Upregulation of dNTP Levels After Telomerase Inactivation Influences Telomerase-Independent Telomere Maintenance Pathway Choice in Saccharomyces cerevisiae

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ABSTRACT In 10–15% of cancers, telomere length is maintained by a telomerase-independent, recombination-mediated pathway called alternative lengthening of telomeres (ALT). ALT mechanisms were first seen, and have been best studied, in telomerase-null Saccharomyces cerevisiae cells called “survivors”. There are two main types of survivors. Type I survivors amplify Y’ subtelomeric elements while type II survivors, similar to the majority of human ALT cells, amplify the terminal telomeric repeats. Both types of survivors require Rad52, a key homologous recombination protein, and Pol32, a non-essential subunit of DNA polymerase δ. A number of additional proteins have been reported to be important for either type I or type II survivor formation, but it is still unclear how these two pathways maintain telomeres. In this study, we performed a genome-wide screen to identify novel genes that are important for the formation of type II ALT-like survivors. We identified 23 genes that disrupt type II survivor formation when deleted. 17 of these genes had not been previously reported to do so. Several of these genes (DUN1, CCR4, and MOT2) are known to be involved in the regulation of dNTP levels. We find that dNTP levels are elevated early after telomerase inactivation and that this increase favors the formation of type II survivors.

KEYWORDS Saccharomyces cerevisiae telomeres telomerase-independent telomere maintenance survivors dNTP levels

Eukaryotic chromosomes have specialized structures at their termini called telomeres. Telomeres prevent natural chromosome ends from being recognized and processed as DNA double-strand breaks in need of repair (Jain and Cooper 2010). Due to incomplete DNA replication and nucleolytic degradation, telomeres shorten with each round of cell division. Telomere shortening is reversed by the action of telomerase, a specialized reverse transcriptase that extends telomeres (Greider and Blackburn 1985). However, most human somatic cells do not express sufficient levels of telomerase to prevent telomere shortening, which has been implicated in human aging (López-Otin et al. 2013). The downregulation of telomerase early during human development has been proposed to function as a barrier to tumorigenesis because cancers cells need to maintain their telomeres to avoid replicative senescence or apoptosis induced by telomere erosion (Hanahan and Weinberg 2011). Most cancer cells overcome this barrier by reactivating telomerase, but 10–15% of cancers employ a telomerase-independent pathway known as alternative lengthening of telomeres (ALT) (Sobinoff and Pickett 2017).

In the budding yeast Saccharomyces cerevisiae, telomerase is constitutively expressed, allowing the maintenance of telomeres 300 ± 75 bp in length (Wellinger and Zakian 2012). The core components of telomerase in S. cerevisiae are a protein catalytic component (Est2) and an RNA subunit (TLC1) (Lingner et al. 1997; Singer and Gottschling 1994). Abrogating telomerase function, for example by deleting either EST2 or TLC1, will cause telomere attrition and, eventually, cell cycle
arrest and replicative senescence. A small subset of cells can overcome senescence and become what are called “survivors” (Lundblad and Blackburn 1993), using telomerase-independent telomere maintenance mechanisms as in ALT cancer cells.

There are two main types of S. cerevisiae survivors: type I and type II. Type I survivors exhibit amplification of the subtelomeric Y' elements; in contrast, type II survivors amplify the terminal (TG1)n telomeric sequences (Lundblad and Blackburn 1993; Teng and Zakian 1999). Type I and type II survivors require Rad52-dependent homologous sequences (Lundblad and Blackburn 1993; Teng and Zakian 1999). Type I and type II survivors require Rad52-dependent homologous recombination (HR) and the DNA polymerase. Type I and type II survivors maintain telomeres via Rad51-dependent BIR. Type II survivors exhibit amplification mechanisms as in ALT cancer cells. Consistent with the importance of end resection for type II survivor formation (Huang et al. 2001; Johnson et al. 2000; Chen et al. 2001; Dilley et al. 2016; Jiang et al. 2005; Zhong et al. 2007).

