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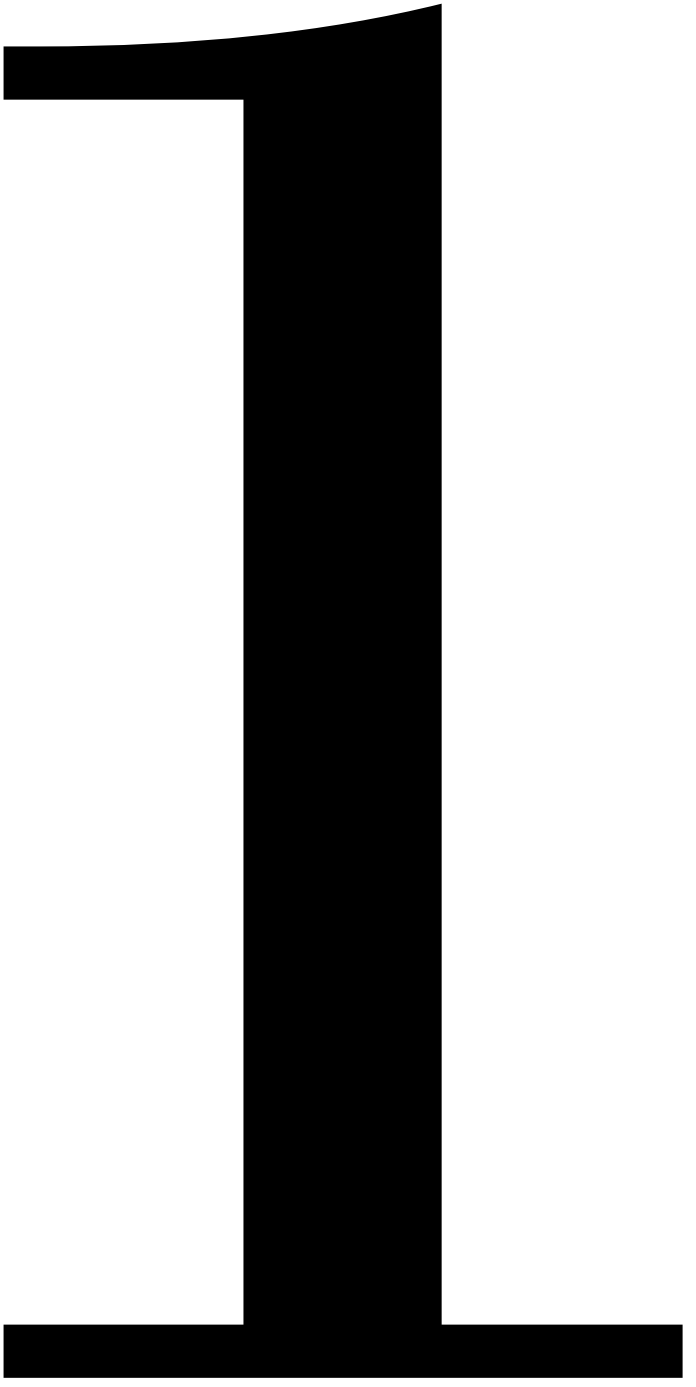


Chapter 1

Introduction

Telomeres and their involvement in ageing and cancer

Eukaryotic chromosomes have specialised structures at their termini called telomeres. Telomeres contain DNA repeats (5'-TTAGGG-3' in mammals) and specialised protein structures which have a capping function to maintain chromosomal stability and cell viability and play a role in facilitating the complete replication of chromosomal termini. Chromosomal capping by telomeres prevents nucleolytic degradation and prevents chromosomal ends from being recognized as DNA double-strand breaks (DSB) [1, 2]. Telomere dysfunction leads to activation of DSB repair mechanisms and the DNA damage response (DDR) pathway, which may cause chromosomal rearrangements, telomere-telomere fusion events, senescence and cell death [1]. Due to nucleolytic degradation and incomplete end replication by the DNA replication machinery, eukaryotic telomeres gradually shorten with each round of cell division, a problem referred to as the end-replication problem [3]. In order to counteract this, a specialized reverse transcriptase called telomerase elongates telomere ends by adding short tandem repeats, using a short region of the RNA subunit of telomerase as a template for iterative reverse transcription [4-6]. In most human somatic cells, telomerase is downregulated and telomeres shorten with each round of cell division, leading to cell ageing and cell death [7]. This telomere attrition is a hallmark of ageing in humans [8]. In 85-90% of cancers, telomerase is reactivated to elongate telomeres [9]. The **A**lternative **L**engthening of **T**elomeres (ALT) pathway, which is a telomerase-independent mechanism, elongates telomeres in 10-15% of cancers by using a recombination-based mechanism [10]. This recombination-mediated telomere mechanism was first discovered in the budding yeast *Saccharomyces cerevisiae*. These cells are called post-senescence survivors (or just 'survivors') and require certain recombination proteins to elongate telomeres in the absence of telomerase [11].

Telomerase

Telomerase was discovered by Greider and Blackburn in 1985 as the 'telomere terminal transferase' enzyme [4]. This unique enzyme contains a protein catalytic subunit (*TERT*) and a template-containing RNA component (*TERC*). The RNA template varies in sequence and length between species. The human *TERC* RNA template contains an 11 base pair (bp) long sequence (5'-CUAACCCUAAC-3') that is complementary to the human 5'-TTAGGG-3' telomeric repeat sequence [12]. The nucleolar protein dyskerin binds to the *TERC* RNA component to stabilize the telomerase complex in humans [13].

Budding yeast telomerase holoenzyme consists of four subunits, the catalytic protein subunit Est2, a large noncoding RNA (called telomerase component 1 (*TLC1*)), and two additional subunits, Est1 and Est3, which are required for telomerase activity *in vivo* [14]. The TLC1 RNA contains a region with the 5'–CACCACACCCACACAC–3' sequence that is complementary to yeast telomeric repeat sequence ($TG_{1–3}$), as well as other regions that serve as a scaffold for the binding of Est2 and accessory proteins [15, 16]. Est1 is needed for the association of Est2 and Est3 to form a functional telomerase holoenzyme [17]. *In vivo*, telomerase is highly regulated and elongates only a small number of telomeres each cell cycle, preferentially the shortest ones [18].

Telomere structure

In most eukaryotic cells telomeres are composed of tandem repeats, but can vary in length depending on species, individual, cell type, chromosome and cellular age (i.e. 350–500 bp in yeast to several kb in mammals) [19-22].

Human telomeric DNA is typically 10-15 kilobase pairs (kb) long. The terminal end consists of a single stranded 3' telomeric overhang that is protected by a t-loop configuration [23]). A t-loop structure is formed by the strand invasion of the telomeric 3′ overhang into the double-stranded DNA of the same telomere [24]. The telomeric repeats are bound by proteins that form the shelterin complex. The shelterin complex has different functions which involves the regulation of telomere length, chromosome end protection and the regulation and recruitment of telomerase [25-27]. The shelterin complex consists of TRF1 and TRF2, TIN2, RAP1, TPP1 and POT1 (see Figure 1A) [23]. POT1 binds specifically to the single-stranded 3' G-rich overhang [28]. TRF1 and TRF2 bind to the double-stranded telomeric DNA and can repress telomere elongation in *cis* [29, 30]. Furthermore, TRF2 is essential for t-loop formation and maintenance at the 3′ telomeric overhang [24, 31]. TPP1 binds to TIN2 and POT1, thereby bridging the double-stranded and single-stranded parts of the telomere [32]. POT1 plays a role in telomere length regulation by competing with telomerase for the access of the 3' overhang [33]. Shelterin is a repressor of the DDR pathway [34]. TRF2 inhibits the DNA damage sensing protein ATM, whereas POT1 inhibits ATR-mediated response [35].

S. cerevisiae telomeric DNA consists of 300 ± 75 bp of $C_{1–3}A/TG_{1–3}$ repetitive sequences with a 12-14 nucleotide-long extension of the G-rich strand to form a 3′ single-stranded overhang [14]. The subtelomeric regions also contain repetitive

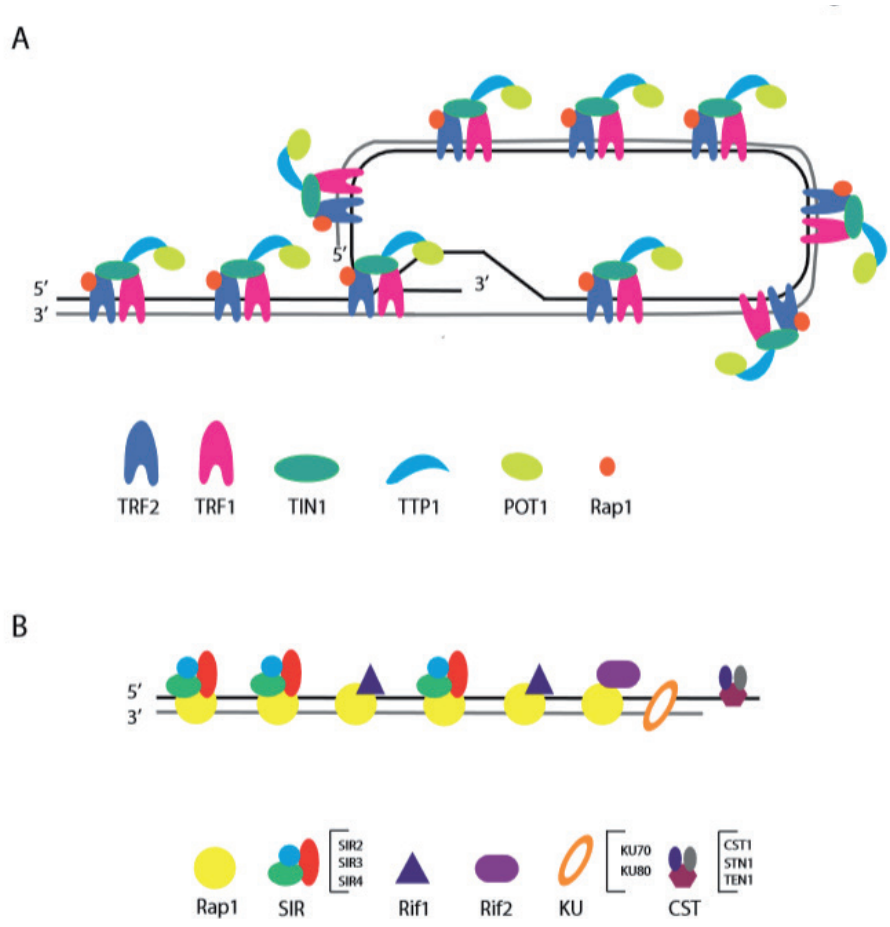


Figure 1. Structure of the human telomere and the *S. cerevisiae* telomere (based on Claussin and Chang, 2016).
A: Structure of a mammalian telomere with the shelterin proteins and the t-loop configuration. B: Structure of a *S. cerevisiae* telomere with binding proteins.

X and Y′ elements. An X element is located at all chromosome ends, while the Y′ elements are located in zero to four tandem copies between an X element and the terminal telomeric repeats (see Figure 2) [36]. In *S. cerevisiae* the double-stranded telomere DNA is bound by the Rap1 protein, which recruits Rif1 and Rif2 and the silent chromatin proteins Sir3 and Sir4 (see Figure 1B) [37-40]. The absence of Rif1 or Rif2, or a C-terminal truncation of Rap1 that prevents the recruitment of Rif1 and Rif2, causes extensive telomere elongation, suggesting that these proteins are involved in telomere length regulation [38, 39]. The Ku heterodimer (consisting

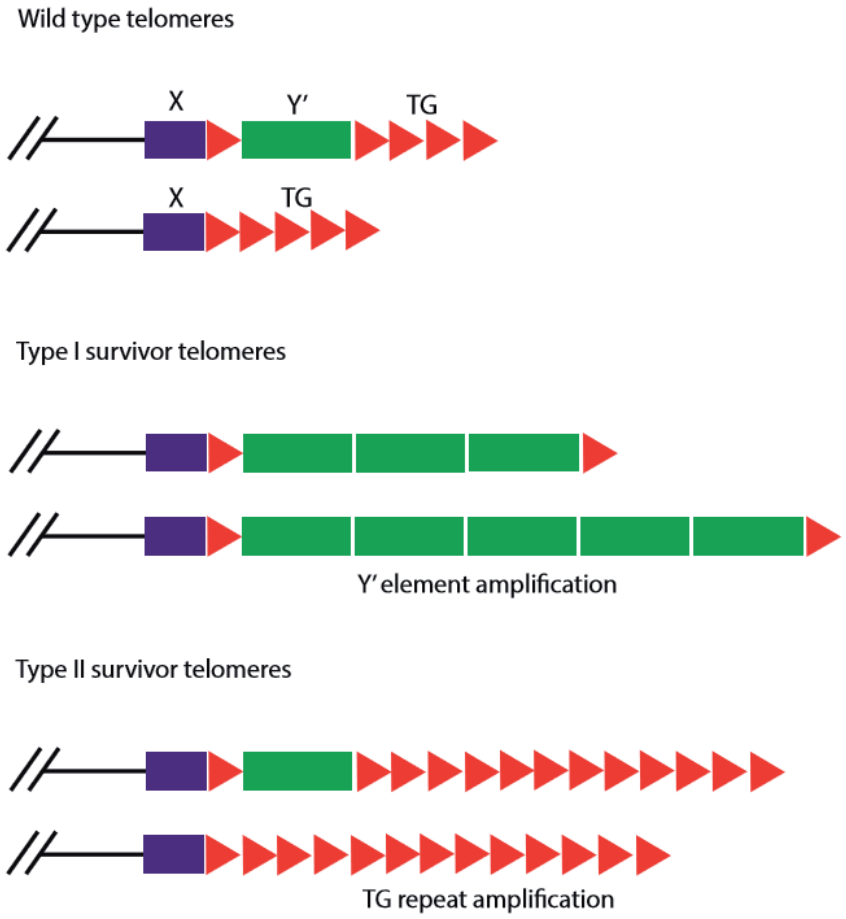


Figure 2. Structure of *S. cerevisiae* telomeres in wild-type telomerase-positive cells, type I survivors and type II survivors (based on Claussin and Chang, 2016).
All telomeres contain an X element, and approximately half to two-thirds also contain one to four Y′ elements. The type I survivors amplify the Y′ elements, even in telomeres that did not originally have an Y′ element. The type II survivors amplify the terminal telomeric repeats.

of Yku70 and Yku80) binds directly to the telomere or by the protein-protein interaction between Yku80 and Sir4 [41]. The yKu complex inhibits 5'–3' end resection and recombination at telomeres [42, 43]. The absence of Ku at telomeres increases the length of the 3' telomeric overhangs [42, 44-46]. The 3' overhang is bound by the CST complex (consisting of Cdc13, Stn1 and Ten1) [47-51]. The *cdc13-1* and *stn1-13* mutant strains show elevated levels of telomeric recombination, extensive telomere resection and have long 3′ overhangs [43, 49, 50, 52].

Telomere length maintenance in the absence of telomerase

In telomerase-deficient yeast cells, telomeres shorten progressively during each cell division due to incomplete end-replication. When the telomeres become very short, cells enter a state that blocks cell division, termed senescence. Using telomerase-independent mechanisms these cells can overcome senescence and maintain their telomere length and function [1, 9]. For *S. cerevisiae*, two main types of survivors are described to date, type I and type II. These types differ in their telomere sequence and recombination proteins utilized [11, 53-55]. Both survivor types are dependent on the Rad52-dependent homologous recombination (HR) protein and the DNA polymerase δ subunit Pol32, which is required for break-induced replication (BIR), suggesting that both survivor pathways occur through recombination-dependent DNA replication [11, 56]. The Pif1 helicase is also important for the generation of survivors [57], likely due to its role in BIR [58, 59]. Cells lacking both telomerase and *RAD52* are severely compromised in forming survivors, although rare Rad52-independent survivors can be formed in some genetic backgrounds [11, 53, 60].

Type I survivors

Type I survivors grow very slow compared to wild-type cells and type II survivors [11]. Also their terminal telomeric repeats are maintained at sizes substantially below those of wild-type cells [61] The type I survivors maintain telomeres by amplification of the subtelomeric Y' elements and short telomeric TG tracts that are sometimes found between the tandem Y' elements (see Figure 2) [11, 54, 55]. Although not all chromosome ends originally contain Y' elements, in type I survivors all chromosome ends have Y' elements [11]. Type I survivors, as well as wild-type cells, contain extra-chromosomal Y' circles that may serve as substrates for Y' recombination [62, 63]. The Y' element contains an open reading frame (ORF) that encodes for a helicase called Y-Help1 that is strongly induced during growth arrest in telomerase-negative cells and may play a role in survivor cells [64]. The amplification of an Y' element could be used as homologous template to allow for sufficient BIR.

In addition to Rad52 and Pol32, the formation of type I survivors is dependent on the homologous recombination proteins Rad51, Rad54, Rad55 and Rad57 [54]. In homology-dependent DSB repair, the Rad51 protein is loaded onto RPA-coated single-stranded DNA (ssDNA) by Rad52, forming a nucleoprotein presynaptic filament that promotes pairing and strand exchange with a homologous duplex

DNA template [65, 66]. The filament formation and stabilization also involves Rad55, Rad57, Rad54 and the Shu complex (consisting of Shu1, Shu2, Psy3 and Csm2) [67-69]. Rad55 forms a heterodimer with Rad57 and stimulates strand exchange by stabilizing the assembly of Rad51 to the ssDNA [70]. The Shu complex interacts indirectly with Rad51 through the Rad51 paralogue Rad55-Rad57 to stimulate Rad51 filament attachment to the ssDNA [71]. Rad54 is a chromatin remodeling protein that is a member of the Swi2/Snf2 family and interacts with Rad51 and stimulates the Rad51 DNA strand exchange activity [72, 73]. Other proteins that have shown to reduce type I survivor formation are the Pif1 helicase and INO80 chromatin remodeling complex [74].

Type II survivors

The growth of type II survivors is comparable to telomerase-positive cells [11, 53]. Type II survivors have long and heterogeneous-sized telomere lengths that can be up to 12 kb in length (see Figure 2) [53]. Type II survivors amplify the $C_{1-3}A/TG_{1-3}$ sequences, is Rad51-independent, and involves the MRX (Mre11, Rad50, Xrs2) complex, Sgs1 and Rad59 [54-56, 75-78]. The MRX complex plays a role in end resection and maintaining the DSB tethered to each other for DSB repair [79]. Rad59 plays a role in single-strand annealing (SSA) between direct DNA repeats [65]. Sgs1 is a helicase of the RecQ family and homologous to the human BLM and WRN helicases and functions in genome stability [65, 80, 81]. Both Sgs1 and the exonuclease Exo1 are involved in DNA end resection and are important for type II survivor formation [76, 82-85]. Extensive end resection might be important to initiate BIR and promote type II survivor formation.

