Suppression of chromosome healing and anti-checkpoint pathways in yeast post-senescence 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 post-senescence survivors that use recombination-based alternative lengthening of telomeres (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) post-senescence 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 pre-senescent telomerase-negative cells. The results indicate that both chromosome healing and anti-checkpoint activity of telomere seeds are suppressed in yeast models of ALT pathways.
**Introduction**

Telomeres, the natural chromosomes 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 \((TG_{1,3})_n\) 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). In order 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 much quicker than at non-seed-containing DSBs (2-3 hours vs >10 hours); 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 anti-checkpoint 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 post-senescence survivors (Lundblad and Blackburn 1993). As ALT involves rapid telomere elongation using other telomeres or shedded 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 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 post-senescence survivors. Surprisingly, we found that both de novo telomere addition at telomere seeds, as well as their anti-checkpoint function, appear to be suppressed in ALT cells, which instead repair such breaks after a very long delay (>21 hours) 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 Supplementary 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) (Ira et al. 2003; Lee et al. 1998) 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 post-senescence survivors, telomerase-negative pre-senescence cultures were back-diluted to approximately $10^5$ cells/mL at 24-hour 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). Pre-senescent cultures were inoculated from overnight starter cultures of freshly deleted \textit{est}2\Delta colonies. Unless stated otherwise, all experiments were carried out in YPR (1% yeast extract, 2% peptone, 2% raffinose) at 30°C. For DSB induction, 3% galactose was added for expression of HO endonuclease. \textit{RIF1/2} overexpression plasmids were provided by Maria-Pia Longhese (Anbalagan \textit{et al.} 2011; Viscardi \textit{et al.} 2003).

\textbf{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 1V/cm, transferred to nylon membranes, incubated with [\textit{\alpha}-\textit{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.

\section*{RESULTS}

\textbf{Impaired chromosome healing in post-senescence survivors:} To investigate chromosome healing in ALT cells, we generated \textit{est}2\Delta 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 at an ectopic \textit{ADE2} locus on chromosome VII (Diede and Gottschling 1999)(Figure 1A). This strain also contains a non-cleavable \textit{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 \textit{et al.} 2005), \textit{de novo} telomere addition to the DSB commenced within \textasciitilde 2 hours in wild type control cells (Figure 1B). In contrast, the TG-seed end of the DSB remained remarkably stable without \textit{de}
novel telomerization or detectable end-resection for at least 21 hours in est2Δ survivors (Figure 1B). Similar results were obtained in several independent type II survivors (Figure 1C) as well as rad50Δ est2Δ type I survivors (Figure 1D). These results indicate that de novo telomere addition is considerably less efficient in post-senescence survivors compared to telomerase-positive cells.

**Prolonged checkpoint activation by telomere seed-containing DSBs in post-senescence 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 hours after HO break induction. In contrast, prolonged Rad53 activation lasting for 4-6 hours was observed in est2Δ survivors (Figure 2A).

As the checkpoint was activated despite lack of noticeable 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 (Supplementary 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 hours after HO induction, indicating that end-resection had progressed past the *SpeI* site ~2.3 kbp distal from the HO site (Supplementary 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 anti-checkpoint that restricts checkpoint activation by the non-TG-seeded end *in trans* to 2-3 hours (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 post-senescence survivors that were maintaining their telomeres by the ALT pathway. In contrast to prolonged Rad53 activation in post-senescence *est2Δ* and *tlc1Δ* survivors, checkpoint activation in pre-senescence 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 post-senescent 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. 2008), these experiments were performed in the presence of the sml1Δ suppressor of rad53Δ lethality (Zhao et al. 1998). sml1Δ by itself neither affected 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 post-senescent survivors.

**Efficient long-term repair of seed-containing DSBs with heterogenous repair products in post-senescence 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 hours, we plated 10-fold serial dilutions of est2Δ survivors on galactose-containing plates and monitored 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 less colonies than on plates lacking galactose (where no break is induced; Figure 4A). Analysis of all 6 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 lane 5 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 WT cells (Supplementary Figure S2). Thus, these results indicate that TG-seeds may also impair homologous recombination repair of DSBs in post-senescence 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 post-senescence survivors, in that both telomere healing and the anti-checkpoint function of seed-containing breaks seem to be suppressed in post-senescence survivors. The finding that the seed-containing DSB end is initially remarkably stable (in contrast to rapid degradation of the non-seeded 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). While 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 only act on initially extremely long telomeres (Grandin and Charbonneau 2009; Lebel et al. 2009) or depend on the absence of Exo1 (Marinelli and Lydall 2004) (which seems functional in our survivors based on efficient resection of the non-seed-containing DSB end; Supplementary 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 (Figs. 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 efficiently recombined in our type II survivors (Supplementary 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
anti-checkpoint function as such is indeed telomerase-independent, but only in pre-senescent cells, whereas it is compromised once these cells have switched to the ALT pathway (Figure 2). However, even in survivors the anti-checkpoint may still be partially active, as Rad53 activation in response to the seed DSB was turned off after about 6 hours, which is considerably earlier than checkpoint adaptation to an irreparable break after about 10-12 hours (Pellicioli et al. 2001). A possible explanation for the suppression of the anti-checkpoint 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 anti-checkpoint factor that may antagonize Rad53 activation even independently of its Rap1-dependent telomere capping function (Ribeyre and Shore 2012; Xue et al. 2011). However, neither overexpression of Rif1 nor Rif2 restored the telomere seed anti-checkpoint function in our survivors (Supplementary 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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Figure legends

Figure 1  Absence of *de novo* telomere elongation in post-senescent survivors. (A) Schematic diagram of the assay system. Triangles indicate telomeres, circles indicate centromeres, and short black 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 *est2Δ* type II survivors (B,C) and a type I survivor (D) at the indicated times after HO induction. PRE, chr. VII fragment before HO cut; INT, internal control band from chr. XV; the arrowhead in B points to *de novo* telomeres in the WT.

Figure 2  Persistent checkpoint activation in type II post-senescence survivors. (A-C) Western blot analysis of extracts of the 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 pre-senescent cells (bottom panel); wild type and *est2Δ* survivor cultures (top panel) were treated at the same time with pre-senescent cells for comparison.

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

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 panel A probed with the *ADE2* probe as indicated in Figure 1.
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