Sgs1 and Exo1, which are needed for processive resection of DNA ends (Mimitou and Symington 2008; Zhu et al. 2008), are also important for type II survivor formation (Huang et al. 2001; Johnson et al. 2001; Maringele and Lydall 2004; Bertuch and Lundblad 2004). Consistent with the importance of end resection for type II survivor formation, the sgs1Δ-D664A mutation (Bernstein et al. 2009; Bernstein et al. 2013), which is competent for recombination repair but defective in resection, also prevents the formation of type II survivors (Hardy et al. 2014). Similarly, type II survivor formation is hindered by the deletion of FUN30, which encodes a chromatin remodeler that promotes end resection (Costelloe et al. 2012). BLM, a human homolog of Sgs1, has also been implicated in facilitating telomere maintenance in ALT cells (Stavropoulos et al. 2002).

Several additional proteins have also been implicated in the formation of type II survivors. These include the Tel1 and Mec1 DNA damage checkpoint kinases: in the absence of either Mec1 or Tel1, type II survivor formation is impaired, and is completely abolished in mec1Δ tel1Δ double mutants (Tsai et al. 2002). Furthermore, the RNA polymerase II degradation factor Def1, the B-type cyclin Cbl2, the tRNA modification protein Sua5, and Mdt4/Pin4, which interacts with the DNA damage kinase Rad53, are also important for type II survivor formation (Chen et al. 2005; Grandin and Charbonneau 2003; Meng et al. 2010; Pike and Heierhorst 2007). An analysis of 280 genes known to alter telomere length homeostasis when deleted further identified 22 genes that are important for type II survivor formation, including genes encoding members of the nonsense mediated decay pathway, the DNA repair protein Rad6, and the KEOPS complex (Hu et al. 2013). However, it is still unclear how most of these proteins function in the formation of type II survivors, and whether there are more proteins involved in this process.

In this study, we performed a genome-wide screen to identify novel genes that are important for the formation of type II survivors. We identified 23 genes, 17 of which were not previously reported to be involved in type II survivor formation. Several of these genes are involved in the regulation of intracellular deoxyribonucleoside triphosphate (dNTP) levels. We show that dNTP levels are increased early after inactivation of telomerase, and that this increase is important to generate type II survivors.

MATERIALS AND METHODS

Yeast strains and growth conditions

Standard yeast media and growth conditions were used (Treco and Lundblad 2001; Sherman 2002). With the exception of MCY610 and the yeast knockout (YKO) collection (Giaever et al. 2002), all yeast strains used in this study are RAD5 derivatives of W303 (Thomas and Rothstein 1989; Zhao et al. 1998) and are listed in Table 1. MCY610 has a hybrid BY4741 and W303 genetic background. Generation of survivors on agar plates and in liquid culture was performed as previously described (van Mourik et al. 2016).

SGA screening procedure

The est2Δ and rad51Δ deletions were introduced into the strains of the YKO collection using synthetic genetic array (SGA) methodology (Tong and Boone 2006). The MATA can1ΔSTE2pr-Sp his5 est2ΔnatMX his3 leu2 by1Δ RAD5 rad51ΔURA3 TRP1 ura3 query strain for the screen was derived from the sporulation of MCY610. The pinning steps were performed using a ROTOR HD4 Singer Instruments, Somerset, UK) with a 384-density format. The final est2ΔnatMX rad51ΔURA3 xxxXkanMX triple mutants (where xxxXkanMX represents a deletion of a gene from the YKO collection) were quadruplicated (i.e., the plate density was increased to 1536), and the resulting four colonies per strain were individually streaked on YPD plates, followed by incubation at 30°C for 3 days. The strains were re-streaked 5-6 times until senescence was observed and survivors were formed, or until senescence was observed but no survivors formed.

Telomere Southern blot

Yeast genomic DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega), digested with XhoI, separated on a 1% (w/v) agarose gel, and transferred to a Hybond-N+ membrane (GE Healthcare). The membrane was hybridized to a telomere-specific (5’-CACCCACCCACACACCCACACCA-3’) digoxigenin-labeled probe.

Measurement of dNTP levels

dNTP levels were measured as previously described (Watt et al. 2016).