Studies have identified additional genes that are important to generate type II survivors. Fun30, a chromatin remodeler that promotes end resection, RNA polymerase II degradation factor Def2, B-type cyclin Clb2, tRNA modification protein Sua5, and the Mdt4/Pin4 protein, which interacts with the DNA damage kinase Rad53, are important for type II survivor formation [84, 86-90]. The Tel1 and Mec1 DNA damage PI3K checkpoint kinases are also involved in type II survivor generation. In the absence of either Mec1 or Tel1, telomerase-negative cells are impaired in the formation of type II survivors, and completely abolished in the *tlc1Δ mec1Δ tel1Δ sml1Δ* strain (*sml1Δ* suppresses the lethality associated with *mec1Δ*), which can form only type I survivors [91]. A screen 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 [74]. The complete mechanism for type II survivor formation and maintenance still needs to be elucidated.

The genetic requirement for type I and type II survivors, as described above, are not always strict. Grandin and Charbonneau (2003) showed that in a telomerase-negative background, *cdc13-1* and *yku70Δ* mutants form type II survivors by the recombination of $TG_{1\text{-}3}$ sequences accomplished by the Rad51 pathway when the *RAD50/RAD59* pathway has been inactivated [88].

Furthermore, telomere length affects the ratio of type I vs. type II survivor formation. Deletion of *RIF1* and *RIF2* causes telomere lengthening [75]. In a telomerase-null background, deletion of Rif1 and/or Rif2 accelerates senescence without increasing the shortening rate. This causes the cell to senesce with longer telomeres, and these long telomeres promote the formation of type II survivors [92].

Break-induced replication and survivors

The precise mechanism of telomere elongation in the absence of telomerase is unclear. However, the evidence to date strongly implicates the recombination-mediated DNA replication known as break-induced replication (BIR) [56]. BIR is a HR process to initiate DNA replication when only one end of a DSB shares homology with a donor sequence or to initiate DNA replication in the absence of an origin of replication [61]. There are two BIR pathways: one is Rad51-dependent and one is independent of Rad51, but requires the MRX complex and Rad59 [93]. Similarly, the formation of type I survivors is dependent on Rad51 (and Rad54 and Rad57, which function in the same pathway as Rad51), whereas type II survivors require the MRX complex and Rad59 [55, 75], suggesting that type I and type II survivors maintain telomeres via Rad51-dependent and Rad51-independent BIR, respectively. The Rad51-dependent BIR pathway used for type I survivor formation extends telomeres through the amplification of subtelomeric Y' elements [11]. The movement of Y' elements that are added to new chromosome ends is likely due to homology mediated recombination [94]. The Rad51-independent BIR pathway used for type II survivor formation extends the telomeres by amplification of the $C_{1\text{–}3}A/TG_{1\text{–}3}$ sequences and are unstable and heterogeneous in length [53, 54]. A proposed mechanism for telomere elongation in type I and type II survivors is the 'roll-and spread' mechanism that involves both rolling circle synthesis and

intertelomeric BIR events [61]. A study in *Kluyveromyces lactis* proposed a model where extrachromosomal circular DNA containing telomeric sequences (t-circles) are generated at the time of senescence from a single end via a recombination event [95]. The t-circle can be used as template to enable rapid TG amplification using rolling circle synthesis at a short telomere end. T-circles have also been found in human ALT cells [96, 97] and *S. cerevisiae* type I survivors that had Y'-containing circles and type II survivors that had heterogeneously sized circles of telomeric repeats [63, 98, 99].

Type I survivors arise more frequently that type II survivors, but grow slower and easily convert to type II survivors [53]. This may be due to the fact that Rad51-dependent BIR is more efficient that Rad51-independent BIR, which may explain the higher frequency of type I survivors [100, 101]. Since *S. cerevisiae* survivor formation requires BIR, this model can be used to identify genes involved in BIR.

Characteristics and mechanisms of ALT cancer cells

ALT cancer cells are characterized by highly heterogeneous telomere lengths [10, 102, 103], abundant extrachromosomal telomeric repeat DNA (ECTR) [96, 104], elevated levels of telomeric sister chromatid exchanges (T-SCE) [105] and promyelocytic leukemia (PML) bodies, also known as ALT-associated PML bodies (APB) [106]. APBs are nuclear structures that contain extrachromosomal telomeric circles (t-circles) and shelterin proteins [96, 107]. Furthermore, APBs contain DNA damage response factors and recombination proteins (i.e. Rad51, Rad52, BLM, WRN, and the MRN complex) and telomere-specific binding proteins (TRF1, TRF2) [106, 107].

ALT cells, as well as *S. cerevisiae* survivors, display high levels of long non-coding telomeric repeat containing RNA (TERRA) [108-112]. TERRA is located at APBs and has been implicated as a regulator of ALT telomere recombination [113].

Human ALT cells have similar features as seen in the *S. cerevisiae* type II survivors (i.e. long, heterogeneous telomere length, requirement for the MRN complex and the Sgs1-homolog, BLM, and t-circles) [10, 96, 103, 114-117]. However, variant cell lines have been reported that lack APBs and long telomeres, but contain telomeres with subtelomeric elements [118, 119].

The mechanism underlying ALT in cancer cell development and maintenance is still unclear. However, it has been demonstrated that ALT cell activation could be

triggered by different factors. The inactivation of the ATP-dependent chromatin remodelers ATRX (alpha-thalassemia/mental retardation X-linked) and DAXX (death associated protein-6) are the most common genetic alterations found in ALT cells [120, 121]. ATRX and DAXX act together to assemble the telomeric regions into H3.3-containing nucleosomes [122-124]. Deletion of ATRX or DAXX could affect the H3.3 loading at telomeres and therefore disturb re-assembly of telomeric heterochromatin and cause further genome instability [125]. Another study found that the disruption of the histone chaperone paralogs ASF1a and ASF1b induce ALT activity and ALT features (i.e. telomere length heterogeneity, ECTRs and APB formation). ASF1 is a nucleosome assembly factor that transfers the H3.1-H4 and H3.3-H4 histone dimers into nucleosomes [126]. A recent study by Dilley *et al* shed more light on the BIR mechanism that might be used in ALT cancer cells to elongate and maintain telomere length. The authors created targeted DSBs in telomeres by fusing the Fok1 nuclease enzyme, which cleaves DNA, to the telomere-binding protein TRF1. They observed a ten-fold increase in telomeric DNA synthesis after TRF1–Fok1 induction in ALT cells and long tracks of telomeric repeats, which are consistent with DNA synthesis occurring through the break-induced replication mechanism. Furthermore, is was shown that POLD3, which is the human homolog of Pol32, is required for telomere synthesis during ALT. Dilley and colleagues also found that break-induced telomere synthesis was Rad51-independent in ALT cells. Instead, a complex that consists of the polymerase POLδ and the proteins PCNA and RFC1-5 was detected at DNA damage sites in ALT cells and was required for the break-induced telomere synthesis. Overall, the authors provided evidence for a Rad51-independent mechanism of mammalian break-induced telomere synthesis and ALT telomere maintenance [127].

Outline

Telomere length is maintained by ALT mechanisms in around 10-15% of cancers [10]. These ALT mechanisms are similar to survivor mechanisms in yeast, the type II pathway in particular, suggesting that *S. cerevisiae* can be used as a model organism to study ALT.

The main aim of my PhD was to identify novel genes and underlying mechanisms that are involved in survivor formation in *S. cerevisiae*. The BIR mechanism has been proposed as the mechanism for survivor formation in the absence of telomerase. The Shu complex, which consists of Shu1, Shu2, Psy3 and Csm2, promotes Rad51-

dependent homologous recombination and may also play a role in BIR. In **Chapter 2** we investigate if the Shu complex is involved in type II survivor formation. Surprisingly, we found that the Shu complex does not influence survivor formation. In **Chapter 3** we performed a genome-wide screen to identify novel genes that are involved in type II survivor formation. We identified 17 novel genes that influence type II survivor formation. Furthermore, we noticed that several of these genes are known to be involved in the regulation of deoxyribonucleoside triphosphate (dNTP) levels. We investigated the role of one of these genes, *DUN1*, which encodes a DNA damage checkpoint kinase and positive regulator of dNTP levels, and showed that dNTP levels are increased early after inactivation of telomerase in a Dun1-dependent manner, and that this increase is important to generate type II survivors. In **Chapter 4**, a high-throughput genome-wide screen was performed to identify novel genes that are involved in type I survivor formation. In **Chapter 5**, I discuss the implications of my findings and future perspectives.

References

1. Cesare, A.J. and R.R. Reddel, *Telomere uncapping and alternative lengthening of telomeres.* Mech Ageing Dev, 2008. **129**(1-2): p. 99-108.
2. Jain, D. and J.P. Cooper, *Telomeric strategies: means to an end.* Annu Rev Genet, 2010. **44**: p. 243-69.
3. Lingner, J., J.P. Cooper, and T.R. Cech, *Telomerase and DNA end replication: no longer a lagging strand problem?* Science, 1995. **269**(5230): p. 1533-4.
4. Greider, C.W. and E.H. Blackburn, *Identification of a specific telomere terminal transferase activity in Tetrahymena extracts.* Cell, 1985. **43**(2 Pt 1): p. 405-13.
5. Greider, C.W. and E.H. Blackburn, *The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity.* Cell, 1987. **51**(6): p. 887-98.
6. Greider, C.W. and E.H. Blackburn, *A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis.* Nature, 1989. **337**(6205): p. 331-7.
7. Sharpless, N.E. and R.A. DePinho, *Telomeres, stem cells, senescence, and cancer.* J Clin Invest, 2004. **113**(2): p. 160-8.
8. Lopez-Otin, C., et al., *The hallmarks of aging.* Cell, 2013. **153**(6): p. 1194-217.
9. Shay, J.W. and S. Bacchetti, *A survey of telomerase activity in human cancer.* Eur J Cancer, 1997. **33**(5): p. 787-91.
10. Bryan, T.M., et al., *Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines.* Nat Med, 1997. **3**(11): p. 1271-4.
11. Lundblad, V. and E.H. Blackburn, *An alternative pathway for yeast telomere maintenance rescues est1- senescence.* Cell, 1993. **73**(2): p. 347-60.
12. Feng, J., et al., *The RNA component of human telomerase.* Science, 1995. **269**(5228): p. 1236-41.
13. Mitchell, J.R., E. Wood, and K. Collins, *A telomerase component is defective in the human disease dyskeratosis congenita.* Nature, 1999. **402**(6761): p. 551-5.
14. Wellinger, R.J. and V.A. Zakian, *Everything you ever wanted to know about Saccharomyces cerevisiae telomeres: beginning to end.* Genetics, 2012. **191**(4): p. 1073-105.
15. Singer, M.S. and D.E. Gottschling, *TLC1: template RNA component of Saccharomyces cerevisiae telomerase.* Science, 1994. **266**(5184): p. 404-9.
16. Zappulla, D.C. and T.R. Cech, *RNA as a flexible scaffold for proteins: yeast telomerase and beyond.* Cold Spring Harb Symp Quant Biol, 2006. **71**: p. 217-24.
17. Osterhage, J.L., J.M. Talley, and K.L. Friedman, *Proteasome-dependent degradation of Est1p regulates the cell cycle-restricted assembly of telomerase in Saccharomyces cerevisiae.* Nat Struct Mol Biol, 2006. **13**(8): p. 720-8.
18. Teixeira, M.T., et al., *Telomere length homeostasis is achieved via a switch between telomerase-extendible and -nonextendible states.* Cell, 2004. **117**(3): p. 323-35.
19. Shampay, J., J.W. Szostak, and E.H. Blackburn, *DNA sequences of telomeres maintained in yeast.* Nature, 1984. **310**(5973): p. 154-7.
20. McEachern, M.J. and E.H. Blackburn, *A conserved sequence motif within the exceptionally diverse telomeric sequences of budding yeasts.* Proc Natl Acad Sci U S A, 1994. **91**(8): p. 3453-7.
21. de Lange, T., et al., *Structure and variability of human chromosome ends.* Mol Cell Biol, 1990. **10**(2): p. 518-27.
22. Starling, J.A., et al., *Extensive telomere repeat arrays in mouse are hypervariable.* Nucleic Acids Res, 1990. **18**(23): p. 6881-8.
23. de Lange, T., *Shelterin-Mediated Telomere Protection.* Annu Rev Genet, 2018.
24. Griffith, J.D., et al., *Mammalian telomeres end in a large duplex loop.* Cell, 1999. **97**(4): p. 503-14.

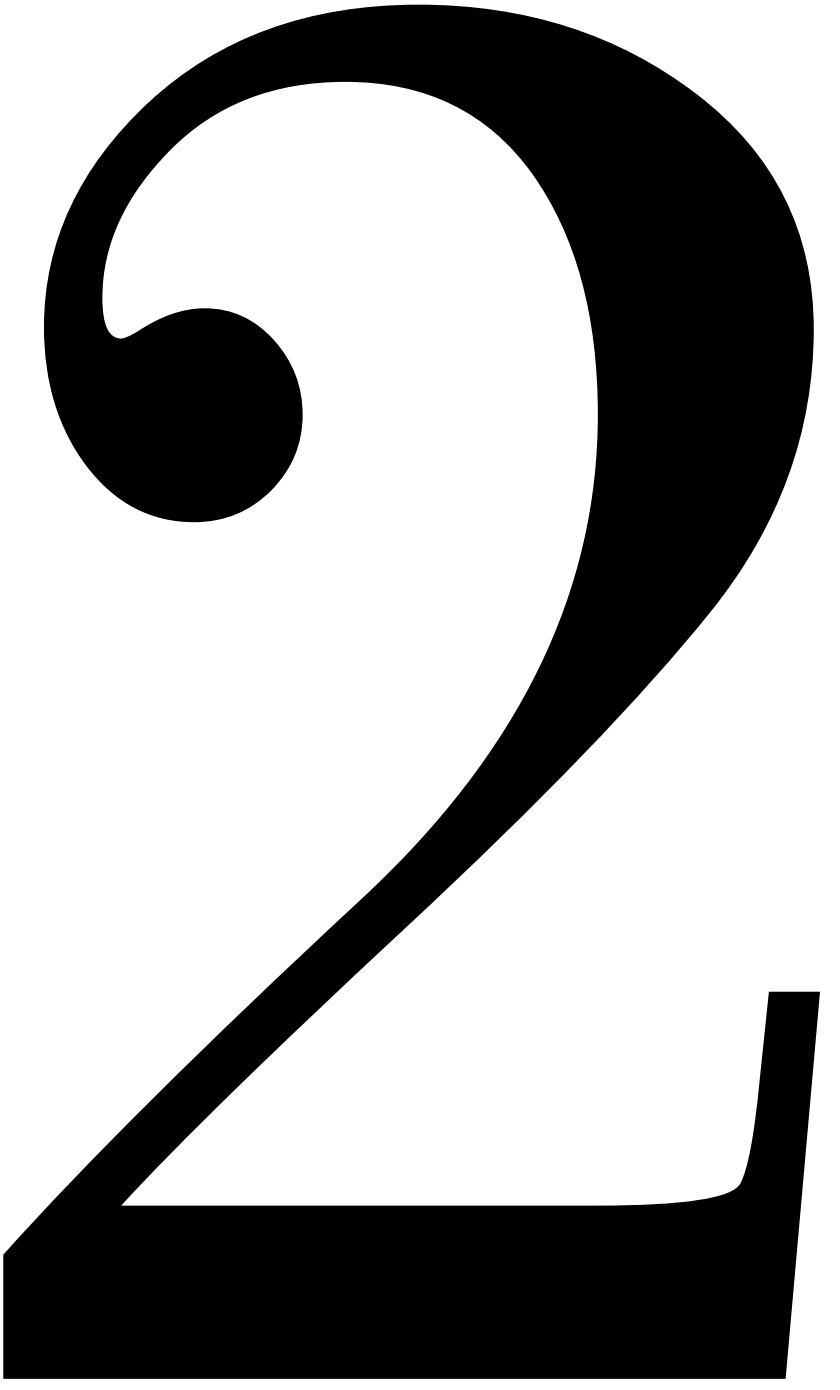
25. Smogorzewska, A. and T. de Lange, *Regulation of telomerase by telomeric proteins.* Annu Rev Biochem, 2004. **73**: p. 177-208.
26. de Lange, T., *Shelterin: the protein complex that shapes and safeguards human telomeres.* Genes Dev, 2005. **19**(18): p. 2100-10.
27. Chen, L.Y., D. Liu, and Z. Songyang, *Telomere maintenance through spatial control of telomeric proteins.* Mol Cell Biol, 2007. **27**(16): p. 5898-909.
28. Baumann, P. and T.R. Cech, *Pot1, the putative telomere end-binding protein in fission yeast and humans.* Science, 2001. **292**(5519): p. 1171-5.
29. Broccoli, D., et al., *Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2.* Nat Genet, 1997. **17**(2): p. 231-5.
30. Ancelin, K., et al., *Targeting assay to study the cis functions of human telomeric proteins: evidence for inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2.* Mol Cell Biol, 2002. **22**(10): p. 3474-87.
31. Doksani, Y., et al., *Super-resolution fluorescence imaging of telomeres reveals TRF2-dependent T-loop formation.* Cell, 2013. **155**(2): p. 345-356.
32. Loayza, D. and T. De Lange, *POT1 as a terminal transducer of TRF1 telomere length control.* Nature, 2003. **423**(6943): p. 1013-8.
33. O'Sullivan, R.J. and J. Karlseder, *Telomeres: protecting chromosomes against genome instability.* Nat Rev Mol Cell Biol, 2010. **11**(3): p. 171-81.
34. Palm, W. and T. de Lange, *How shelterin protects mammalian telomeres.* Annu Rev Genet, 2008. **42**: p. 301-34.
35. Denchi, E.L. and T. de Lange, *Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1.* Nature, 2007. **448**(7157): p. 1068-71.
36. Chan, C.S. and B.K. Tye, *Organization of DNA sequences and replication origins at yeast telomeres.* Cell, 1983. **33**(2): p. 563-73.
37. Gilson, E., et al., *Distortion of the DNA double helix by RAP1 at silencers and multiple telomeric binding sites.* J Mol Biol, 1993. **231**(2): p. 293-310.
38. Hardy, C.F., L. Sussel, and D. Shore, *A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation.* Genes Dev, 1992. **6**(5): p. 801-14.
39. Wotton, D. and D. Shore, *A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in Saccharomyces cerevisiae.* Genes Dev, 1997. **11**(6): p. 748-60.
40. Moretti, P., et al., *Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1.* Genes Dev, 1994. **8**(19): p. 2257-69.
41. Larcher, M.V., et al., *Ku Binding on Telomeres Occurs at Sites Distal from the Physical Chromosome Ends.* PLoS Genet, 2016. **12**(12): p. e1006479.
42. Polotnianka, R.M., J. Li, and A.J. Lustig, *The yeast Ku heterodimer is essential for protection of the telomere against nucleolytic and recombinational activities.* Curr Biol, 1998. **8**(14): p. 831-4.
43. DuBois, M.L., et al., *A quantitative assay for telomere protection in Saccharomyces cerevisiae.* Genetics, 2002. **161**(3): p. 995-1013.
44. Gravel, S., et al., *Yeast Ku as a regulator of chromosomal DNA end structure.* Science, 1998. **280**(5364): p. 741-4.
45. Mimitou, E.P. and L.S. Symington, *Ku prevents Exo1 and Sgs1-dependent resection of DNA ends in the absence of a functional MRX complex or Sae2.* EMBO J, 2010. **29**(19): p. 3358-69.
46. Bonetti, D., et al., *Shelterin-like proteins and Yku inhibit nucleolytic processing of Saccharomyces cerevisiae telomeres.* PLoS Genet, 2010. **6**(5): p. e1000966.
47. Lin, J.J. and V.A. Zakian, *The Saccharomyces CDC13 protein is a single-strand TG1-3 telomeric DNA-binding protein in vitro that affects telomere behavior in vivo.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13760-5.
48. Nugent, C.I., et al., *Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance.* Science, 1996. **274**(5285): p. 249-52.

