOTTON and SHORE 1997) was assessed by Southern blot using a Y'-probe as described (Pike and Heierhorst 2007). **(B)** Western blots as in Fig. 2 of the indicated strains containing the empty-vector control or *RIF1* or *RIF2* overexpression constructs as indicated.

Table S1 Yeast strains used in this study

Strain	Genotype	Reference
Y219	<i>ade1 leu2-3,112 lys5 trp1::hisG ura3-52 hmlΔ::ADE1 hmrΔ::ADE1 ade3::GAL-HO</i>	(Lee <i>et al.</i> 1998)
Y496	<i>ade1 leu2-3,112 lys5 trp1::hisG ura3-52 hmlΔ::ADE1 hmrΔ::ADE1 ade3::GAL-HO</i> <i>MATa-inc arg5,6::MATa-HPH</i>	(Ira <i>et al.</i> 2003)
Y585	<i>MATa-inc ade2-101 lys2-801 his3-Δ200 trp1-Δ63 ura3-52 leu2-Δ1::GAL1-HO-LEU2 VII-L::ADE2-TG(1-3)-HO site-LYS2</i>	(Diede and Gottschling 2001)
Y1537	Y585 <i>rad50Δ::NAT</i>	This study
Y1591	Y585 <i>est2Δ::KAN</i> type II survivor	This study
Y1592	Y585 <i>est2Δ::KAN</i> type II survivor	This study
Y1596	Y585 <i>tel1Δ::NAT</i>	This study
Y1602	Y585 <i>sml1Δ::HIS3</i>	This study
Y1615	Y585 <i>tlc1Δ::NAT</i> type II survivor	This study
Y1616	Y585 <i>sml1Δ::HIS3 est2Δ::KAN</i> type II survivor	This study
Y1621	Y585 <i>tel1Δ::NAT est2Δ::KAN</i> type II survivor	This study
Y1629	Y496 <i>est2Δ::KAN</i> type II survivor	This study
Y1636	Y585 <i>rad9Δ::NAT</i>	This study
Y1637	Y585 <i>est2Δ::KAN</i> type II survivor <i>rad9Δ::NAT</i>	This study
Y1643	Y585 <i>sml1Δ::HIS3 est2Δ::KAN</i> type II survivor <i>rad53-K227A</i>	This study

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