Data and reagent availability

Data are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

RESULTS AND DISCUSSION

Screening for novel genes that are important for type II survivor formation

To identify genes that are important for type II survivor formation, we screened the yeast knockout (YKO) collection for gene deletions that
impair the ability of est2Δ rad51Δ strains to form type II survivors. We used synthetic genetic array (SGA) methodology (Tong and Boone 2000) to create a library of MATα est2Δ rad51Δ xxxx mutants, where xxxx is a deletion of a nonessential gene from the YKO collection (Figure 1). Deletion of RAD51 prevents type I survivor formation (Teng et al. 2000; Chen et al. 2001), allowing us to screen for genes important for type II survivor formation. Each est2Δ rad51Δ xxxx triple mutant was quadruplicated by replica-pinning, and each replicate was then serially propagated on agar plates to follow senescence and survivor formation (i.e., each est2Δ rad51Δ xxxx strain was tested four times for its ability to form survivors). 32 triple mutants failed to form survivors in all four replicates, 100 failed to form survivors in three of the four replicates, and 403 failed to form survivors in two of the replicates. All 132 that failed to form survivors in at least three of the four replicates, plus 40 randomly selected that failed to form survivors in two of the four replicates, were further tested by repeating the serial propagation procedure with multiple isolates of single mutants (est2Δ), double mutants (est2Δ rad51Δ, est2Δ xxxx, rad51Δ xxxx) and triple mutants (est2Δ rad51Δ xxxx) obtained by tetrad dissection of spore-pollinated diploids. This allowed us to compare the phenotypic growth between the selected mutants (e.g., to ensure that loss of viability upon serial propagation was not the result of a synthetic genetic interaction between rad51Δ and xxxx) and to validate the hits. In this second test, 26 triple mutants failed to form survivors in >50% of the multiple isolates. Only one mutant of these 26 was from the 40 that failed to form survivors in two of four replicates in the original screen, so we did

Table 1 Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCY610</td>
<td>MATaα can1STE2pr-HIS3/can1STE2pr-Sp-his5 lyp1α/lyp1Δ rad51ΔURA3 /RAD51 rad50ΔkanMX/RAD50</td>
<td>This study</td>
</tr>
<tr>
<td>YPM7</td>
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<td>This study</td>
</tr>
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<td>This study</td>
</tr>
<tr>
<td>YPM9</td>
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<td>This study</td>
</tr>
<tr>
<td>YPM10</td>
<td>MATaα est2ΔURA3/EST2 rad51ΔnatMX/RAD51 mrd2ΔkanMX/NMD2</td>
<td>This study</td>
</tr>
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<td>This study</td>
</tr>
<tr>
<td>YPM12</td>
<td>MATaα est2ΔURA3/EST2 rad51ΔnatMX/RAD51 dun1ΔTRP1/DUN1 sm1ΔHIS3/SML1</td>
<td>This study</td>
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<tr>
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<td>This study</td>
</tr>
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<td>This study</td>
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<tr>
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<tr>
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<td>This study</td>
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<td>MATα est2ΔURA3 type II survivor</td>
<td>This study</td>
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<td>YPM56</td>
<td>MATα est2ΔURA3 type II survivor</td>
<td>This study</td>
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<tr>
<td>MCY775</td>
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<td>This study</td>
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<td>MCY784</td>
<td>MATα est2ΔURA3 type II survivor</td>
<td>This study</td>
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<td>MCY785</td>
<td>MATα est2ΔURA3 sm1ΔHIS3 type II survivor</td>
<td>This study</td>
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<td>MATα est2ΔURA3 sm1ΔHIS3 type II survivor</td>
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<td>This study</td>
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<td>MATα est2ΔURA3 type II survivor</td>
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<td>YPM61</td>
<td>MATα est2ΔURA3 dun1ΔTRP1 type II survivor</td>
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<td>YPM62</td>
<td>MATα est2ΔURA3 dun1ΔTRP1 type II survivor</td>
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<tr>
<td>YPM65</td>
<td>MATα est2ΔURA3 dun1ΔTRP1 type II survivor</td>
<td>This study</td>
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</table>
not test any additional genes from this group. Importantly, the 26 included strains with a deletion of \( \text{RAD52}, \text{RAD50}, \text{RAD59}, \text{SGS1}, \text{CLB2}, \) or \( \text{NMD2}, \) which are all known to be required for type II survivor formation (Lundblad and Blackburn 1993; Teng et al. 2000; Chen et al. 2001; Huang et al. 2001; Johnson et al. 2001; Grandin and Charbonneau 2003; Hu et al. 2013), as well as \( \text{RMI1} \) and \( \text{YLR235C} \) (which overlaps the

