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
To identify genes that are important for type II survivor formation, we screened the yeast knockout (YKO) collection for gene deletions that

**RESULTS AND DISCUSSION**

**Measurement of dNTP levels**

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

**Data and reagent availability**

Data are stored on a searchable database. A list of 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 MATa est2Δ rad51Δ xxxΔ mutants, where xxxΔ 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 xxxΔ 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 xxxΔ 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 xxxΔ, rad51Δ xxxΔ) and triple mutants (est2Δ rad51Δ xxxΔ) obtained by tetrad dissection of sporulated 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 xxxΔ) 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

| Strain name | Relevant genotype | Source |
|-------------|-------------------|--------|
| MCY610      | MATa/α can1ΔSTE2pr-HIS3/can1ΔSTE2pr-Sp-his5 lyp1Δ/lyp1Δ rad51ΔURA3 /RAD51 rad2ΔnatMX/EST2 TRP1/trp1-1 ADE2/ADE2 his3Δ1/his3 leu2Δ0/leu2 ura3Δ0/ura3 RAD5/RAD5-535 | This study |
| YPM7        | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rad50ΔkanMX/RAD50 | This study |
| YPM8        | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rad92ΔkanMX/RAD59 | This study |
| YPM9        | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 | This study |
| YPM10       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 mrd2ΔkanMX/NMD2 | This study |
| YPM11       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rgi1ΔkanMX/RI11 | This study |
| YPM12       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 dun1ΔTRP1/DUN1 sm1ΔHIS3/SML1 | This study |
| YPM13       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 cib2ΔkanMX/CLB2 | This study |
| YPM20       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 vps2ΔkanMX/VPS25 | This study |
| YPM21       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 lsm1ΔkanMX/LSM1 | This study |
| YPM29       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 mti1ΔkanMX/RMI1 | This study |
| YPM30       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 spt2ΔkanMX/SPT20 | This study |
| YPM31       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 cdc55ΔkanMX/CDC55 | This study |
| YPM32       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 chk1ΔkanMX/CHK1 | This study |
| YPM33       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 pph3ΔkanMX/PPH3 | This study |
| YPM34       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 mot2ΔkanMX/MOT2 | This study |
| YPM35       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rpn4ΔkanMX/VPN4 | This study |
| YPM36       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 ylr358cΔkanMX/YLR358C | This study |
| YPM37       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 mm3ΔkanMX/RRM3 | This study |
| YPM38       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 tsc3ΔkanMX/TSC3 | This study |
| YPM39       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 pxp1ΔkanMX/PXP1 | This study |
| YPM40       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 mtc7ΔkanMX/MTC7 | This study |
| YPM41       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 d0a4ΔkanMX/DOA4 | This study |
| YPM42       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 cik1ΔkanMX/CIK1 | This study |
| YPM43       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 ured2ΔkanMX/URE2 | This study |
| YPM44       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 vma22ΔkanMX/VMA22 | This study |
| YPM45       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rpl8ΔkanMX/RPL8B | This study |
| YPM48       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 ylr235cΔkanMX/YLR235C | This study |
| YPM51       | MATa/α est2ΔURA3/EST2 rad51ΔnatMX/RAD51 ccr4ΔkanMX/CCR4 | This study |
| YPM55       | MATa/α est2ΔURA3 type II survivor | This study |
| YPM56       | MATa/α est2ΔURA3 type II survivor | This study |
| MCY775      | MATa/α est2ΔURA3/EST2 dun1ΔTRP1/DUN1 sm1ΔHIS3/SML1 | This study |
| MCY783      | MATa/α est2ΔURA3 type II survivor | This study |
| MCY784      | MATa/α est2ΔURA3 type II survivor | This study |
| MCY785      | MATa/α est2ΔURA3 sml1ΔHIS3 type II survivor | This study |
| MCY786      | MATa/α est2ΔURA3 sml1ΔHIS3 type II survivor | This study |
| MCY788      | MATa/α est2ΔURA3 dun1ΔTRP1 sml1ΔHIS3 type II survivor | This study |
| YPM60       | MATa/α est2ΔURA3 type II survivor | This study |
| YPM61       | MATa/α est2ΔURA3 dun1ΔTRP1 type II survivor | This study |
| YPM62       | MATa/α est2ΔURA3 dun1ΔTRP1 type II survivor | This study |
| YPM63       | MATa/α est2ΔURA3 dun1ΔTRP1 type II survivor | This study |
| YPM64       | MATa/α est2ΔURA3 dun1ΔTRP1 sm1ΔHIS3 type II survivor | This study |
| YPM65       | MATa/α est2ΔURA3 dun1ΔTRP1 sm1ΔHIS3 type II survivor | This study |