49. Grandin, N., S.I. Reed, and M. Charbonneau, *Stn1, a new Saccharomyces cerevisiae protein, is implicated in telomere size regulation in association with Cdc13.* Genes Dev, 1997. **11**(4): p. 512-27.
50. Grandin, N., C. Damon, and M. Charbonneau, *Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13.* EMBO J, 2001. **20**(5): p. 1173-83.
51. Gao, H., et al., *RPA-like proteins mediate yeast telomere function.* Nat Struct Mol Biol, 2007. **14**(3): p. 208-14.
52. Garvik, B., M. Carson, and L. Hartwell, *Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint.* Mol Cell Biol, 1995. **15**(11): p. 6128-38.
53. Teng, S.C. and V.A. Zakian, *Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in Saccharomyces cerevisiae.* Mol Cell Biol, 1999. **19**(12): p. 8083-93.
54. Le, S., et al., *RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase.* Genetics, 1999. **152**(1): p. 143-52.
55. Chen, Q., A. Ijpma, and C.W. Greider, *Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events.* Mol Cell Biol, 2001. **21**(5): p. 1819-27.
56. Lydeard, J.R., et al., *Break-induced replication and telomerase-independent telomere maintenance require Pol32.* Nature, 2007. **448**(7155): p. 820-3.
57. Dewar, J.M. and D. Lydall, *Pif1- and Exo1-dependent nucleases coordinate checkpoint activation following telomere uncapping.* EMBO J, 2010. **29**(23): p. 4020-34.
58. Saini, N., et al., *Migrating bubble during break-induced replication drives conservative DNA synthesis.* Nature, 2013. **502**(7471): p. 389-92.
59. Wilson, M.A., et al., *Pif1 helicase and Polδ promote recombination-coupled DNA synthesis via bubble migration.* Nature, 2013. **502**(7471): p. 393-6.
60. Lebel, C., et al., *Telomere maintenance and survival in saccharomyces cerevisiae in the absence of telomerase and RAD52.* Genetics, 2009. **182**(3): p. 671-84.
61. McEachern, M.J. and J.E. Haber, *Break-induced replication and recombinational telomere elongation in yeast.* Annu Rev Biochem, 2006. **75**: p. 111-35.
62. Horowitz, H. and J.E. Haber, *Identification of autonomously replicating circular subtelomeric Y' elements in Saccharomyces cerevisiae.* Mol Cell Biol, 1985. **5**(9): p. 2369-80.
63. Larrivee, M. and R.J. Wellinger, *Telomerase- and capping-independent yeast survivors with alternate telomere states.* Nat Cell Biol, 2006. **8**(7): p. 741-7.
64. Yamada, M., et al., *Y'-Help1, a DNA helicase encoded by the yeast subtelomeric Y' element, is induced in survivors defective for telomerase.* J Biol Chem, 1998. **273**(50): p. 33360-6.
65. Krogh, B.O. and L.S. Symington, *Recombination proteins in yeast.* Annu Rev Genet, 2004. **38**: p. 233-71.
66. Ogawa, T., et al., *Similarity of the yeast RAD51 filament to the bacterial RecA filament.* Science, 1993. **259**(5103): p. 1896-9.
67. Sung, P., et al., *Rad51 recombinase and recombination mediators.* J Biol Chem, 2003. **278**(44): p. 42729-32.
68. Mankouri, H.W., H.P. Ngo, and I.D. Hickson, *Shu proteins promote the formation of homologous recombination intermediates that are processed by Sgs1-Rmi1-Top3.* Mol Biol Cell, 2007. **18**(10): p. 4062-73.
69. Sung, P., *Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase.* Genes Dev, 1997. **11**(9): p. 1111-21.
70. Hays, S.L., A.A. Firmenich, and P. Berg, *Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins.* Proc Natl Acad Sci U S A, 1995. **92**(15): p. 6925-9.

71. Godin, S., et al., *The Shu complex interacts with Rad51 through the Rad51 paralogues Rad55-Rad57 to mediate error-free recombination.* Nucleic Acids Res, 2013. **41**(8): p. 4525-34.
72. Mazina, O.M. and A.V. Mazin, *Human Rad54 protein stimulates DNA strand exchange activity of hRad51 protein in the presence of Ca2+.* J Biol Chem, 2004. **279**(50): p. 52042-51.
73. Raschle, M., et al., *Multiple interactions with the Rad51 recombinase govern the homologous recombination function of Rad54.* J Biol Chem, 2004. **279**(50): p. 51973-80.
74. Hu, Y., et al., *Telomerase-null survivor screening identifies novel telomere recombination regulators.* PLoS Genet, 2013. **9**(1): p. e1003208.
75. Teng, S.C., et al., *Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process.* Mol Cell, 2000. **6**(4): p. 947-52.
76. Huang, P., et al., *SGS1 is required for telomere elongation in the absence of telomerase.* Curr Biol, 2001. **11**(2): p. 125-9.
77. Johnson, F.B., et al., *The Saccharomyces cerevisiae WRN homolog Sgs1p participates in telomere maintenance in cells lacking telomerase.* EMBO J, 2001. **20**(4): p. 905-13.
78. Tsukamoto, Y., A.K. Taggart, and V.A. Zakian, *The role of the Mre11-Rad50-Xrs2 complex in telomerase- mediated lengthening of Saccharomyces cerevisiae telomeres.* Curr Biol, 2001. **11**(17): p. 1328-35.
79. Longhese, M.P., et al., *Mechanisms and regulation of DNA end resection.* EMBO J, 2010. **29**(17): p. 2864-74.
80. Bachrati, C.Z. and I.D. Hickson, *RecQ helicases: suppressors of tumorigenesis and premature aging.* Biochem J, 2003. **374**(Pt 3): p. 577-606.
81. Gangloff, S., et al., *The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a potential eukaryotic reverse gyrase.* Mol Cell Biol, 1994. **14**(12): p. 8391-8.
82. Mimitou, E.P. and L.S. Symington, *Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing.* Nature, 2008. **455**(7214): p. 770-4.
83. Zhu, Z., et al., *Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends.* Cell, 2008. **134**(6): p. 981-94.
84. Maringele, L. and D. Lydall, *EXO1 plays a role in generating type I and type II survivors in budding yeast.* Genetics, 2004. **166**(4): p. 1641-9.
85. Bertuch, A.A. and V. Lundblad, *EXO1 contributes to telomere maintenance in both telomerase-proficient and telomerase-deficient Saccharomyces cerevisiae.* Genetics, 2004. **166**(4): p. 1651-9.
86. Costelloe, T., et al., *The yeast Fun30 and human SMARCAD1 chromatin remodellers promote DNA end resection.* Nature, 2012. **489**(7417): p. 581-4.
87. Chen, Y.B., et al., *Def1p is involved in telomere maintenance in budding yeast.* J Biol Chem, 2005. **280**(26): p. 24784-91.
88. Grandin, N. and M. Charbonneau, *Mitotic cyclins regulate telomeric recombination in telomerase-deficient yeast cells.* Mol Cell Biol, 2003. **23**(24): p. 9162-77.
89. Meng, F.L., et al., *Sua5p is required for telomere recombination in Saccharomyces cerevisiae.* Cell Res, 2010. **20**(4): p. 495-8.
90. Pike, B.L. and J. Heierhorst, *Mdt1 facilitates efficient repair of blocked DNA double-strand breaks and recombinational maintenance of telomeres.* Mol Cell Biol, 2007. **27**(18): p. 6532-45.
91. Tsai, Y.L., et al., *Involvement of replicative polymerases, Tel1p, Mec1p, Cdc13p, and the Ku complex in telomere-telomere recombination.* Mol Cell Biol, 2002. **22**(16): p. 5679-87.
92. Chang, M., J.C. Dittmar, and R. Rothstein, *Long telomeres are preferentially extended during recombination-mediated telomere maintenance.* Nat Struct Mol Biol, 2011. **18**(4): p. 451-6.
93. Anand, R.P., S.T. Lovett, and J.E. Haber, *Break-induced DNA replication.* Cold Spring Harb Perspect Biol, 2013. **5**(12): p. a010397.
94. Louis, E.J., *Corrected sequence for the right telomere of Saccharomyces cerevisiae chromosome III.* Yeast, 1994. **10**(2): p. 271-4.

95. McEachern, M.J., D.H. Underwood, and E.H. Blackburn, *Dynamics of telomeric DNA turnover in yeast.* Genetics, 2002. **160**(1): p. 63-73.
96. Cesare, A.J. and J.D. Griffith, *Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops.* Mol Cell Biol, 2004. **24**(22): p. 9948-57.
97. Wang, H.M., Y.F. Xia, and X.Y. Wu, *[ATM and telomere instability].* Yi Chuan Xue Bao, 2004. **31**(2): p. 212-6.
98. Groff-Vindman, C., et al., *Recombination at long mutant telomeres produces tiny single- and double-stranded telomeric circles.* Mol Cell Biol, 2005. **25**(11): p. 4406-12.
99. Lin, C.Y., et al., *Extrachromosomal telomeric circles contribute to Rad52-, Rad50-, and polymerase delta-mediated telomere-telomere recombination in Saccharomyces cerevisiae.* Eukaryot Cell, 2005. **4**(2): p. 327-36.
100. Davis, A.P. and L.S. Symington, *RAD51-dependent break-induced replication in yeast.* Mol Cell Biol, 2004. **24**(6): p. 2344-51.
101. Malkova, A., et al., *RAD51-dependent break-induced replication differs in kinetics and checkpoint responses from RAD51-mediated gene conversion.* Mol Cell Biol, 2005. **25**(3): p. 933-44.
102. Murnane, J.P., et al., *Telomere dynamics in an immortal human cell line.* EMBO J, 1994. **13**(20): p. 4953-62.
103. Bryan, T.M., et al., *Telomere elongation in immortal human cells without detectable telomerase activity.* EMBO J, 1995. **14**(17): p. 4240-8.
104. Nabetani, A. and F. Ishikawa, *Unusual telomeric DNAs in human telomerase-negative immortalized cells.* Mol Cell Biol, 2009. **29**(3): p. 703-13.
105. Londono-Vallejo, J.A., et al., *Alternative lengthening of telomeres is characterized by high rates of telomeric exchange.* Cancer Res, 2004. **64**(7): p. 2324-7.
106. Yeager, T.R., et al., *Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body.* Cancer Res, 1999. **59**(17): p. 4175-9.
107. Nittis, T., L. Guittat, and S.A. Stewart, *Alternative lengthening of telomeres (ALT) and chromatin: is there a connection?* Biochimie, 2008. **90**(1): p. 5-12.
108. Azzalin, C.M., et al., *Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends.* Science, 2007. **318**(5851): p. 798-801.
109. Benetti, R., et al., *Role of TRF2 in the assembly of telomeric chromatin.* Cell Cycle, 2008. **7**(21): p. 3461-8.
110. Schoeftner, S. and M.A. Blasco, *Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II.* Nat Cell Biol, 2008. **10**(2): p. 228-36.
111. Balk, B., et al., *Telomeric RNA-DNA hybrids affect telomere-length dynamics and senescence.* Nat Struct Mol Biol, 2013. **20**(10): p. 1199-205.
112. Lovejoy, C.A., et al., *Loss of ATRX, genome instability, and an altered DNA damage response are hallmarks of the alternative lengthening of telomeres pathway.* PLoS Genet, 2012. **8**(7): p. e1002772.
113. Episkopou, H., et al., *Alternative Lengthening of Telomeres is characterized by reduced compaction of telomeric chromatin.* Nucleic Acids Res, 2014. **42**(7): p. 4391-405.
114. Jiang, W.Q., et al., *Suppression of alternative lengthening of telomeres by Sp100-mediated sequestration of the MRE11/RAD50/NBS1 complex.* Mol Cell Biol, 2005. **25**(7): p. 2708-21.
115. Bhattacharyya, S., et al., *Telomerase-associated protein 1, HSP90, and topoisomerase IIalpha associate directly with the BLM helicase in immortalized cells using ALT and modulate its helicase activity using telomeric DNA substrates.* J Biol Chem, 2009. **284**(22): p. 14966-77.
116. Wang, R.C., A. Smogorzewska, and T. de Lange, *Homologous recombination generates T-loop-sized deletions at human telomeres.* Cell, 2004. **119**(3): p. 355-68.
117. Henson, J.D., et al., *DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity.* Nat Biotechnol, 2009. **27**(12): p. 1181-5.

118. Fasching, C.L., K. Bower, and R.R. Reddel, *Telomerase-independent telomere length maintenance in the absence of alternative lengthening of telomeres-associated promyelocytic leukemia bodies.* Cancer Res, 2005. **65**(7): p. 2722-9.
119. Marciniak, R.A., et al., *A novel telomere structure in a human alternative lengthening of telomeres cell line.* Cancer Res, 2005. **65**(7): p. 2730-7.
120. Heaphy, C.M., et al., *Altered telomeres in tumors with ATRX and DAXX mutations.* Science, 2011. **333**(6041): p. 425.
121. Wu, G., et al., *Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas.* Nat Genet, 2012. **44**(3): p. 251-3.
122. Drane, P., et al., *The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3.* Genes Dev, 2010. **24**(12): p. 1253-65.
123. Lewis, P.W., et al., *Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres.* Proc Natl Acad Sci U S A, 2010. **107**(32): p. 14075-80.
124. Wong, L.H., et al., *ATRX interacts with H3.3 in maintaining telomere structural integrity in pluripotent embryonic stem cells.* Genome Res, 2010. **20**(3): p. 351-60.
125. Apte, M.S. and J.P. Cooper, *Life and cancer without telomerase: ALT and other strategies for making sure ends (don't) meet.* Crit Rev Biochem Mol Biol, 2017. **52**(1): p. 57-73.
126. O'Sullivan, R.J., et al., *Rapid induction of alternative lengthening of telomeres by depletion of the histone chaperone ASF1.* Nat Struct Mol Biol, 2014. **21**(2): p. 167-74.
127. Dilley, R.L., et al., *Break-induced telomere synthesis underlies alternative telomere maintenance.* Nature, 2016. **539**(7627): p. 54-58.



Chapter 2

Recombination-mediated telomere maintenance in *Saccharomyces cerevisiae* is not dependent on the Shu complex

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Abstract

In cells lacking telomerase, telomeres shorten progressively during each cell division due to incomplete end-replication. When the telomeres become very short, cells enter a state that blocks cell division, termed senescence. A subset of these cells can overcome senescence and maintain their telomeres using telomerase-independent mechanisms. In *Saccharomyces cerevisiae*, these cells are called 'survivors' and are dependent on Rad52-dependent homologous recombination and Pol32-dependent break-induced replication. There are two main types of survivors: type I and type II. The type I survivors require Rad51 and maintain telomeres by amplification of subtelomeric elements, while the type II survivors are Rad51-independent, but require the MRX complex and Sgs1 to amplify the $C_{1-3}A/TG_{1-3}$ telomeric sequences. Rad52, Pol32, Rad51, and Sgs1 are also important to prevent accelerated senescence, indicating that recombination processes are important at telomeres even before the formation of survivors. The Shu complex, which consists of Shu1, Shu2, Psy3, and Csm2, promotes Rad51-dependent homologous recombination and has been suggested to be important for break-induced replication. It also promotes the formation of recombination intermediates that are processed by the Sgs1-Top3-Rmi1 complex, as mutations in the *SHU* genes can suppress various *sgs1*, *top3*, and *rmi1* mutant phenotypes. Given the importance of recombination processes during senescence and survivor formation, and the involvement of the Shu complex in many of the same processes during DNA repair, we hypothesized that the Shu complex may also have functions at telomeres. Surprisingly, we find that this is not the case: the Shu complex does not affect the rate of senescence, does not influence survivor formation, and deletion of *SHU1* does not suppress the rapid senescence and type II survivor formation defect of a telomerase-negative *sgs1* mutant. Altogether, our data suggest that the Shu complex is not important for recombination processes at telomeres.

Introduction

Telomeres are nucleoprotein structures at the ends of linear chromosomes that help a cell distinguish a natural chromosome end from a DNA double-strand break (DSB) [1]. In *Saccharomyces cerevisiae*, the telomeric DNA consists of 300 ± 75 bp of $C_{1-3}A/TG_{1-3}$ repetitive sequences, with the G-rich strand extending to form a 3′ single-stranded overhang [2]. The subtelomeric regions also contain middle repetitive X and Y′ elements. An X element is found at all chromosome ends, while the Y′ elements are found in zero to four tandem copies between an X element and the terminal telomeric repeats [3]. Telomeres are maintained by a specialized reverse transcriptase called telomerase, whose core subunits are a catalytic protein component (Est2) and an RNA subunit (TLC1), which can extend telomeres by adding TG_{1-3} repeats to the 3′ overhang [4, 5]. In cells lacking telomerase, telomeres shorten progressively during each cell division due to incomplete end-replication and nucleolytic degradation [6]. When the telomeres become very short, cells enter a state that blocks cell division, termed senescence. A subset of these cells can overcome senescence and maintain their telomeres using recombination-based processes, becoming 'survivors' [7]. There are two main types of survivors: type I and type II. Both types require Rad52-dependent homologous recombination (HR). Type I survivors also require Rad51, Rad54, and Rad57, and maintain telomeres by amplification of subtelomeric Y′ elements [7, 8]. Formation of type II survivors, which exhibit amplification of the $C_{1-3}A/TG_{1-3}$ sequences, is Rad51-independent, but requires the MRX complex (Mre11, Rad50, and Xrs2), Rad59, and Sgs1 [8-11]. Both types of survivors also require the DNA polymerase δ subunit Pol32, which is required for break-induced replication (BIR) [12]. BIR can be Rad51-dependent or Rad51-independent, suggesting that type I and type II survivors maintain telomeres through Rad51-dependent BIR and Rad51-independent BIR, respectively [13, 14]. Telomerase-negative cells lacking Rad52, Rad51, Rad54, Rad57, Sgs1, or Pol32 also senesce very rapidly, indicating that these proteins are important at telomeres even before the emergence of survivors [7, 10, 11, 15, 16].