<table>
<thead>
<tr>
<th>Table 2 Genes identified that are important for type II survivor formation</th>
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<tr>
<td><strong>Gene</strong></td>
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<tr>
<td><strong>in BY4741 background</strong></td>
</tr>
<tr>
<td>CCR4</td>
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<tr>
<td>CDC55</td>
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<td>CHK1</td>
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<td>CLB2</td>
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<td>DOA4</td>
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<td>DUN1</td>
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<td>LSM1</td>
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<td>RGI1</td>
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<td>RMI1</td>
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<td>RPL8B</td>
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<td>RPN4</td>
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<tr>
<td>RRM3</td>
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<tr>
<td>SGS1</td>
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<td>SPT20</td>
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<tr>
<td>VMA22</td>
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<tr>
<td>YLR235C</td>
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<td>YLR355C</td>
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</table>

\(^a\)These est2Δ rad51Δ xxxΔ triple mutants were obtained either from the original screen, where four isolates were generated using SGA methodology, or by tetrad dissection of sporulated diploids.

\(^b\)CCR4 was not identified in the original screen, but was tested in the W303 background due to its functional connection with MOT2.
SML1

dNTP levels. To test this hypothesis, we examined whether a deletion of
vors. If so, the compromised ability of cells lacking Dun1 or the Ccr4-Not
tant for generating type II survivors suggests that the ability to upregulate
was not identi-
plex, a key regulator of eukaryotic gene expression that is required for
regulate the Crt1-dependent inhibition of the
Dif1, three negative regulators of ribonucleotide reductase (RNR)
(Zhao and Rothstein 2002; Huang

We noticed that two of the identi-
ed genes, DUN1 and MOT2, are involved in the regulation of dNTP levels. Dun1 is a DNA damage
checkpoint kinase that phosphorylates and inhibits Sml1, Crt1, and
Dif1, three negative regulators of ribonucleotide reductase (RNR)
(Zhao and Rothstein 2002; Huang et al. 1998; Lee et al. 2008). The
RNR complex catalyzes the rate limiting step in dNTP synthesis (Hofer
et al. 2012). Mot2 (also known as Not4) is part of the Ccr4-Not com-
plex, a key regulator of eukaryotic gene expression that is required for
transcriptional induction of RNR genes in response to DNA damage or
replication stress (Mulder et al. 2005). Ccr4 and Dun1 cooperate to
regulate the Crt1-dependent inhibition of the RNR genes in response to
DNA replication stress (Woolstencroft et al. 2006). Although CCR4
was not identified in our screen, we found that est2Δ rad51Δ ccr4Δ
triple mutants were unable to form survivors (Table 2).

The finding that both Dun1 and the Ccr4-Not complex are impor-
tant for generating type II survivors suggests that the ability to upregulate
intracellular dNTP levels is important for the formation of type II survi-
vors. If so, the compromised ability of cells lacking Dun1 or the Ccr4-Not
complex to form type II survivors should be suppressed by increasing
dNTP levels. To test this hypothesis, we examined whether a deletion of
SML1 could suppress the defect in survivor formation of est2Δ rad51Δ
dun1Δ cells. Sml1 inhibits RNR by binding to Rnr1, the large subunit of
RNR (Zhao et al. 1998; Chabes et al. 1999). Cells lacking Dun1 have a
twofold decrease in dNTP levels, but sml1Δ and dun1Δ sml1Δ mutants
both have a 2.5-fold increase in dNTP levels (Fasullo et al. 2010; Zhao
et al. 1998; Gupta et al. 2013). An est2Δ/EST2 rad51Δ/RAD51 dun1Δ/
DUN1 sml1Δ/SML1 diploid was sporulated to generate haploid meiotic
progeny, which were serially propagated in liquid medium to monitor
senescence and survivor formation. We found that deletion of SML1 largely
suppresses the dun1Δ type II survivor formation defect (Figure 2),
suggesting that decreased dNTP levels hinder the formation of type II
survivors.

dNTP pools are upregulated in telomerase-null pre-
senescent cells and in type II survivors