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not test any additional genes from this group. Importantly, the 26 included strains with a deletion of \( \text{RAD52, RAD50, RAD59, SGS1, CLB2, or NMD2} \), which are all known to be required for type II survivor formation (Lundblad and Blackburn 1993; Teng \textit{et al.} 2000; Chen \textit{et al.} 2001; Huang \textit{et al.} 2001; Johnson \textit{et al.} 2001; Grandin and Charbonneau 2003; Hu \textit{et al.} 2013), as well as \( \text{RMI1 and YLR235C} \) (which overlaps the

| Table 2 Genes identified that are important for type II survivor formation |

| Gene | Fraction of \( \text{est2} \Delta \text{rad51} \Delta \text{xxx} \Delta \) that are able to form survivors in \( \text{BY4741} \) background\(^{a}\) | Fraction of \( \text{est2} \Delta \text{rad51} \Delta \text{xxx} \Delta \) that are able to form survivors in \( \text{W303} \) background | Reference |
|------|---------------------------------|---------------------------------|----------|
| \( \text{CCR4} \)\(^b\) | 0/10 (0%) | 2/9 (22%) | Grandin and Charbonneau 2003 |
| \( \text{CDC55} \) | 0/12 (0%) | 2/10 (20%) | |
| \( \text{CHK1} \) | 5/14 (36%) | 2/10 (20%) | |
| \( \text{CLB2} \) | 2/14 (14%) | | |
| \( \text{DOA4} \) | 5/14 (36%) | 3/10 (30%) | |
| \( \text{DUN1} \) | 2/12 (17%) | 1/25 (4%) | |
| \( \text{LSM1} \) | 5/14 (36%) | 0/7 (0%) | |
| \( \text{MOT2} \) | 0/10 (0%) | 1/4 (25%) | |
| \( \text{NMD2} \) | 0/12 (0%) | | |
| \( \text{PPH3} \) | 2/12 (17%) | 2/10 (20%) | |
| \( \text{RAD50} \) | 2/10 (20%) | | |
| \( \text{RAD52} \) | 0/11 (0%) | | |
| \( \text{RAD59} \) | 4/11 (36%) | | |
| \( \text{RGI1} \) | 0/4 (0%) | 2/10 (20%) | |
| \( \text{RMI1} \) | 1/7 (14%) | 0/10 (0%) | |
| \( \text{RPL8B} \) | 1/8 (13%) | 2/10 (20%) | |
| \( \text{RPN4} \) | 1/9 (11%) | 3/10 (30%) | |
| \( \text{RRM3} \) | 4/12 (33%) | 3/10 (30%) | |
| \( \text{SGS1} \) | 0/11 (0%) | | |
| \( \text{SPT20} \) | 0/5 (0%) | 0/10 (0%) | |
| \( \text{VMA22} \) | 1/10 (10%) | 3/10 (30%) | |
| \( \text{YLR235C} \) | 1/16 (6%) | 0/10 (0%) | |
| \( \text{YLR358C} \) | 1/5 (20%) | 4/9 (44%) | |

\(^{a}\)These \( \text{est2} \Delta \text{rad51} \Delta \text{xxx} \Delta \) triple mutants were obtained either from the original screen, where four isolates were generated using SGA methodology, or by tetrad dissection of sporulated diploids.

\(^{b}\)\( \text{CCR4} \) was not identified in the original screen, but was tested in the \( \text{W303} \) background due to its functional connection with \( \text{MOT2} \).
could suppress the defect in survivor formation of 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/sml1Δ diploid was sporulated to generate haploid meiotic progeny, which were serially propagated in liquid medium to monitor senescence and survivor formation. We find 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-senescent est2Δ cells and remain elevated in type II survivors. Deletion of dun1Δ abolishes this increase, a phenotype that is suppressed by an additional deletion of SML1. These observations suggest that telomere shortening in telomerase-negative cells triggers an increase 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 telomerase inactivation (ETI); within ~35 population doublings after the generation 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 (Ipma and Greider 2003; Xie et al. 2015). In addition, a recent study has shown that ETI cells experience replication stress, resulting 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
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 telomeres 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 run with each sample as a control.
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