The Shu complex, which consists of Shu1, Shu2, Psy3, and Csm2, interacts indirectly with Rad51 through the Rad51 paralogues Rad55-Rad57 to stimulate Rad51 filament attachment to the single-stranded DNA, which is essential for the homology recognition and strand invasion steps of HR [17-19]. When any of these four genes are deleted, a higher rate of mutations and increased number of genome rearrangements are observed [20, 21]. The Shu complex also promotes the formation of recombination intermediates that are processed by the Sgs1-Top3-

Rmi1 complex, as mutations in the *SHU* genes can suppress various *sgs1*, *top3*, and *rmi1* mutant phenotypes [21, 22].

Given the role of the Shu complex in recombination-mediated processes, and the role of recombination proteins in senescence and survivor formation [23], we hypothesized that the Shu complex also functions during senescence and survivor formation. Surprisingly, we find that the Shu complex affects neither the rate of senescence nor survivor formation significantly. Furthermore, the deletion of *SHU1* does not suppress the rapid senescence and type II survivor formation defect of a telomerase-negative *sgs1Δ* mutant. Taken together, our findings suggest that the Shu complex does not normally function in recombination-mediated processes at telomeres.

Materials and methods

Yeast strains and growth conditions

Standard yeast media and growth conditions were used [24, 25]. Strains used in this study are listed in Table 1 and all are *RAD5* derivatives of W303 (*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5*) [26, 27].

Table 1. Yeast strains used in this study.

Strain name	Relevant genotype
MCY574	*MAT***a**/α *est2ΔURA3/EST2 shu1ΔHIS3/SHU1*
MCY575	*MAT***a**/α *tlc1ΔHIS3/TLC1 shu2ΔURA3/SHU2*
MCY576	*MAT***a**/α *tlc1ΔHIS3/TLC1 psy3ΔkanMX/PSY3*
MCY577	*MAT***a**/α *tlc1ΔHIS3/TLC1 csm2ΔkanMX/CSM2*
YPM1	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔkanMX/RAD51 shu1ΔHIS3/SHU1*
YPM2	*MAT***a**/α *tlc1ΔHIS3/TLC1 rad51ΔkanMX/RAD51 shu2ΔURA3/SHU2*
YPM3	*MAT***a**/α *est2ΔURA3/EST2 rad59ΔkanMX/RAD59 shu1ΔHIS3/SHU1*
YPM4	*MAT***a**/α *tlc1ΔHIS3/TLC1 rad59ΔkanMX/RAD59 shu2ΔURA3/SHU2*
YPM5	*MAT***a**/α *est2ΔURA3/EST2 sgs1ΔnatMX/SGS1 shu1ΔHIS3/SHU1*

Liquid culture senescence assay

Senescence assays in liquid culture were performed essentially as previously described [28, 29]. Each senescence assay started with *est2Δ/EST2* or *tlc1Δ/TLC1* heterozygous diploids that were propagated for at least 50 generations before sporulation to ensure that telomeres were at a stable equilibrium length. Freshly dissected

spores were allowed to form colonies on YPD agar plates after 2 days of growth at 30°C. Cells from these colonies were serially passaged in liquid YPD medium at 24-h intervals. For each passage, the cell density of each culture was measured and the cultures were diluted back into fresh YPD medium at a cell density of 2 x 10^5 cells/ml. Senescence was plotted with respect to population doublings (PDs). PD was used as a metric rather than time (e.g. days in culture) because senescence caused by telomere shortening is related to cell division, not time. In addition, the use of PDs prevents mutations that only alter the rate of cell division from being mistakenly interpreted as having an effect on the rate of senescence.

Generation of survivors on agar plates

Diploids were propagated and sporulated as in the liquid culture senescence assays. Cells from freshly dissected spores were streaked on YPD plates and grown at 30°C for 3 days. Individual colonies were restreaked for 5–6 times to allow for survivor generation.

Telomere PCR and telomere length measurements

Yeast genomic DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega). Y′ telomeres and telomere VI-R were amplified by PCR as previously described [30, 31]. Telomere PCR products were separated by agarose gel electrophoresis and average telomere length was determined as previously described [32].

Telomere genomic blot

Genomic DNA was isolated, digested with *Xho*I, separated on a 1% (w/v) agarose gel, and transferred to a Hybond-N^+ membrane (GE Healthcare). The membrane was then hybridized to a telomere-specific (5′-CACCACACCCACACACCACACCCACA-3′) digoxigenin-labeled probe.

Results and discussion

The Shu complex does not affect senescence or survivor formation

To investigate whether the Shu complex plays a role during the process of senescence and in the formation of survivors in telomerase-negative cells, we first performed liquid culture senescence assays. Diploid strains that are deleted for one copy

of either *EST2* or *TLC1* and also one copy of one of the four *SHU* genes were sporulated and the haploid progeny were propagated in liquid culture for several days (see Materials and Methods). In each case, the rate of senescence and survivor formation of *est2Δ* or *tlc1Δ* mutants was not affected by deletion of any of the *SHU* genes (Figure 1). Since all four *shu* mutants behaved similarly, subsequent experiments were performed with only one or two *shu* mutants.

We next determined whether the Shu complex influences telomere length homeostasis or telomere shortening in the absence of telomerase. We measured the telomere length of wild type, *shu1Δ*, *est2Δ*, and *est2Δ shu1Δ* haploid strains approximately 35 generations after the sporulation of an *est2Δ/EST2 shu1Δ/SHU1* diploid. Deletion of *SHU1* did not affect either telomere length homeostasis of telomerase-positive cells or the telomere shortening rate of *est2Δ* cells (Figure 2).

Although our liquid culture senescence assays revealed that telomerase-negative *shu* mutants could form survivors (Figure 1), we wished to determine whether both

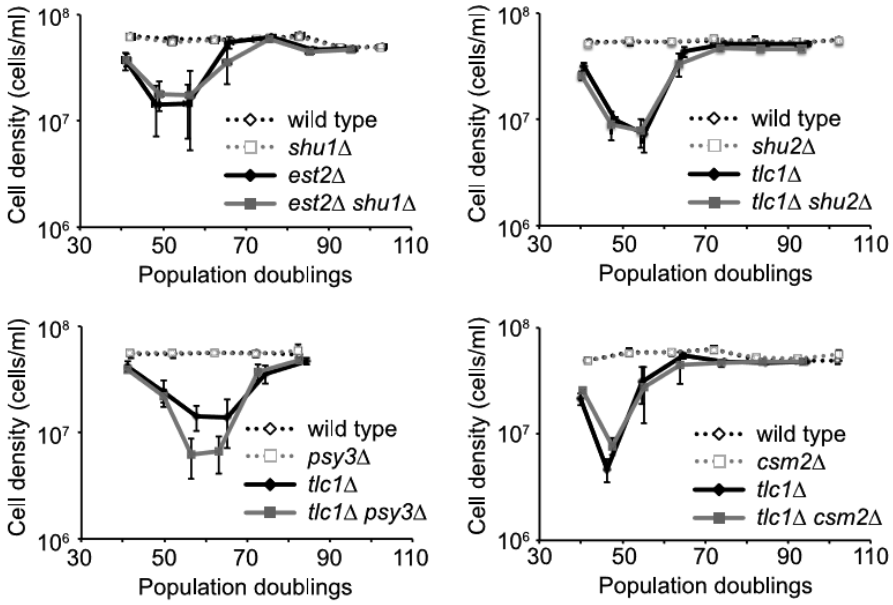

Figure 1. The Shu complex does not influence the rate of senescence or survivor formation. *est2Δ/EST2 shu1Δ/SHU1* (top left), *tlc1Δ/TLC1 shu2Δ/SHU2* (top right), *tlc1Δ/TLC1 psy3Δ/PSY3* (bottom left), and *tlc1Δ/TLC1 csm2Δ/CSM2* (bottom right) diploid strains were sporulated to generate the indicated haploid strains, which were subjected to a liquid culture senescence assay as described in the Materials and Methods. For each experiment, 2–3 isolates of each telomerase-positive strain and 4–5 isolates of each telomerase-negative strain were followed. The mean cell densities and standard errors of the means are shown.

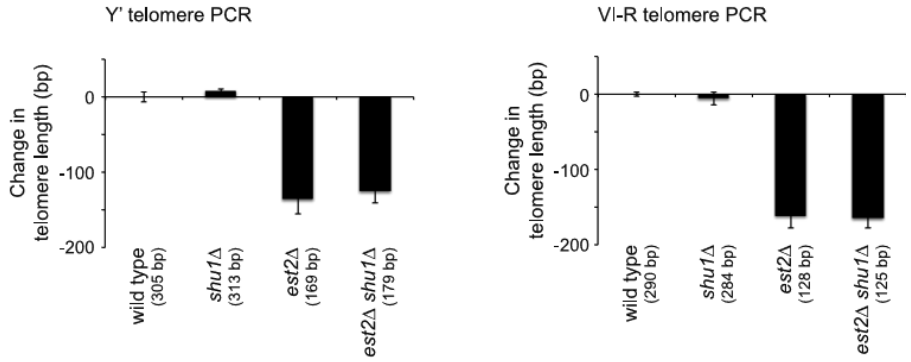

Figure 2. Deletion of *SHU1* does not affect telomere length in the presence or absence of telomerase.

Strains of the indicated genotypes, generated from the sporulation of an *est2Δ/EST2 shu1Δ/SHU1* diploid, were assayed for telomere length by Y′ and VI-R telomere PCR after being passaged for approximately 35 generations. The change in telomere length, compared to wild-type telomere length, was quantified and plotted. Mean ± standard error for 3–4 independent isolates for each genotype are shown. Raw mean telomere length values are given in parentheses.

types of survivors could be formed. We constructed *est2Δ rad51Δ shu1Δ*, *tlc1Δ rad51Δ shu2Δ*, *est2Δ rad59Δ shu1Δ*, and *tlc1Δ rad59Δ shu2Δ* strains and passaged them several times on solid medium. Since Rad51 is required for the growth of type I survivors [8], we can test whether deletion of *SHU1* or *SHU2* prevents type II survivor formation in a *rad51Δ* background. Likewise, since Rad59 is required for the growth of type II survivors [8], we can test whether deletion of *SHU1* or *SHU2* prevents type I survivor formation in a *rad59Δ* background. All mutants were able to recover from senescence and form survivors (data not shown), indicating that neither type I nor type II survivors depend on the Shu complex for their formation.

To further validate that the Shu complex does not affect type I or type II survivor formation, we analyzed by genomic blot the telomeres of *est2Δ* and *est2Δ shu1Δ* survivors generated by serial passaging on solid medium after the sporulation of an *est2Δ/EST2 shu1Δ/SHU1* diploid strain. 71 *est2Δ* single mutants and 69 *est2Δ shu1Δ* double mutants were followed. Both *est2Δ* and *est2Δ shu1Δ* survivors were able to form type I and type II survivors, and for both genotypes, type I survivors were more abundant (Table 2), as previously reported [9, 33]. We did observe a small increase in type II survivor formation in the absence of *SHU1*, but this effect is not statistically significant ($X^2 = 1.49$, $P = 0.11$). Thus, we conclude that the Shu complex does not play a major role in type I or type II survivor formation.

Table 2. Type II survivor frequencies in *est2Δ* and *est2Δ shu1Δ* cells.

Genotype	Type II frequency
est2Δ	5.6% (4/71)
est2Δ shu1Δ	13.0% (9/69)

Deletion of SHU1 does not rescue the rapid senescence and type II survivor formation defect in est2Δ sgs1Δ cells

Telomerase-negative cells lacking Sgs1 senesce rapidly and fail to form type II survivors [10, 11]. Since mutations in *SHU* genes can rescue various aspects of the sgs1 mutant phenotype [21], we investigated whether the rapid senescence and type II survivor formation defect of telomerase-negative *sgs1Δ* mutants could be rescued by the deletion of *SHU1*. An *est2Δ/EST2 sgs1Δ/SGS1 shu1Δ/SHU1* diploid was sporulated to generate haploid meiotic progeny that were followed in a liquid culture senescence assay. The *est2Δ sgs1Δ* and *est2Δ sgs1Δ shu1Δ* mutants senesce at the same rate, and faster than an *est2Δ* single mutant (Figure 3A). The telomeres of the survivors were also analyzed by genomic blotting (Figure 3B). Type I survivors exhibit short telomeres and strong hybridization at 5.2 kb and 6.7 kb, which is due to amplification of the tandemly repeated Y′ short and Y′ long elements, respectively. The telomeres of type II survivor are extended and very heterogeneous in size. Since type II survivors grow much better than type I survivors, they outcompete the type I survivors in a liquid culture senescence assay [9, 33]. Thus, all *est2Δ* and *est2Δ shu1Δ* survivors generated this way are type II. The *est2Δ sgs1Δ* strains formed only type I survivors, as expected because deletion of *SGS1* prevents type II survivor formation [10, 11]. Deletion of *SHU1* did not rescue the inability of *est2Δ sgs1Δ* mutants to form type II survivors. Taken together, these results indicate that the Shu complex does not function upstream of Sgs1 with regards to senescence and survivor formation.

Overall, our findings indicate that the Shu complex does not play an important role during senescence and survivor formation. This result is surprising given the role of recombination proteins in these processes. In particular, the Shu complex is known to promote Rad51 filament formation [17-19], and Rad51 is needed to prevent rapid senescence and for type I survivor formation [8, 15], but telomerase-negative *shu* mutants do not show a similar phenotype (Figure 1 and Table 2). However, *shu* mutants are much less sensitive to DNA damaging agents than *rad51Δ* and *rad52Δ* mutants. In addition, spontaneous Rad51 focus formation is only down twofold in a

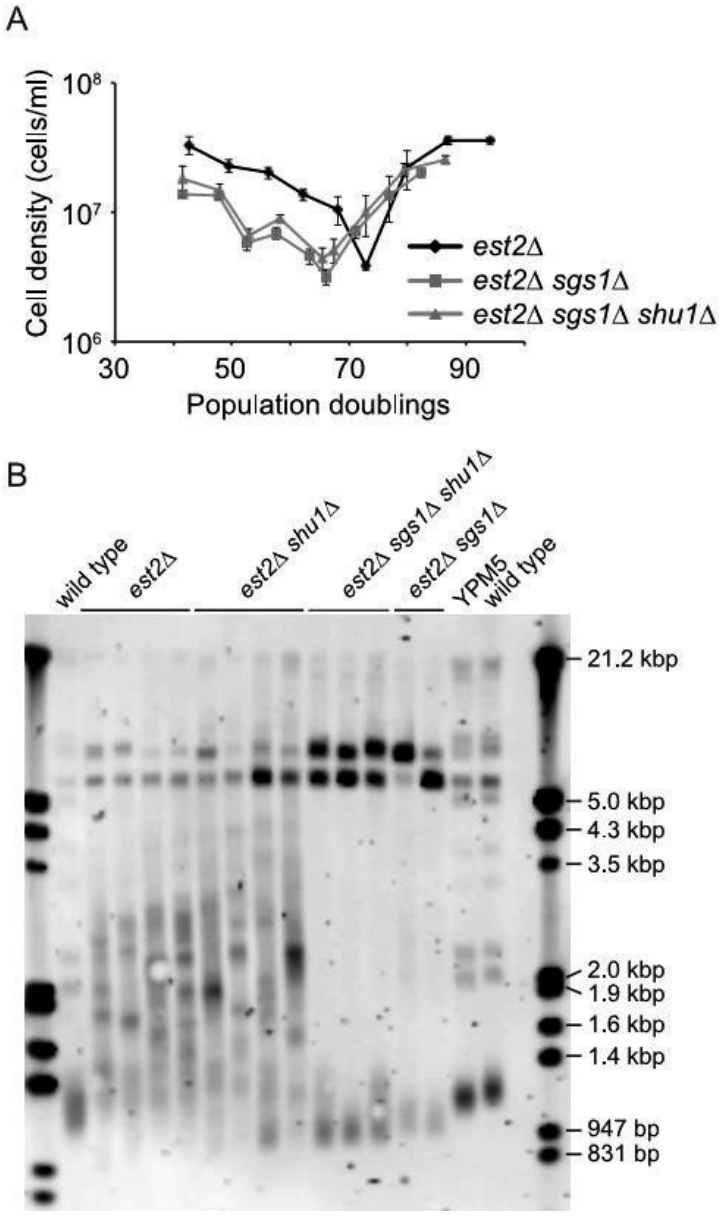

Figure 3. Rapid senescence and type II survivor formation defect of *est2Δ sgs1Δ* cells are not rescued by deletion of *SHU1*.

(A) Strains for the indicated genotypes, generated from the sporulation of an *est2Δ/EST2 sgs1Δ/SGS1 shu1Δ/SHU1* (YPM5) diploid, were subjected to a liquid culture senescence assay. (B) A telomere genomic blot was performed on genomic DNA from strains of the indicated genotypes. The *est2Δ*, *est2Δ shu1Δ*, *est2Δ sgs1Δ shu1Δ*, *est2Δ sgs1Δ* strains were first passaged for 8 days in a liquid culture senescence assay to generate survivors. A haploid wild-type strain is included (on both sides of the blot), along with the YPM5 diploid.

shu1Δ strain [34], and while the Shu complex stimulates the loading of Rad51 onto RPA-coated single-stranded DNA in vitro, it is not absolutely required [19]. Thus, in the absence of the Shu complex, suboptimal Rad51 filament formation may be sufficient to delay senescence and promote survivor formation in telomerase-null cells. Nevertheless, it has recently been observed that the deletion of *PSY3* partially suppresses telomere elongation in *cdc9-1* mutants [35], indicating that the Shu complex may have a role at telomeres in certain situations.

Our work raises intriguing questions about what substrates the Shu complex acts on. It has been suggested that the Shu complex functions in BIR [35, 36]. If so, it would be interesting to determine why it does not apparently affect BIR-mediated survivor formation. Of course, cells may regulate BIR differently at telomeres than at DSBs. Alternatively, telomeres resemble one-ended DSBs, and the Shu complex may only function when both ends of a DSB are present. If this is the case, it will be interesting to figure out how the Shu complex differentiates between one-ended and two-ended DSBs. Finally, while the role of recombination in telomerase-independent telomere maintenance is clear, it is much less obvious why recombination proteins are needed to prevent accelerated senescence. The discovery that the Shu complex is not important during senescence implies that only some recombination activities are important, which adds another piece to solving this puzzle.