To confirm our hypothesis that dNTP levels are important for type II
survivor formation, we measured dNTP pools in pre-senescent cells
(approximately 35 generations after the loss of telomerase) and in type II
survivors (Figure 3A). Survivor type was determined by a telomere
Southern blot (Figure 3B). We find that dNTP levels are increased in
pre-senescence est2Δ cells and remain elevated in type II survivors.
Deletion of Dun1 abolishes this increase, a phenotype that is sup-
pressed by an additional deletion of SML1. These observations suggest
that telomere shortening in telomerase-negative cells triggers an in-
crease in dNTP levels that facilitates the generation of type II survivors.
Interestingly, an est2Δ dun1Δ mutant can still form type II survivors,
albeit at a reduced efficiency. This indicates that while an increase in
dNTP levels promotes the initial formation of type II survivors, it is not
needed for maintenance of the survivors.

The elevation in dNTP levels occurs relatively early after telo-
merase inactivation (ETI; within ~35 population doublings after the
formation of est2Δ haploid meiotic progeny), well before a majority of
cells become senescent. Consistent with this observation, the
DNA damage response and expression of RNR3 is induced in ETI
cells (Ijpm and Greider 2003; Xie et al. 2015). In addition, a recent
study has shown that ETI cells experience replication stress, result-
ing in a dependence on the DNA damage response for viability that
is alleviated by elevating dNTP pools via a deletion of SML1 (Jay
et al. 2016). Taken together, these findings indicate that replication
stress occurs in the absence of telomerase, leading to an

Figure 2 Deletion of SML1 suppresses the type II survivor forma-
tion defect of a est2Δ rad51Δ dun1Δ strain. (A) Senescence and survivor
formation were monitored in liquid culture by serial passaging of indi-
vidual isolates of est2Δ rad51Δ dun1Δ (n = 19, red lines) and est2Δ rad51Δ dun1Δ sml1Δ (n = 20, blue lines), derived from the
sporulation of YPM12. (B) Percentage of est2Δ rad51Δ dun1Δ and est2Δ rad51Δ dun1Δ sml1Δ cul-
tures from panel A that were able to form survivors. Error bars repre-
sent exact binomial 95% confidence intervals; p-value was determined us-
ing Fisher’s exact test.

Genes involved in the regulation of dNTP pools are im-
portant for type II survivor formation

We noticed that two of the identified genes, DUN1 and MOT2, are involved in the regulation of dNTP levels. Dun1 is a DNA damage
checkpoint kinase that phosphorylates and inhibits Sml1, Crt1, and
Dif1, three negative regulators of ribonucleotide reductase (RNR)
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SML1 could suppress the defect in survivor formation of est2Δ rad51Δ
dun1Δ.
upregulation of dNTP levels that promotes the formation of type II survivors. Interestingly, we find that dNTP levels stay elevated in type II survivors (Figure 3), despite these cells looking similar to telomerase-positive wild-type cells in terms of growth rate as well as telomere movement and localization (Teng and Zakian 1999; Straatman and Louis 2007). This observation may be due to the fact that dNTP levels are elevated during BIR (Deem et al. 2011), which is required both to prevent accelerated senescence in pre-senescent cells and for telomere elongation in survivors (Fallet et al. 2014; Lydeard et al. 2007).

In summary, this work has identified novel genes important for the formation of type II survivors. We show that dNTP levels increase early after the loss of telomerase, promoting the formation of type II survivors. However, the increased dNTP levels are not required for the maintenance of type II survivors. Given the similarities between type II survivors and human ALT cancer cells, these findings may help us design more effective strategies to combat cancers that use ALT to maintain telomeres.

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Figure 3 dNTP levels are upregulated in est2Δ pre-senescent cells and type II survivors. (A) Strains of the indicated genotypes were assayed for dNTP levels. Data are represented as mean ± SE (B) Representative telomere Southern blot of survivors generated by serial propagation in liquid culture of haploid meiotic progeny derived from the sporulation of MCY775. Type I survivors exhibit short telomeres and strong hybridization at 5.2 kb and 6.7 kb due to amplification of the tandemly repeated Y’ short and Y’ long elements, respectively. The telomes of type II survivors are extended and very heterogeneous in size. The black arrow indicates a 1.8 kb DNA fragment, generated from the BsmAI-digestion of plasmid pYt103 (Shampay et al. 1984). This fragment contains telomeric sequences and was ran with each sample as a control.

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