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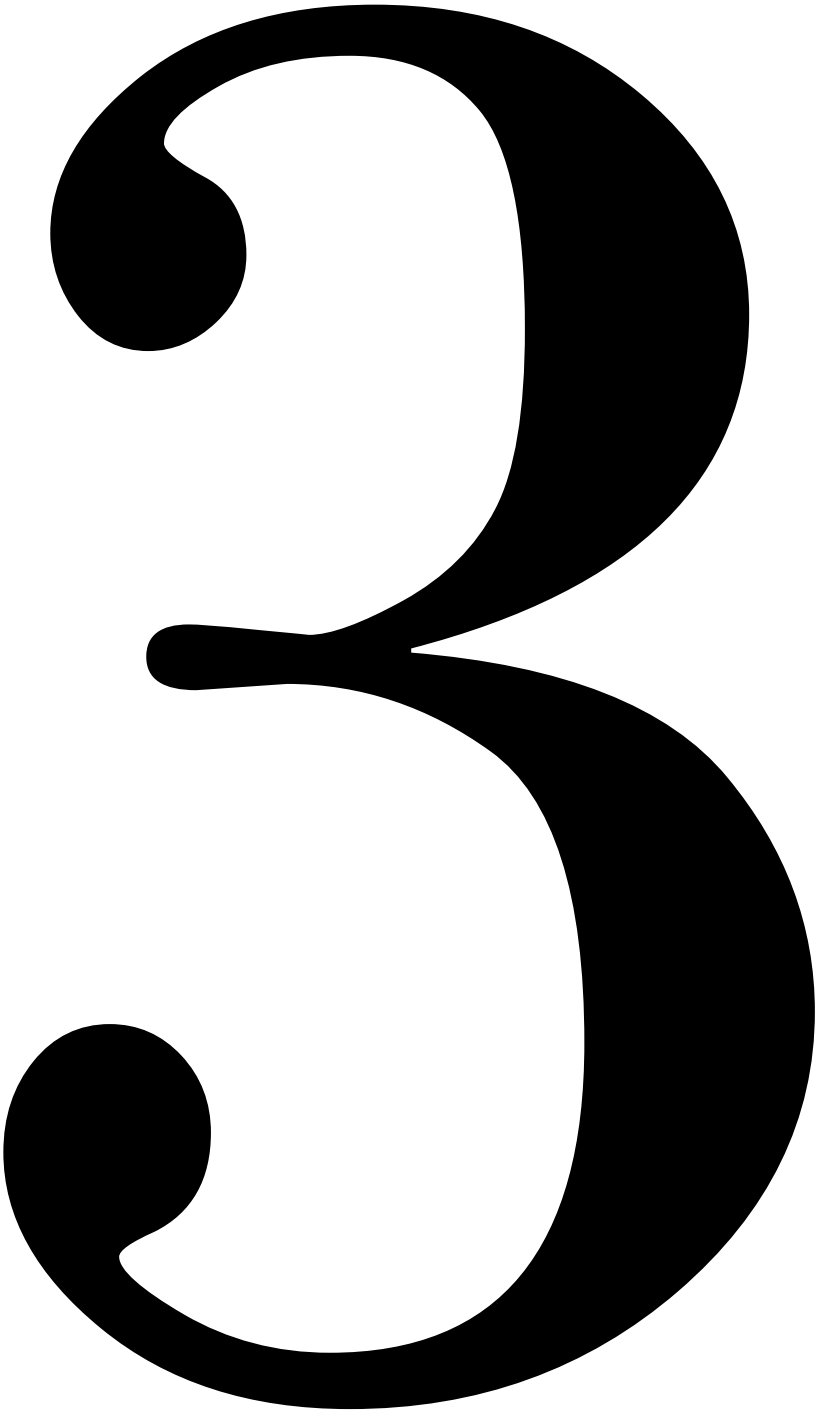
Author contributions

Conceived and designed the experiments: MC. Performed the experiments: PMM JJ DA CC MC. Analyzed the data: PMM JJ DA CC RR MC. Contributed reagents/materials/analysis tools: PMM MC. Wrote the paper: PMM MC.

References

1. de Lange, T., *How telomeres solve the end-protection problem.* Science, 2009. **326**(5955): p. 948-52.
2. Wellinger, R.J. and V.A. Zakian, *Everything you ever wanted to know about Saccharomyces cerevisiae telomeres: beginning to end.* Genetics, 2012. **191**(4): p. 1073-105.
3. Chan, C.S. and B.K. Tye, *Organization of DNA sequences and replication origins at yeast telomeres.* Cell, 1983. **33**(2): p. 563-73.
4. Lingner, J., et al., *Three Ever Shorter Telomere (EST) genes are dispensable for in vitro yeast telomerase activity.* Proc Natl Acad Sci U S A, 1997. **94**(21): p. 11190-5.
5. Singer, M.S. and D.E. Gottschling, *TLC1: template RNA component of Saccharomyces cerevisiae telomerase.* Science, 1994. **266**(5184): p. 404-9.
6. Lundblad, V. and J.W. Szostak, *A mutant with a defect in telomere elongation leads to senescence in yeast.* Cell, 1989. **57**(4): p. 633-43.
7. Lundblad, V. and E.H. Blackburn, *An alternative pathway for yeast telomere maintenance rescues est1- senescence.* Cell, 1993. **73**(2): p. 347-60.
8. Chen, Q., A. Ijpma, and C.W. Greider, *Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events.* Mol Cell Biol, 2001. **21**(5): p. 1819-27.
9. Teng, S.C., et al., *Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process.* Mol Cell, 2000. **6**(4): p. 947-52.
10. Huang, P., et al., *SGS1 is required for telomere elongation in the absence of telomerase.* Curr Biol, 2001. **11**(2): p. 125-9.
11. Johnson, F.B., et al., *The Saccharomyces cerevisiae WRN homolog Sgs1p participates in telomere maintenance in cells lacking telomerase.* EMBO J, 2001. **20**(4): p. 905-13.
12. Lydeard, J.R., et al., *Break-induced replication and telomerase-independent telomere maintenance require Pol32.* Nature, 2007. **448**(7155): p. 820-3.
13. Davis, A.P. and L.S. Symington, *RAD51-dependent break-induced replication in yeast.* Mol Cell Biol, 2004. **24**(6): p. 2344-51.
14. Signon, L., et al., *Genetic requirements for RAD51- and RAD54-independent break-induced replication repair of a chromosomal double-strand break.* Mol Cell Biol, 2001. **21**(6): p. 2048-56.
15. Le, S., et al., *RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase.* Genetics, 1999. **152**(1): p. 143-52.
16. Fallet, E., et al., *Length-dependent processing of telomeres in the absence of telomerase.* Nucleic Acids Res, 2014. **42**(6): p. 3648-65.
17. Godin, S., et al., *The Shu complex interacts with Rad51 through the Rad51 paralogues Rad55-Rad57 to mediate error-free recombination.* Nucleic Acids Res, 2013. **41**(8): p. 4525-34.
18. Sasanuma, H., et al., *A new protein complex promoting the assembly of Rad51 filaments.* Nat Commun, 2013. **4**: p. 1676.
19. Gaines, W.A., et al., *Promotion of presynaptic filament assembly by the ensemble of S. cerevisiae Rad51 paralogues with Rad52.* Nat Commun, 2015. **6**: p. 7834.
20. Huang, M.E., et al., *A genomewide screen in Saccharomyces cerevisiae for genes that suppress the accumulation of mutations.* Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11529-34.
21. Shor, E., J. Weinstein, and R. Rothstein, *A genetic screen for top3 suppressors in Saccharomyces cerevisiae identifies SHU1, SHU2, PSY3 and CSM2: four genes involved in error-free DNA repair.* Genetics, 2005. **169**(3): p. 1275-89.
22. Mankouri, H.W., H.P. Ngo, and I.D. Hickson, *Shu proteins promote the formation of homologous recombination intermediates that are processed by Sgs1-Rmi1-Top3.* Mol Biol Cell, 2007. **18**(10): p. 4062-73.

23. Claussin, C. and M. Chang, *The many facets of homologous recombination at telomeres.* Microbial Cell, 2015. **2**(9): p. 308-321.
24. Treco, D.A. and V. Lundblad, *Preparation of yeast media.* Curr Protoc Mol Biol, 2001. **Chapter 13**: p. Unit13 1.
25. Sherman, F., *Getting started with yeast.* Methods Enzymol, 2002. **350**: p. 3-41.
26. Thomas, B.J. and R. Rothstein, *Elevated recombination rates in transcriptionally active DNA.* Cell, 1989. **56**(4): p. 619-30.
27. Zhao, X., E.G. Muller, and R. Rothstein, *A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools.* Mol Cell, 1998. **2**(3): p. 329-40.
28. Chang, M., J.C. Dittmar, and R. Rothstein, *Long telomeres are preferentially extended during recombination-mediated telomere maintenance.* Nat Struct Mol Biol, 2011. **18**(4): p. 451-6.
29. Chang, M. and R. Rothstein, *Rif1/2 and Tel1 function in separate pathways during replicative senescence.* Cell Cycle, 2011. **10**(21): p. 3798-9.
30. Poschke, H., et al., *Rif2 promotes a telomere fold-back structure through Rpd3L recruitment in budding yeast.* PLoS Genet, 2012. **8**(9): p. e1002960.
31. Chang, M., M. Arneric, and J. Lingner, *Telomerase repeat addition processivity is increased at critically short telomeres in a Tel1-dependent manner in Saccharomyces cerevisiae.* Genes Dev, 2007. **21**(19): p. 2485-94.
32. Gupta, A., et al., *Telomere length homeostasis responds to changes in intracellular dNTP pools.* Genetics, 2013. **193**(4): p. 1095-105.
33. Teng, S.C. and V.A. Zakian, *Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in Saccharomyces cerevisiae.* Mol Cell Biol, 1999. **19**(12): p. 8083-93.
34. Bernstein K.A., et al., *The Shu complex, which contains Rad51 paralogues, promotes DNA repair through inhibition of the Srs2 anti-recombinase*. Mol Biol Cell, 2011. **22**(9): p. 1599-607. pmid:21372173
35. Vasianovich, Y., L.A. Harrington, and S. Makovets, *Break-induced replication requires DNA damage-induced phosphorylation of Pif1 and leads to telomere lengthening.* PLoS Genet, 2014. **10**(10): p. e1004679.
36. Anand, R.P., S.T. Lovett, and J.E. Haber, *Break-induced DNA replication.* Cold Spring Harb Perspect Biol, 2013. **5**(12): p. a010397.



Chapter 3

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.

Introduction

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 [1]. 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 [2]. However, most human somatic cells do not express sufficient levels of telomerase to prevent telomere shortening, which has been implicated in human aging [3]. 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 [4]. 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) [5].

In the budding yeast *Saccharomyces cerevisiae*, telomerase is constitutively expressed, allowing the maintenance of telomeres 300 ± 75 bp in length [6]. The core components of telomerase in *S. cerevisiae* are a protein catalytic component (Est2) and an RNA subunit (TLC1) [7, 8]. 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" [9], 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 $(TG_{1\text{-}3})_n$ telomeric sequences [9, 10]. Type I and type II survivors require Rad52-dependent homologous recombination (HR) and the DNA polymerase δ subunit Pol32, which is required for break-induced replication (BIR), suggesting that both survivor pathways occur through recombination-dependent DNA replication [9, 11]. The Pif1 helicase is also important for the generation of type I and type II survivors [12], likely due to its role in BIR [13, 14]. There are two BIR pathways: one is Rad51-dependent and one is independent of Rad51, but requires the MRX complex (consisting of Mre11, Rad50, and Xrs2) and Rad59 [15]. Similarly, the formation of type I survivors is dependent on Rad51 (and Rad54 and Rad57, which function in the same pathway as Rad51), whereas type II survivors require the MRX

complex and Rad59 [16, 17], suggesting that type I and type II survivors maintain telomeres via Rad51-dependent and Rad51-independent BIR, respectively.

Type II survivors resemble the majority of human ALT cells in that both are characterized by long and heterogeneous-sized telomere length [10, 18, 19], extrachromosomal circular DNA containing telomeric sequence [20-22], and telomere maintenance by Rad51-independent BIR requiring the MRX (or MRN—Mre11, Rad50, Nbs1—in humans) complex [16, 17, 23-25].

Sgs1 and Exo1, which are needed for processive resection of DNA ends [26, 27], are also important for type II survivor formation [28-31]. Consistent with the importance of end resection for type II survivor formation, the *sgs1-D664Δ* mutation [32, 33], which is competent for recombination repair but defective in resection, also prevents the formation of type II survivors [34]. Similarly, type II survivor formation is hindered by the deletion of *FUN30*, which encodes a chromatin remodeler that promotes end resection [35]. BLM, a human homolog of Sgs1, has also been implicated in facilitating telomere maintenance in ALT cells [36].

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 [37]. Furthermore, the RNA polymerase II degradation factor Def1, the B-type cyclin Clb2, the tRNA modification protein Sua5, and Mdt4/Pin4, which interacts with the DNA damage kinase Rad53, are also important for type II survivor formation [38-41]. 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 [42]. 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.

Material and method

Yeast strains and growth conditions

Standard yeast media and growth conditions were used [43, 44]. With the exception of MCY610 and the yeast knockout (YKO) collection [45], all yeast strains used in this study are *RAD5* derivatives of W303 [46, 47] 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 [48].

SGA screening procedure

The *est2Δ* and *rad51Δ* deletions were introduced into the strains of the YKO collection using synthetic genetic array (SGA) methodology [49]. The *MAT*α *can1ΔSTE2pr-Sp_his5 est2ΔnatMX his3 leu2 lyp1Δ RAD5 rad51ΔURA3 TRP1 ura3* query strain for the screen was derived from the sporulation of MCY610. The pinning steps were performed using a ROTOR HDA (Singer Instruments, Somerset, UK) with a 384-density format. The final *est2ΔnatMX rad51ΔURA3 xxxΔkanMX* triple mutants (where *xxxΔkanMX* 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′-CACCACACCCACACACCACACCCACA-3′) digoxygenin-labelled probe.

Measurement of dNTP levels

dNTP levels were measured as previously described [50].

Table 1. Yeast strains used in this study.

Strain name	Relevant genotype	Source
MCY610	*MAT***a**/ *can1ΔSTE2pr-HIS3/can1ΔSTE2pr-Sp_his5 lyp1Δ/lyp1Δ rad51ΔURA3 /RAD51 est2ΔnatMX/EST2 TRP1/trp1-1 ADE2/ADE2 his3Δ1/his3 leu2Δ0/leu2 ura3Δ0/ura3 RAD5/rad5-535*	This study
CCY6	*MAT***a**/α *est2ΔURA3/EST2*	Clémence Claussin
CCY16	*MAT***a**/α *est2ΔURA3/EST2 rad52ΔnatMX/RAD52*	(Claussin and Chang 2016)
YPM7	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rad50ΔkanMX/RAD50*	This study
YPM8	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rad59ΔkanMX/RAD59*	This study
YPM9	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51*	This study
YPM10	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 nmd2ΔkanMX/NMD2*	This study
YPM11	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rgi1ΔkanMX/RGI1*	This study
YPM12	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 dun1ΔTRP1/DUN1 sml1ΔHIS3/SML1*	This study
YPM17	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 clb2ΔkanMX/CLB2*	This study
YPM20	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 vps25ΔkanMX/VPS25*	This study
YPM21	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 lsm1ΔkanMX/LSM1*	This study
YPM29	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rmi1ΔkanMX/RMI1*	This study
YPM30	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 spt20ΔkanMX/SPT20*	This study
YPM31	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 cdc55ΔkanMX/CDC55*	This study
YPM32	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 chk1ΔkanMX/CHK1*	This study
YPM33	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 pph3ΔkanMX/PPH3*	This study
YPM34	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 mot2ΔkanMX/MOT2*	This study
YPM35	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rpn4ΔkanMX/RPN4*	This study
YPM36	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 ylr358cΔkanMX/YLR358C*	This study
YPM37	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rrm3ΔkanMX/RRM3*	This study

YPM38	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 tsc3ΔkanMX/TSC3*	This study
YPM39	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 pxp1ΔkanMX/PXP1*	This study
YPM40	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 mtc7ΔkanMX/MTC7*	This study
YPM41	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 doa4ΔkanMX/DOA4*	This study
YPM42	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 cik1ΔkanMX/CIK1*	This study
YPM43	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 ure2ΔkanMX/URE2*	This study
YPM44	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 vma22ΔkanMX/VMA22*	This study
YPM45	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 rpl8bΔkanMX/RPL8B*	This study
YPM48	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 ylr235cΔkanMX/YLR235C*	This study
YPM51	*MAT***a**/α *est2ΔURA3/EST2 rad51ΔnatMX/RAD51 ccr4ΔkanMX/CCR4*	This study
YPM55	*MAT*α *est2ΔURA3* type II survivor	This study
YPM56	*MAT*α *est2ΔURA3* type II survivor	This study
MCY775	*MAT***a**/α *est2ΔURA3/EST2 dun1ΔTRP1/DUN1 sml1ΔHIS3/SML1*	This study
MCY783	*MAT***a** *est2ΔURA3* type II survivor	This study
MCY784	*MAT***a** *est2ΔURA3* type II survivor	This study
MCY785	*MAT***a** *est2ΔURA3 sml1ΔHIS3* type II survivor	This study
MCY786	*MAT***a** *est2ΔURA3 sml1ΔHIS3* type II survivor	This study
MCY788	*MAT***a** *est2ΔURA3 dun1ΔTRP1 sml1ΔHIS3* type II survivor	This study
YPM60	*MAT***a** *est2ΔURA3* type II survivor	This study
YPM61	*MAT***a** *est2ΔURA3 dun1ΔTRP1* type II survivor	This study
YPM62	*MAT***a** *est2ΔURA3 dun1ΔTRP1* type II survivor	This study
YPM63	*MAT***a** *est2ΔURA3 dun1ΔTRP1* type II survivor	This study
YPM64	*MAT***a** *est2ΔURA3 dun1ΔTRP1 sml1ΔHIS3* type II survivor	This study
YPM65	*MAT***a** *est2ΔURA3 dun1ΔTRP1 sml1ΔHIS3* type II survivor	This study

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 [49] 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 [16, 17], 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).

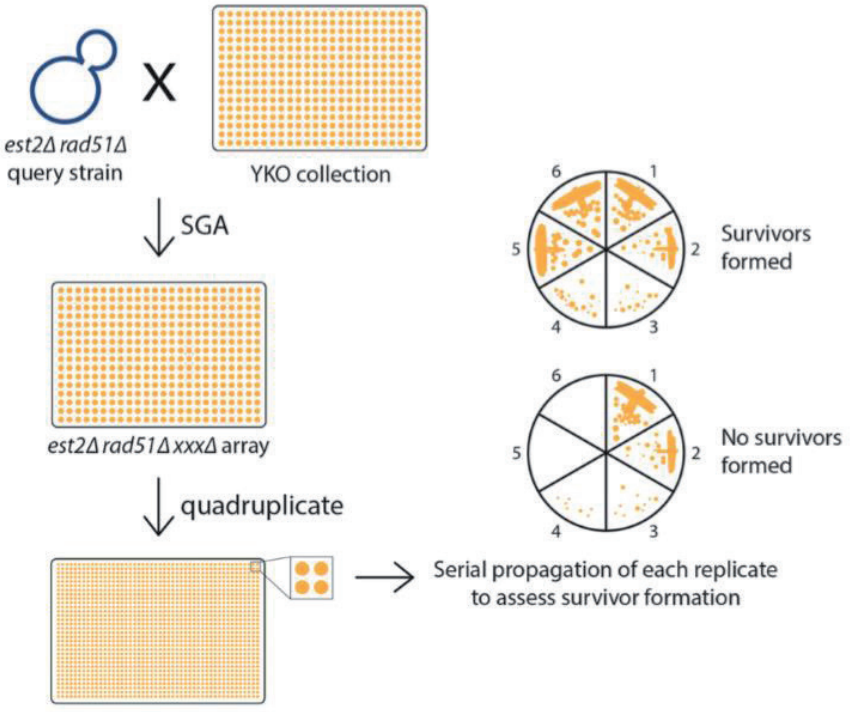

Figure 1. Screening approach for identifying genes important for type II survivor formation. A *MAT est2Δ rad51Δ* query strain is crossed to an ordered array of *MAT***a** viable yeast deletion mutants to generate an array of *est2Δ rad51Δ xxxΔ* triple mutants via SGA methodology. The triple mutant strains were then quadruplicated by replica-pinning onto fresh agar plates. The resulting four colonies of each *est2Δ rad51Δ xxxΔ* triple mutant was then serially propagated up to six times on sectored YPD plates.

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 not test any additional genes from this group. Importantly, the 26 included strains with a deletion of *RAD52*, *RAD50*, *RAD59*, *SGS1*, *CLB2*, or *NMD2*, which are all known to be required for type II survivor formation [9, 16, 17, 28, 29, 39, 42], as well as *RMI1* and *YLR235C* (which overlaps the *TOP3* open reading frame so that deletion of *YLR235C* likely results in a *top3* hypomorph). Like Sgs1, Top3 is also required for type II survivor formation [51]. Sgs1, Top3, and Rmi1 form an evolutionarily conserved complex [52, 53], so not surprisingly, we find that Rmi1 is also important for type II survivor formation.

To further validate that these genes are important for type II survivor formation, we knocked out each gene in an *est2Δ/EST2 rad51Δ/RAD51* diploid strain of a different genetic background (W303). Once again, we generated haploid meiotic progeny from these diploid strains and serially propagated multiple isolates of each genotype on agar plates to monitor senescence and survivor formation. Overall, 23 genes were identified that are important in type II survivor formation, and of those, 17 genes were not previously reported to be involved in survivor formation (Table 2).

Genes involved in the regulation of dNTP pools are important 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

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

	Fraction of *est2Δ rad51Δ xxxΔ* that are able to form survivors		
Gene	**in BY4741 background**[a]	**in W303 background**	**Reference**
CCR4[b]		0/10 (0%)	
CDC55	0/12 (0%)	2/9 (22%)	
CHK1	5/14 (36%)	2/10 (20%)	
CLB2	2/14 (14%)		Grandin and Charbonneau 2003
DOA4	5/14 (36%)	3/10 (30%)	
DUN1	2/12 (17%)	1/25 (4%)	
LSM1	5/14 (36%)	0/7 (0%)	
MOT2	0/10 (0%)	1/4 (25%)	
NMD2	0/12 (0%)		Hu et al. 2013
PPH3	2/12 (17%)	2/10 (20%)	
RAD50	2/10 (20%)		Chen et al. 2001
RAD52	0/11 (0%)		Lundblad and Blackburn 1993
RAD59	4/11 (36%)		Chen et al. 2001
RGI1	0/4 (0%)	2/10 (20%)	
RMI1	1/7 (14%)	0/10 (0%)	
RPL8B	1/8 (13%)	2/10 (20%)	
RPN4	1/9 (11%)	3/10 (30%)	
RRM3	4/12 (33%)	3/10 (30%)	
SGS1	0/11 (0%)		Huang et al. 2001; Johnson et al. 2001
SPT20	0/5 (0%)	0/10 (0%)	
VMA22	1/10 (10%)	3/10 (30%)	
YLR235C	1/16 (6%)	0/10 (0%)	
YLR358C	1/5 (20%)	4/9 (44%)	

[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*.

phosphorylates and inhibits Sml1, Crt1, and Dif1, three negative regulators of ribonucleotide reductase (RNR) [54-56]. The RNR complex catalyzes the rate limiting step in dNTP synthesis [57]. Mot2 (also known as Not4) is part of the Ccr4-Not complex, a key regulator of eukaryotic gene expression that is required for transcriptional induction of *RNR* genes in response to DNA damage or replication stress [58]. Ccr4 and Dun1 cooperate to regulate the Crt1-dependent inhibition

of the *RNR* genes in response to DNA replication stress [59]. 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 important for generating type II survivors suggests that the ability to upregulate intracellular dNTP levels is important for the formation of type II survivors. 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 [47, 60]. 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 [47, 61, 62]. 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 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.

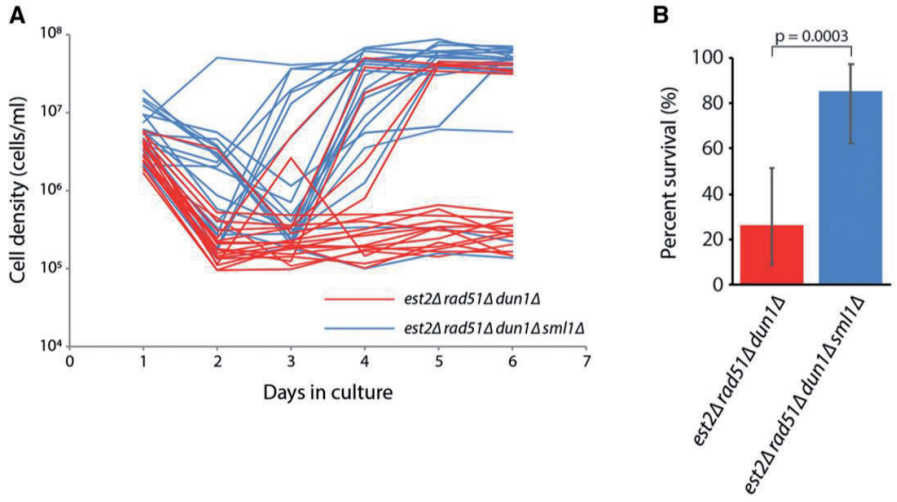

Figure 2. Deletion of *SML1* suppresses the type II survivor formation defect of a *est2Δ rad51Δ dun1Δ* strain.

(A) Senescence and survivor formation were monitored in liquid culture by serial passaging of individual 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 dunΔ* and *est2Δ rad51Δ dun1Δ sml1Δ* cultures from panel A that were able to form survivors. Error bars represent exact binomial 95% confidence intervals; p-value was determined using Fisher's exact test.

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 *DUN1* 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 [63, 64]. 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* [65]. 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 [10, 66]. This observation may be due to the fact that dNTP levels are elevated during BIR [67], which is required both to prevent accelerated senescence in pre-senescent cells and for telomere elongation in survivors [11, 68].

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.

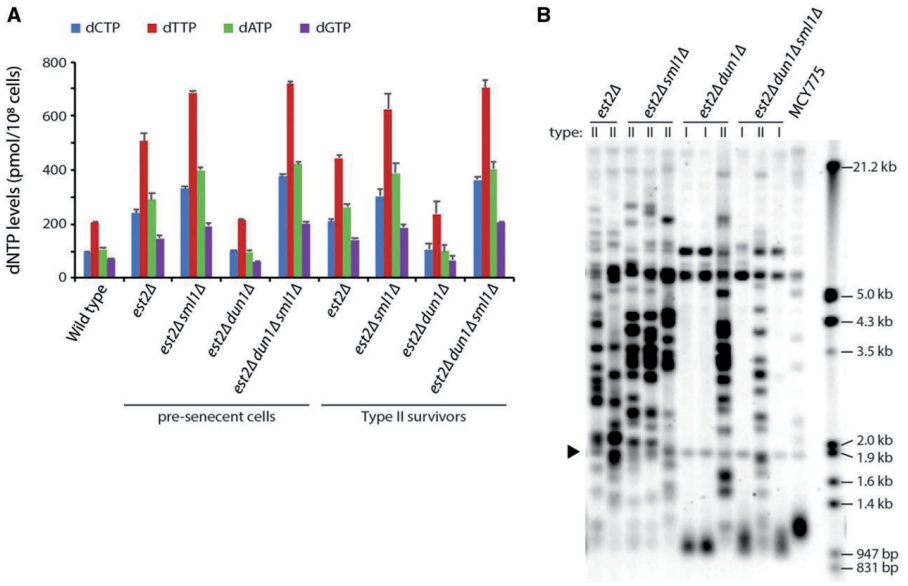

Figure 3. dNTP levels are upregulated in est2Δ pre-senescent cells and type II survivors.
(A) Strains of the indicated genotypes were assayed for dNTP levels. Data are represented as mean SE. (B) Representative telomere Southern blot of survivors generated by serial propagation in liquid culture of haploid 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 ran with each sample as a control.

Acknowledgments

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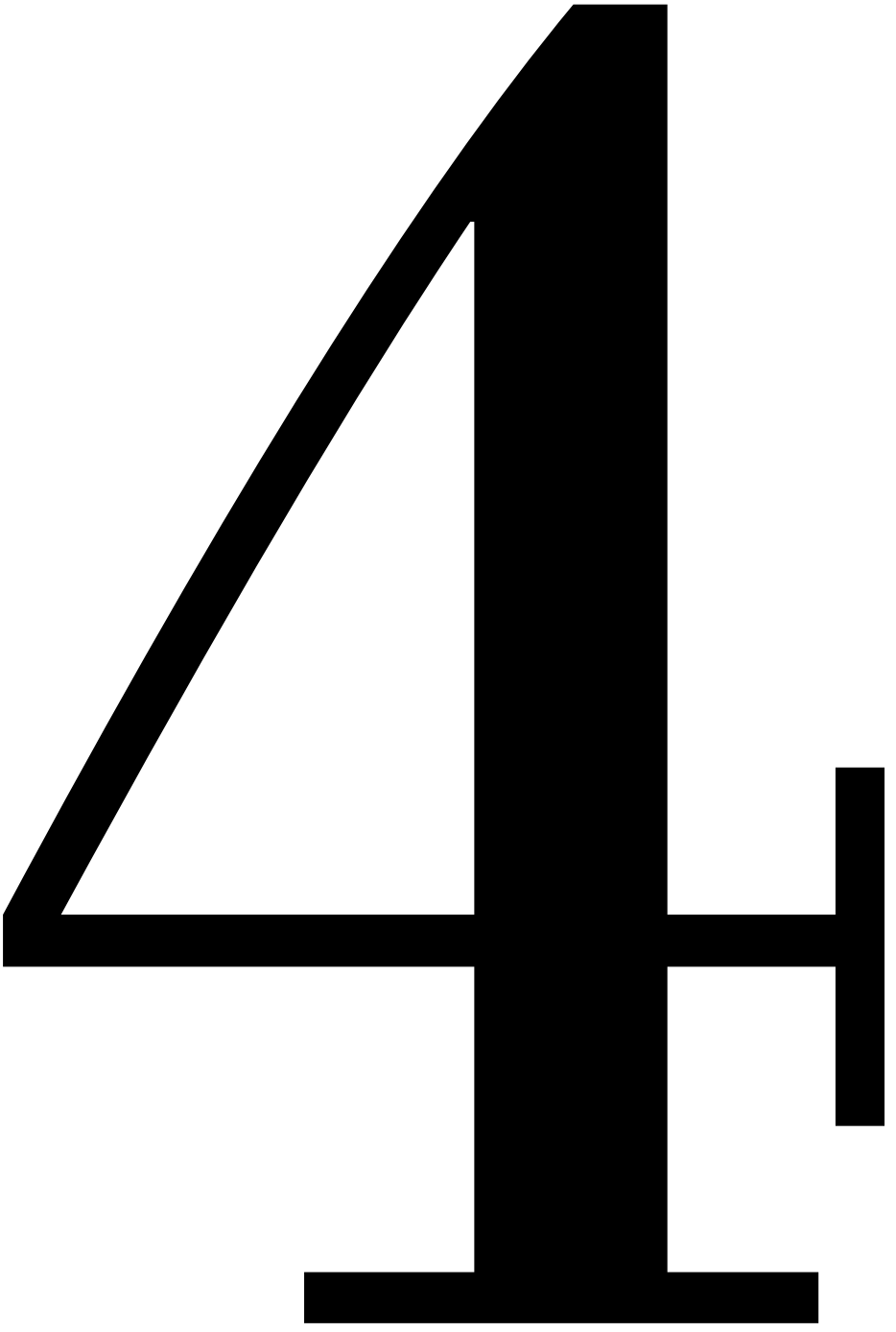
References

1. Jain, D. and J.P. Cooper, *Telomeric strategies: means to an end.* Annual review of genetics, 2010. **44**: p. 243-69.
2. Greider, C.W. and E.H. Blackburn, *Identification of a specific telomere terminal transferase activity in Tetrahymena extracts.* Cell, 1985. **43**(2 Pt 1): p. 405-13.
3. Lopez-Otin, C., et al., *The hallmarks of aging.* Cell, 2013. **153**(6): p. 1194-217.
4. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
5. Sobinoff, A.P. and H.A. Pickett, *Alternative Lengthening of Telomeres: DNA Repair Pathways Converge.* Trends Genet, 2017. **33**(12): p. 921-932.
6. Wellinger, R.J. and V.A. Zakian, *Everything you ever wanted to know about Saccharomyces cerevisiae telomeres: beginning to end.* Genetics, 2012. **191**(4): p. 1073-105.
7. Lingner, J., et al., *Reverse transcriptase motifs in the catalytic subunit of telomerase.* Science, 1997. **276**(5312): p. 561-7.
8. Singer, M.S. and D.E. Gottschling, *TLC1: template RNA component of Saccharomyces cerevisiae telomerase.* Science, 1994. **266**(5184): p. 404-9.
9. Lundblad, V. and E.H. Blackburn, *An alternative pathway for yeast telomere maintenance rescues est1- senescence.* Cell, 1993. **73**(2): p. 347-60.
10. Teng, S.C. and V.A. Zakian, *Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in Saccharomyces cerevisiae.* Mol Cell Biol, 1999. **19**(12): p. 8083-93.
11. Lydeard, J.R., et al., *Break-induced replication and telomerase-independent telomere maintenance require Pol32.* Nature, 2007. **448**(7155): p. 820-3.
12. Dewar, J.M. and D. Lydall, *Pif1- and Exo1-dependent nucleases coordinate checkpoint activation following telomere uncapping.* The EMBO journal, 2010. **29**: p. 4020-4034.
13. Saini, N., et al., *Migrating bubble during break-induced replication drives conservative DNA synthesis.* Nature, 2013. **502**(7471): p. 389-92.
14. Wilson, M.A., et al., *Pif1 helicase and Polδ promote recombination-coupled DNA synthesis via bubble migration.* Nature, 2013. **502**(7471): p. 393-6.
15. Anand, R.P., S.T. Lovett, and J.E. Haber, *Break-induced DNA replication.* Cold Spring Harb Perspect Biol, 2013. **5**(12): p. a010397.
16. Teng, S.C., et al., *Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process.* Mol Cell, 2000. **6**(4): p. 947-52.
17. Chen, Q., A. Ijpma, and C.W. Greider, *Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events.* Mol Cell Biol, 2001. **21**(5): p. 1819-27.
18. Bryan, T.M., et al., *Telomere elongation in immortal human cells without detectable telomerase activity.* EMBO J, 1995. **14**(17): p. 4240-8.
19. Bryan, T.M., et al., *Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines.* Nat Med, 1997. **3**(11): p. 1271-4.
20. Larrivée, M. and R.J. Wellinger, *Telomerase- and capping-independent yeast survivors with alternate telomere states.* Nat Cell Biol, 2006. **8**(7): p. 741-7.
21. Cesare, A.J. and J.D. Griffith, *Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops.* Mol Cell Biol, 2004. **24**(22): p. 9948-57.
22. Henson, J.D., et al., *DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity.* Nat Biotechnol, 2009. **27**(12): p. 1181-5.
23. Dilley, R.L., et al., *Break-induced telomere synthesis underlies alternative telomere maintenance.* Nature, 2016. **539**(7627): p. 54-58.
24. Jiang, W.Q., et al., *Suppression of alternative lengthening of telomeres by Sp100-mediated sequestration of the MRE11/RAD50/NBS1 complex.* Mol Cell Biol, 2005. **25**(7): p. 2708-21.

25. Zhong, Z.H., et al., *Disruption of telomere maintenance by depletion of the MRE11/RAD50/NBS1 complex in cells that use alternative lengthening of telomeres.* J Biol Chem, 2007. **282**(40): p. 29314-22.
26. Mimitou, E.P. and L.S. Symington, *Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing.* Nature, 2008. **455**(7214): p. 770-4.
27. Zhu, Z., et al., *Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends.* Cell, 2008. **134**(6): p. 981-94.
28. Huang, P., et al., *SGS1 is required for telomere elongation in the absence of telomerase.* Curr Biol, 2001. **11**(2): p. 125-9.
29. Johnson, F.B., et al., *The Saccharomyces cerevisiae WRN homolog Sgs1p participates in telomere maintenance in cells lacking telomerase.* EMBO J, 2001. **20**(4): p. 905-13.
30. Maringele, L. and D. Lydall, *EXO1 plays a role in generating type I and type II survivors in budding yeast.* Genetics, 2004. **166**(4): p. 1641-9.
31. Bertuch, A.A. and V. Lundblad, *EXO1 contributes to telomere maintenance in both telomerase-proficient and telomerase-deficient Saccharomyces cerevisiae.* Genetics, 2004. **166**(4): p. 1651-9.
32. Bernstein, K.A., et al., *Sgs1 function in the repair of DNA replication intermediates is separable from its role in homologous recombinational repair.* EMBO J, 2009. **28**(7): p. 915-25.
33. Bernstein, K.A., et al., *Resection activity of the Sgs1 helicase alters the affinity of DNA ends for homologous recombination proteins in Saccharomyces cerevisiae.* Genetics, 2013. **195**(4): p. 1241-51.
34. Hardy, J., et al., *Sgs1 and Sae2 promote telomere replication by limiting accumulation of ssDNA.* Nat Commun, 2014. **5**: p. 5004.
35. Costelloe, T., et al., *The yeast Fun30 and human SMARCAD1 chromatin remodellers promote DNA end resection.* Nature, 2012. **489**(7417): p. 581-4.
36. Stavropoulos, D.J., et al., *The Bloom syndrome helicase BLM interacts with TRF2 in ALT cells and promotes telomeric DNA synthesis.* Hum Mol Genet, 2002. **11**(25): p. 3135-44.
37. Tsai, Y.L., et al., *Involvement of replicative polymerases, Tel1p, Mec1p, Cdc13p, and the Ku complex in telomere-telomere recombination.* Mol Cell Biol, 2002. **22**(16): p. 5679-87.
38. Chen, Y.B., et al., *Def1p is involved in telomere maintenance in budding yeast.* J Biol Chem, 2005. **280**(26): p. 24784-91.
39. Grandin, N. and M. Charbonneau, *Mitotic cyclins regulate telomeric recombination in telomerase-deficient yeast cells.* Mol Cell Biol, 2003. **23**(24): p. 9162-77.
40. Meng, F.L., et al., *Sua5p is required for telomere recombination in Saccharomyces cerevisiae.* Cell Res, 2010. **20**(4): p. 495-8.
41. Pike, B.L. and J. Heierhorst, *Mdt1 facilitates efficient repair of blocked DNA double-strand breaks and recombinational maintenance of telomeres.* Mol Cell Biol, 2007. **27**(18): p. 6532-45.
42. Hu, Y., et al., *Telomerase-null survivor screening identifies novel telomere recombination regulators.* PLoS Genet, 2013. **9**(1): p. e1003208.
43. Treco, D.A. and V. Lundblad, *Preparation of yeast media.* Curr Protoc Mol Biol, 2001. **Chapter 13**: p. Unit13 1.
44. Sherman, F., *Getting started with yeast.* Methods Enzymol, 2002. **350**: p. 3-41.
45. Giaever, G., et al., *Functional profiling of the Saccharomyces cerevisiae genome.* Nature, 2002. **418**(6896): p. 387-91.
46. Thomas, B.J. and R. Rothstein, *Elevated recombination rates in transcriptionally active DNA.* Cell, 1989. **56**(4): p. 619-30.
47. Zhao, X., E.G. Muller, and R. Rothstein, *A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools.* Mol Cell, 1998. **2**(3): p. 329-40.
48. van Mourik, P.M., et al., *Recombination-Mediated Telomere Maintenance in Saccharomyces cerevisiae Is Not Dependent on the Shu Complex.* PLOS ONE, 2016. **11**(3): p. e0151314.

49. Tong, A.H. and C. Boone, *Synthetic genetic array analysis in Saccharomyces cerevisiae.* Methods Mol Biol, 2006. **313**: p. 171-92.
50. Watt, D.L., et al., *Genome-wide analysis of the specificity and mechanisms of replication infidelity driven by imbalanced dNTP pools*. Nucleic Acids Res, 2016. **44**: p. 1669-80. https://doi.org/10.1093/nar/gkv1298.
51. Tsai, H.J., et al., *Involvement of topoisomerase III in telomere-telomere recombination.* J Biol Chem, 2006. **281**(19): p. 13717-23.
52. Chang, M., et al., *RMI1/ NCE4, a suppressor of genome instability, encodes a member of the RecQ helicase/Topo III complex*. EMBO J, 2005. **24**: p. 2024-33. https://doi.org/ 10.1038/sj.emboj.7600684.
53. Mullen, J.R., et al., *Yeast Rmi1/Nce4 controls genome stability as a subunit of the Sgs1-Top3 complex.* Mol Cell Biol, 2005. **25**(11): p. 4476-87.
54. Zhao, X. and R. Rothstein, *The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3746-51.
55. Huang, M., Z. Zhou, and S.J. Elledge, *The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor.* Cell, 1998. **94**(5): p. 595-605.
56. Lee, Y.D., et al., *Dif1 is a DNA-damage-regulated facilitator of nuclear import for ribonucleotide reductase.* Mol Cell, 2008. **32**(1): p. 70-80.
57. Hofer, A., et al., *DNA building blocks: keeping control of manufacture.* Crit Rev Biochem Mol Biol, 2012. **47**(1): p. 50-63.
58. Mulder, K.W., G.S. Winkler, and H.T. Timmers, *DNA damage and replication stress induced transcription of RNR genes is dependent on the Ccr4-Not complex.* Nucleic Acids Res, 2005. **33**(19): p. 6384-92.
59. Woolstencroft, R.N., et al., *Ccr4 contributes to tolerance of replication stress through control of CRT1 mRNA poly(A) tail length.* J Cell Sci, 2006. **119**(Pt 24): p. 5178-92.
60. Chabes, A., V. Domkin, and L. Thelander, *Yeast Sml1, a protein inhibitor of ribonucleotide reductase.* J Biol Chem, 1999. **274**(51): p. 36679-83.
61. Fasullo, M., et al., *Elevated dNTP levels suppress hyper-recombination in Saccharomyces cerevisiae S-phase checkpoint mutants.* Nucleic Acids Res, 2010. **38**(4): p. 1195-203.
62. Gupta, A., et al., *Telomere length homeostasis responds to changes in intracellular dNTP pools.* Genetics, 2013. **193**(4): p. 1095-105.
63. IJpma, A.S. and C.W. Greider, *Short telomeres induce a DNA damage response in Saccharomyces cerevisiae.* Mol Biol Cell, 2003. **14**(3): p. 987-1001.
64. Xie, Z., et al., *Early telomerase inactivation accelerates aging independently of telomere length.* Cell, 2015. **160**(5): p. 928-39.
65. Jay, K.A., D.L. Smith, and E.H. Blackburn, *Early Loss of Telomerase Action in Yeast Creates a Dependence on the DNA Damage Response Adaptor Proteins.* Mol Cell Biol, 2016. **36**(14): p. 1908-19.
66. Straatman, K.R. and E.J. Louis, *Localization of telomeres and telomere-associated proteins in telomerase-negative Saccharomyces cerevisiae.* Chromosome Res, 2007. **15**(8): p. 1033-50.
67. Deem, A., et al., *Break-induced replication is highly inaccurate.* PLoS Biol, 2011. **9**(2): p. e1000594.
68. Fallet, E., et al., *Length-dependent processing of telomeres in the absence of telomerase.* Nucleic Acids Res, 2014. **42**(6): p. 3648-65.
69. Shampay, J., J.W. Szostak, and E.H. Blackburn, *DNA sequences of telomeres maintained in yeast.* Nature, 1984. **310**: p. 154-157.
70. Claussin, C. and M. Chang, *Multiple Rad52-Mediated Homology-Directed Repair Mechanisms Are Required to Prevent Telomere Attrition-Induced Senescence in Saccharomyces cerevisiae.* PLoS Genet, 2016. **12**(7): p. e1006176.



Chapter 4

A genome-wide screen to identify novel genes that are important in the formation of type I survivors in *Saccharomyces cerevisiae*

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Introduction

Telomeres are nucleoprotein structures at the ends of linear chromosomes that help distinguish a natural chromosome end from a DNA double-strand break [1]. Telomerase is a reverse transcriptase that adds telomeric DNA to chromosome ends [2]. Loss of telomerase results in telomere shortening, which eventually causes the cells to senesce. However, a subset of these cells can overcome senescence and maintain their telomeres using telomerase-independent mechanisms. These mechanisms are collectively termed Alternative Lengthening of Telomeres (ALT) and are used in a fraction of human cancers [3].

In *Saccharomyces cerevisiae*, ALT cells are called 'survivors' and require Rad52-dependent homologous recombination and Pol32-dependent break-induced replication [4, 5]. There are two main types of survivors: type I and type II. The type II survivors are Rad51-independent, but require the MRX complex and Sgs1 to amplify the terminal telomeric sequences [4, 6]. Type I survivors require Rad51, Rad54, Rad55 and Rad57 recombination proteins and maintain telomeres by amplification of subtelomeric Y' elements and short telomeric repeats [6, 7]. Furthermore, extra-chromosomal Y' circles have been identified in type I survivors as well as in wild-type cells [8, 9]. A genetic screen performed by Hu and colleagues showed that the Pif1 helicase and INO80 chromatin remodeling complex reduce the formation of type I survivors [10]. Pif1 is likely important because of its role in BIR [11, 12].

Human ALT cancer cells maintain their telomere length by recombination-mediated mechanisms that are most similar to the yeast type II survivors that replicate the telomeric repeats [3, 13]. The human ALT cells are characterized by their long, heterogeneous telomere lengths [3, 13, 14], abundant extrachromosomal telomeric repeat DNA (ECTR) [15] [16], elevated levels of telomeric sister chromatic exchanges (T-SCE) [17] and promyelocytic leukemia (PML) bodies, also known as ALT-associated PML bodies (APB) [18]. However, not all ALT cells look alike. Variant cell lines have been reported that lack APBs and long telomeres, but contain telomeres with subtelomeric elements [19, 20].

Since ALT cells do not always exhibit all the characteristics mentioned above, it is likely that multiple mechanisms are used to maintain telomeres in the absence of telomerase. Just like in yeast cells, that either can form type I or type II survivors in the absence of telomerase, it is important to study both survivor types. In the previous chapter, we performed a genome-wide screen to identify novel genes that are important for

the formation of type II survivors [21]. Here we report a high-throughput, replica pinning-based screening approach to identify novel genes in *S. cerevisiae* that are important in the formation of type I survivors. We identified 34 genes, including several positive controls, which are potentially involved in type I survivor formation.

Material and methods

Yeast strains and growth conditions

Standard yeast media and growth conditions were used [22, 23]. The strain used for the screen is MCY594 *MATa/α can1ΔSTE2pr-SP-his5/can1ΔSTE2pr-SP-his5 lyp1Δ/lyp1Δ his3/his3 leu2/leu2 ura3/ura3 TRP1/TRP1 rad59ΔLEU2/RAD59 est2ΔnatMX/EST2*. MCY594 has a hybrid BY4741 and W303 genetic background.

SGA screening procedure

The *est2Δ* and *rad59Δ* deletions were introduced into the strains of the YKO collection using SGA methodology [24]. A *MATα can1ΔSTE2pr-SP-his5 lyp1Δ his3Δ ura3Δ TRP1 rad59::LEU2 est2ΔnatMX* query strain, derived from the sporulation of MCY594, was used as the query strain. The 384-density format solid agar to solid agar pinning was used to obtain the *MAT***a** *can1ΔSTE2pr-SP-his5 lyp1Δ rad59::LEU2 est2ΔNATMX xxxΔkanMX* mutants by using a ROTOR HDA (Singer Instruments, Somerset, UK). There were four plates for each library plate, which allowed us to test each mutant in quadruplicate for type I survivor formation. After the final selection step the pinning procedure continued on selection plates. The agar plates were incubated for 3 days at 30°C before being photographed and re-pinned. This step was repeated 20 times until senescence and type I survivors were formed, or the mutant died.

Results and discussion

Screen for genes that are important for type I survivor formation

Previously, H-Y. Chang et al. performed a genome-wide analysis to identify genes that affect entry into and recovery from telomere attrition-induced senescence by using the SGA methodology to introduce an *est1Δ* gene deletion into the strains of the yeast knockout collection followed by repeated replica pinning of the resulting strains on agar media to allow for senescence and survivor formation [25]. We performed a similar genome-wide screen to identify gene deletions that prevent

telomerase-deficient *rad59Δ* strains from forming type I survivors. The synthetic genetic array (SGA) methodology was used to create a library of *MAT***a** *est2Δ rad59Δ xxxΔ* mutants, where *xxxΔ* is a deletion in a nonessential gene (Figure 1) [24]. *EST2* encodes the catalytic subunit of telomerase. Deleting *RAD59* prevents type II survivor formation, allowing us to screen for genes important for type I survivor formation. After the creation of the library, we continued with a high-throughput screening approach; the mutants from the pinning plate were in quadruplicate and re-pinned twenty times, with three days of growth after each round of pinning to allow the mutants to senescence and form type I survivors (i.e. each *est2Δ rad59Δ xxxΔ* strain was tested four times for its ability to form survivors).

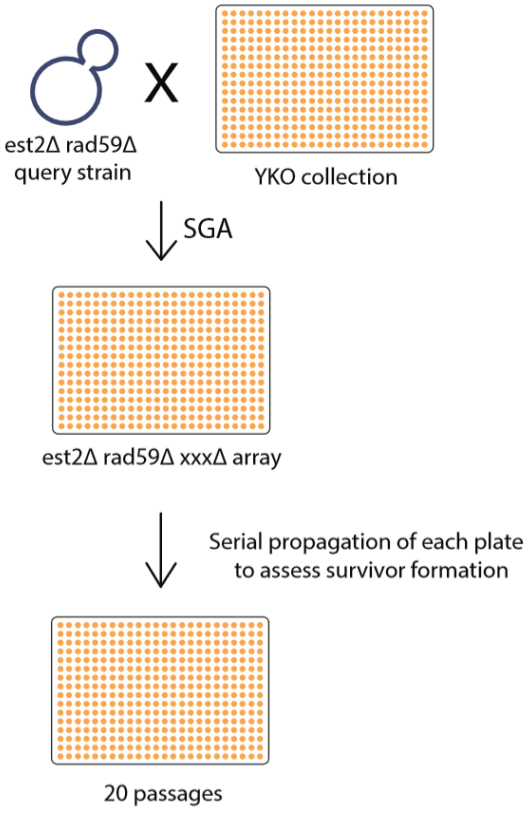

Figure 1. Screening approach for identifying genes important for type I survivor formation [26]. A *MAT*α *est2Δ rad59Δ* query strain is crossed to an ordered array of *MAT***a** viable yeast deletion mutants to generate an array of *est2Δ rad59Δ xxxΔ* triple mutants via SGA methodology. Each plate was present in quadruplicate from the start of the SGA using the 384 pinning format, allowing screening of each mutant four times for survivor formation. The resulting *est2Δ rad59Δ xxxΔ* triple mutant was serially propagated by replica-pinning onto fresh selection plates up to twenty times. After each passage the plate was photographed to follow the senescence and survivor formation of each mutant.

With this screen we identified in total 34 genes that are potentially involved in type I survivor formation or were previously published to play a role in type I survivor formation (*RAD52*, *RAD54*, *RAD55*, *YKU70*, *YKU80*; Table 1). 17 triple mutants failed to form survivors in all four replicates and 17 failed to form survivors in

Table 4. Genes identified that are important for type I survivor formation.

Gene	Fraction of *est2Δ rad59Δ xxxΔ* that are able to form survivors	Reference
ARP8	0/4 (0%)	
BEM2	1/4 (25%)	
BRO1	0/4 (0%)	
BUD6	1/4 (25%)	
CAP2	1/4 (25%)	
CIK1	0/4 (0%)	
CLB2	1/4 (25%)	
COS10	1/4 (25%)	
CPA2	0/4 (0%)	
EOS1	1/4 (25%)	
HIS6	1/4 (25%)	
HSL1	1/4 (25%)	
HSL7	0/4 (0%)	
IST1	1/4 (25%)	
ISY1	1/4 (25%)	
MDM20	0/4 (0%)	
MOT2	0/4 (0%)	
PKR1	0/4 (0%)	
RAD52	1/4 (25%)	Lundblad and Blackburn 1993
RAD54	0/4 (0%)	Le et al. 1999
RAD55	1/4 (25%)	Le et al. 1999
RPN4	0/4 (0%)	
RSM25	1/4 (25%)	
SCW11	0/4 (0%)	
SNF8	0/4 (0%)	
SPT7	0/4 (0%)	
STE14	0/4 (0%)	
SWI3	1/4 (25%)	
TIR3	1/4 (25%)	
WHI3	1/4 (25%)	
YET3	0/4 (0%)	
YKU70	0/4 (0%)	Grandin and Charbonneau 2003
YKU80	0/4 (0%)	Grandin and Charbonneau 2003
YOR022C	1/4 (25%)	

Table 2. GO term analysis of the identified genes in this study.

GO ID	Term	Num list annotation	List size	Cluster frequency	Total num annotation	Population size	Genome frequency	Annotated genes
GO:0006325	chromatin organization	7	34	20.59%	308	6439	4.78%	*ARP8, BUD6, RAD54, SPT7, SWI3, YKU70, YKU80*
GO:0006281	DNA repair	7	34	20.59%	302	6439	4.69%	*ARP8, RAD52, RAD54, RAD55, RPN4, YKU70, YKU80*
GO:0007010	cytoskeleton organization	6	34	17.65%	268	6439	4.16%	*BEM2, BUD6, CAP2, CIK1, CLB2, MDM20*
GO:0006974	cellular response to DNA damage stimulus	6	34	17.65%	235	6439	3.65%	*ARP8, RAD52, RAD54, RAD55, YKU70, YKU80*
GO:0006310	DNA recombination	6	34	17.65%	251	6439	3.90%	*ARP8, RAD52, RAD54, RAD55, YKU70, YKU80*
GO:0000278	mitotic cell cycle	6	34	17.65%	373	6439	5.79%	*BUD6, CIK1, CLB2, HSL1, HSL7, WHI3*
GO:0042221	response to chemical	6	34	17.65%	564	6439	8.76%	*BRO1, EOS1, RPN4, SNF8, STE14, SWI3*

The identified genes in this study were analyzed using SGD gene ontology slim mapper (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). The "Yeast Go Slim: process" was used to determine the list according to biological processes.

three of the four replicates. Interestingly, deletions of seven genes (*RAD52*, *RAD54*, *RAD55*, *CIK1*, *PKR1*, *RPN4*, *YKU80*) were previously found in a genome-wide analysis to behave similarly to a rad52Δ est1Δ archetype, characterized by accelerated senescence and a defect in the ability to form survivors [25]. Of these, as noted above, *RAD52*, *RAD54*, *RAD55*, and *YKU80*, have been previously implicated in type I survivor formation. Thus, the remaining genes (*CIK1*, *PKR1*, and *RPN4*) would be particularly interesting to study further in future studies.

We also performed a Gene Ontology (GO) slim analysis on these genes (see Table 2). Interestingly, the genes *ARP8* and *BUD6* show up multiple times in the top GO slim categories alongside other genes that were previously published to be involved in type I survivor formation. Arp8 is a nuclear actin-related protein involved in chromatin remodeling and Bud6 is an actin-interacting protein [28, 29]. Jokhun and colleagues showed that the intrinsically dynamic nature of the actin cytoskeleton is important in relaying extracellular signals to telomeres and therefore can be important in the maintenance of telomere integrity. Furthermore, they demonstrated that increased cytoskeletal dynamics resulted in increased turnover of the telomere protein TRF1 in mouse embryonic fibroblasts, which could potentially alter the exposure of telomeric sequences to other regulatory molecules [30]. Also, DNA damage-induced nuclear actin filaments may facilitate movement of chromatin and repair factors after DNA damage [31]. In the absence of Arp8 or Bud6, actin-dependent movement of shortened telomeres in senescent cells might be required for the Y′ amplification associated with type I survivor formation.

The identified genes need to be further validated for their importance in type I survivor formation. This can be performed by deleting the gene of interest in the W303 background strain that is more commonly used in the telomere field. Also, testing the hits in another strain background will ensure we obtain type I survivor genes that are important regardless of the strain background. The candidate genes will be deleted in a W303 strain background strain *MAT***a**/α *est2Δ/EST2 rad59Δ/RAD59*, followed by sporulation and tetrad dissection to select for single, double and triple mutants and streaking on YPD for analysis of entry into and recovery from senescence.

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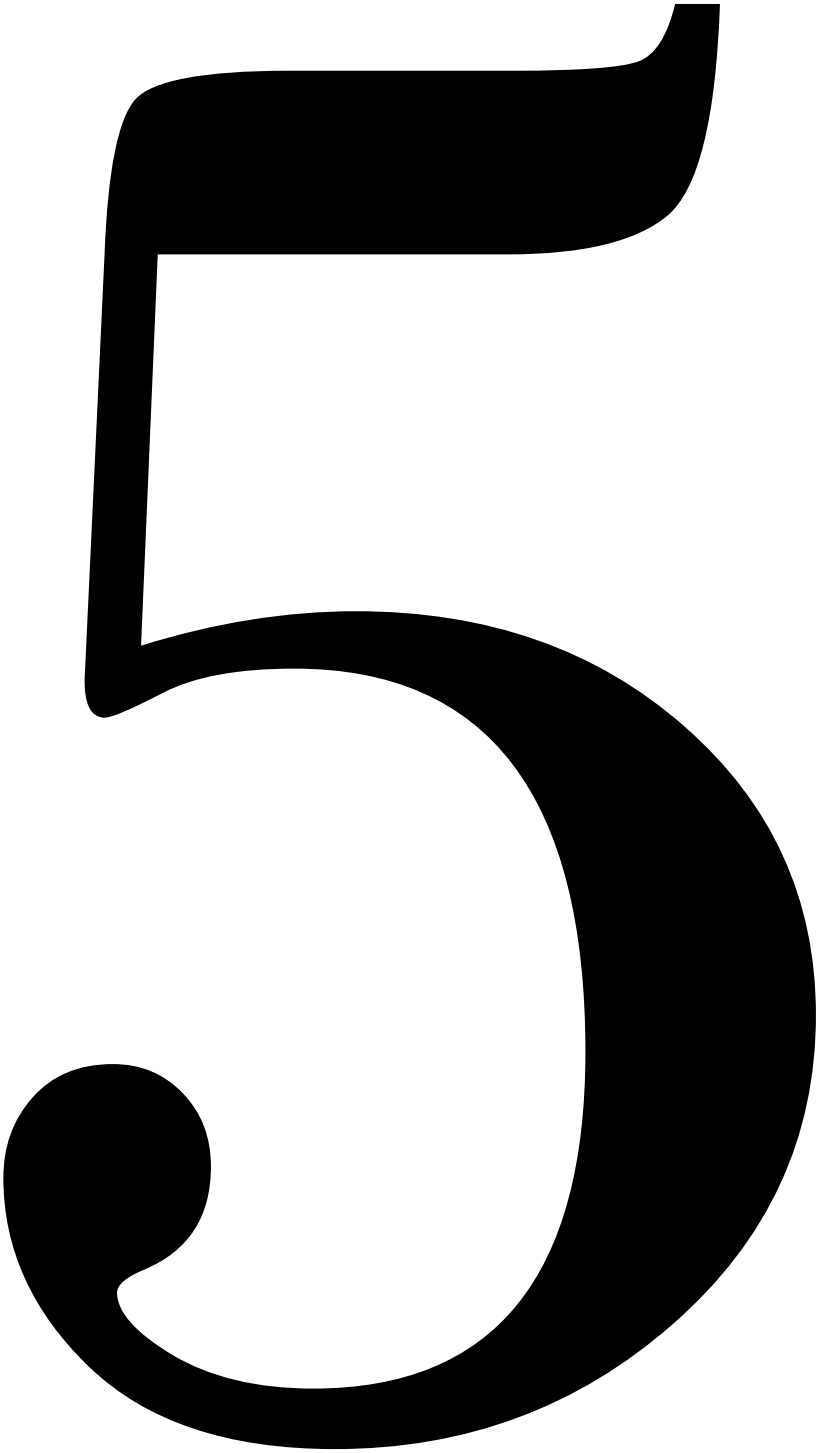
References

1. de Lange, T., *How telomeres solve the end-protection problem.* Science, 2009. **326**(5955): p. 948-52.
2. Greider, C.W. and E.H. Blackburn, *Identification of a specific telomere terminal transferase activity in Tetrahymena extracts.* Cell, 1985. **43**(2 Pt 1): p. 405-13.
3. Bryan, T.M., et al., *Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines.* Nat Med, 1997. **3**(11): p. 1271-4.
4. Lundblad, V. and E.H. Blackburn, *An alternative pathway for yeast telomere maintenance rescues est1- senescence.* Cell, 1993. **73**(2): p. 347-60.
5. Lydeard, J.R., et al., *Break-induced replication and telomerase-independent telomere maintenance require Pol32.* Nature, 2007. **448**(7155): p. 820-3.
6. Chen, Q., A. Ijpma, and C.W. Greider, *Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events.* Mol Cell Biol, 2001. **21**(5): p. 1819-27.
7. Le, S., et al., *RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase.* Genetics, 1999. **152**(1): p. 143-52.
8. Larrivee, M. and R.J. Wellinger, *Telomerase- and capping-independent yeast survivors with alternate telomere states.* Nat Cell Biol, 2006. **8**(7): p. 741-7.
9. Horowitz, H. and J.E. Haber, *Identification of autonomously replicating circular subtelomeric Y' elements in Saccharomyces cerevisiae.* Mol Cell Biol, 1985. **5**(9): p. 2369-80.
10. Hu, Y., et al., *Telomerase-null survivor screening identifies novel telomere recombination regulators.* PLoS Genet, 2013. **9**(1): p. e1003208.
11. Saini, N., et al., *Migrating bubble during break-induced replication drives conservative DNA synthesis.* Nature, 2013. **502**(7471): p. 389-92.
12. Wilson, M.A., et al., *Pif1 helicase and Polδ promote recombination-coupled DNA synthesis via bubble migration.* Nature, 2013. **502**(7471): p. 393-6.
13. Bryan, T.M., et al., *Telomere elongation in immortal human cells without detectable telomerase activity.* EMBO J, 1995. **14**(17): p. 4240-8.
14. Murnane, J.P., et al., *Telomere dynamics in an immortal human cell line.* EMBO J, 1994. **13**(20): p. 4953-62.
15. Cesare, A.J. and J.D. Griffith, *Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops.* Mol Cell Biol, 2004. **24**(22): p. 9948-57.
16. Nabetani, A. and F. Ishikawa, *Unusual telomeric DNAs in human telomerase-negative immortalized cells.* Mol Cell Biol, 2009. **29**(3): p. 703-13.
17. Londono-Vallejo, J.A., et al., *Alternative lengthening of telomeres is characterized by high rates of telomeric exchange.* Cancer Res, 2004. **64**(7): p. 2324-7.
18. Yeager, T.R., et al., *Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body.* Cancer Res, 1999. **59**(17): p. 4175-9.
19. Fasching, C.L., K. Bower, and R.R. Reddel, *Telomerase-independent telomere length maintenance in the absence of alternative lengthening of telomeres-associated promyelocytic leukemia bodies.* Cancer Res, 2005. **65**(7): p. 2722-9.
20. Marciniak, R.A., et al., *A novel telomere structure in a human alternative lengthening of telomeres cell line.* Cancer Res, 2005. **65**(7): p. 2730-7.
21. van Mourik, P.M., et al., *Upregulation of dNTP Levels After Telomerase Inactivation Influences Telomerase-Independent Telomere Maintenance Pathway Choice in Saccharomyces cerevisiae.* G3 (Bethesda), 2018.
22. Treco, D.A. and V. Lundblad, *Preparation of yeast media.* Curr Protoc Mol Biol, 2001. **Chapter 13**: p. Unit13 1.
23. Sherman, F., *Getting started with yeast.* Methods Enzymol, 2002. **350**: p. 3-41.

24. Tong, A.H. and C. Boone, *Synthetic genetic array analysis in Saccharomyces cerevisiae.* Methods Mol Biol, 2006. **313**: p. 171-92.
25. Chang, H.Y., et al., *Genome-wide analysis to identify pathways affecting telomere-initiated senescence in budding yeast.* G3 (Bethesda), 2011. **1**(3): p. 197-208.
26. van Mourik, P.M., et al., *Upregulation of dNTP Levels After Telomerase Inactivation Influences Telomerase-Independent Telomere Maintenance Pathway Choice in Saccharomyces cerevisiae.* G3 (Bethesda), 2018. **8**(8): p. 2551-2558.
27. Grandin, N. and M. Charbonneau, *The Rad51 pathway of telomerase-independent maintenance of telomeres can amplify TG1-3 sequences in yku and cdc13 mutants of Saccharomyces cerevisiae.* Mol Cell Biol, 2003. **23**(11): p. 3721-34.
28. Poch, O. and B. Winsor, *Who's who among the Saccharomyces cerevisiae actin-related proteins? A classification and nomenclature proposal for a large family.* Yeast, 1997. **13**(11): p. 1053-8.
29. Amberg, D.C., et al., *Aip3p/Bud6p, a yeast actin-interacting protein that is involved in morphogenesis and the selection of bipolar budding sites.* Mol Biol Cell, 1997. **8**(4): p. 729-53.
30. Jokhun, D.S., Y. Shang, and G.V. Shivashankar, *Actin Dynamics Couples Extracellular Signals to the Mobility and Molecular Stability of Telomeres.* Biophys J, 2018. **115**(7): p. 1166-1179.
31. Belin, B.J., T. Lee, and R.D. Mullins, *DNA damage induces nuclear actin filament assembly by Formin -2 and Spire-(1/2) that promotes efficient DNA repair. [corrected].* Elife, 2015. **4**: p. e07735.



Chapter 5

General discussion

Introduction

One of the hallmarks of ageing and cancer cell development is telomere dysfunction [1]. Cancer cells acquire the ability to divide indefinitely and elongate the telomeres by either upregulating telomerase or by activating the ALT pathway [2, 3]. The mechanism that underlies the activation of ALT and the maintenance of telomere length in ALT cancer cells remains unclear. The understanding of these processes is important to help identify new hallmarks of ALT cancers cells and for the development of new therapeutic strategies that might benefit diagnosis and prognosis for patients. The human ALT cells have similar features to *S. cerevisiae* telomerase-independent survivors. In the research described in this thesis, we aimed to identify novel genes and underlying mechanisms that are involved in survivor formation in *S. cerevisiae*.

The Shu complex is not essential for survivor formation

The Shu complex promotes Rad51-dependent HR [4-6]. Given the role of recombination proteins in senescence and survivor formation, we explored in chapter 2 if the Shu complex also functions during senescence and survivor formation. To do so, we deleted genes of the Shu complex (*SHU1*, *SHU2*, *CSM2*, *PSY3*) in telomerase-null cells. The mutants showed a similar phenotypic growth and telomere length as the control (telomerase-null cells) and were able to form both type I and type II survivors. Sgs1 is required for type II survivor formation and prevents accelerated senescence in the absence of telomerase [7, 8]. Since deletion of the SHU genes can rescue various aspects of the sgs1 mutant phenotype [9], we tested if this could also suppress the *sgs1* defects in senescence and survivor formation. We showed that the deletion of the *SHU* genes: 1) does not prevent the rapid senescing phenotype of the *tlc1 sgs1* mutant; 2) does not rescue the type II survivor formation defect in *SGS1* deleted cells. Overall, these findings indicate that the Shu complex does not play a role in senescence or survivor formation. The Shu complex is involved in HR-mediated DSB repair by stimulating Rad51 filament formation. However, the Shu complex does not directly interact with Rad51. The Csm2-Psy3 heterodimer is responsible for the DNA binding activity of the Shu complex; preferably to forked DNA and to a lesser extent the 3'overhang (Sasanuma 2013; She 2012; Godin 2013), suggesting that the Shu complex promotes HR at specific replication fork blocking lesions (Godin 2016). The findings of chapter 2 indicate that the Shu complex is most likely not involved in the BIR pathway that is used in survivor formation.

A genome-wide screen to identified novel genes that are important for survivor formation

In chapter 3 and chapter 4 we describe a genome-wide screening approach to identify novel genes that are important for the formation of type I and type II survivors. Chapter 3 describes the genome-wide screen to identify novel genes that are important for the formation of type II ALT-like survivors. The yeast knockout (YKO) collection was used to identify gene deletions that impair the ability of *est2Δ rad51Δ* strains to form type II survivors. We used the synthetic genetic array (SGA) methodology [10] to create a library of *MAT***a** *est2Δ rad51Δ xxxΔ* mutants, where *xxxΔ* is a deletion in a nonessential gene. This was continued by serially propagating the mutants 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). We identified 23 genes, 17 of which were not previously reported to be involved in type II survivor formation. Chapter 4 describes a genome-wide screen to identify novel genes that are important for the formation of type I survivors. The synthetic genetic array (SGA) methodology was used to create a library of *MAT***a** *est2Δ rad59Δ xxxΔ* mutants. After the creation of the library, we continued propagating the mutants by multiple rounds of high-throughput replica pinning. We identified 34 genes, including several positive controls, which are potentially, involved in type I survivor formation. These identified genes need to be further validated for their importance in type I survivor formation. However, the improvements of this optimized screening approach are that the YKO can be screened all at once, costs less materials and reagents, and can be done in less time.

dNTP Levels influence the Telomerase-independent Telomere Maintenance Pathway Choice

With the type II survivor genome-wide screen we identified genes (*DUN1*, *MOT2*, *CCR4*) that are involved in the DNA damage pathway and the regulation of intracellular dNTP levels. Dun1, a Chk2 orthologue, is a checkpoint kinase that regulates downstream targets in response to DNA damage that are negative regulators of ribonucleotide reductase (i.e. Sml1, Crt1, and Dif1) [11-13]. We showed that the deletion of *SML1* largely suppresses the *dun1Δ* type II survivor formation defect, suggesting that decreased dNTP levels hinder the formation of type II survivors. Furthermore, we measured dNTP pools in pre-senescent cells and in type II survivors and found that dNTP levels are increased early after the

loss of telomerase and are still increased in type II survivors, but is not required for the maintenance of survivors.

These findings suggest that a short telomere in a senescing cells can activate the DNA damage response pathway and Dun1 and therefore up-regulate the dNTP levels. Also, short telomeres would have less Rap1 that might affect replication fork progression though the telomere. For instance, fission yeast Taz1 and the human TRF1 proteins, which are analogue of Rap1, are crucial for efficient replication fork progression through the telomere [14, 15].

However, another possibility is that the dNTP level upregulation early after telomerase loss is not related to telomere length. There might be other roles for telomerase other than elongating telomeres. For instance, the absence of telomerase might cause Dun1 activation. This hypothesis can be tested by growing a mutant strain or pre-growing the cells on ethanol to over-elongate the telomeres and measuring the dNTP levels in *est2Δ* cells when the telomeres are still long [16].

Furthermore, we assume that the deletion of *SML1* rescues the *dun1Δ* type II survivor formation defect because of an increase in dNTP levels, but we did not exclude any other roles for *SML1*. This could be easily tested by overexpressing *RNR1*, which is an independent method to increase dNTP levels, to see if the *est2Δ dun1Δ* mutant phenotype can be rescued.

As mentioned previously, we also identified *NOT4* and *CCR4* that are part of the Ccr4-Not complex. The Ccr4-Not complex has different roles in transcription regulation and is conserved in all eukaryotes [17]. The Not4 subunit is required for transcriptional induction of genes encoding the RNR complex by facilitating recruitment of various transcription factors. Both Ccr4 and Dun1 collaborate to inhibit Crt1 activity and to promote induction of the DNA damage gene regulon after replication stress [18]. The Crr4-Not complex has also been implicated in maintaining heterochromatin integrity at subtelomeres and heterochromatin islands in fission yeast *Schizosaccharomyces pombe* [19].

It has already been suggested that the Ccr4-Not complex is involved in cancer, although it is unclear how it is contributing to cancer development [20-22]. A recent study in *Drosophila melanogaster* showed that a reduction in Not3 expression, the human CNOT3 orthologue, results in a significant increase in tumor incidence and the overexpression of Not3 leading to suppressed tumor formation. The downregulation of other subunits of the Ccr4-Not complex also enhanced tumor

formation. They suggested that the function of the Ccr4-Not complex as a tumor suppressor is likely due to its role in mRNA metabolism [23]. It would be interesting to determine if these tumor cells are ALT tumor cells. This might indicate that the Crr4-Not complex plays a role in the telomere length regulation and ALT cancer cell development.

For future plans, it would be also interesting to explore the role of *NOT4* and *CCR4* in survivor formation and maintenance in more detail. Deletions of the other subunits of the Ccr4-Not complex (*not2Δ*, *not3Δ* and *not5Δ*), which were not identified in our screen but are likely false negatives, could be tested as well, to see if they are able to form survivors and effect telomere length. Also, measuring dNTP levels in the deletion mutants of the Ccr4-Not complex during senescence and in survivors could give more insight in the role of these genes and to see if dNTP level regulation is the cause of the survivor formation defect.

dNTP level regulation and the influence on ALT cancer cells

ALT cell lines are not uniform and can differ in characteristics and mechanisms that are used to elongate the telomere, making it difficult to target and treat ALT cancer cells. It is known that alterations in dNTP levels can lead to enhanced mutagenesis and cell proliferation resulting in cancer development. Cancer cells must increase dNTP levels to ensure rapid amplification of the genome [24, 25]. DNA damage and DDR proteins play a role in the regulation of dNTP levels in cancer cells [26-28]. One of the first DNA damage-induced enzymes identified was RNR [29]. Chemotherapeutic agents that target the nucleotide metabolism (especially RNR) are commonly used in the treatment of several types of cancer [30]. However, it is known that common therapeutic RNR inhibitors (i.e. gemcitabine, hydroxyurea) can cause drug resistance, short half-life and toxicity to cells [25]. Combination therapies and finding new chemotherapeutic targets can help reduce the survival of cancer cells that can bypass dNTP starvation. Further research is needed to understand the mechanisms that regulate the dNTP levels and their homeostasis and how it affects telomere elongation in ALT cancer cells.

Formation versus maintenance of survivors

The molecular mechanism through which ALT occurs and is maintained is still poorly understood. However, there is a difference in the DNA repair and

recombination proteins that are involved in the formation and maintenance of ALT cells [31, 32]. As described in chapter 4, we highlighted the essence of dNTP levels in the formation and maintenance of type II survivors. Even though upregulation of dNTP upregulation is essential for type II survivor formation, type II survivor can proliferate with dNTP levels comparable to wild-type cells. However, dNTPs might still be important for survivors to keep elongated telomeres. It would be interesting to test the importance of dNTPs levels in survivors. This can be tested by deleting *RNR1* in *est2Δ* survivor cells, which causes a decrease in dNTP levels. If dNTP levels are not essential for survivors then *est2Δrnr1Δ* survivor cells are able to continue proliferation and maintain telomere length.

The general aim of this thesis was to identify novel genes and underlying mechanisms that are involved in survivor formation in *S. cerevisiae*. We have found novel genes that are essential for type I and type II survivor formation that were not previously mentioned. Furthermore, we propose that dNTP levels play an important role in type II survivor formation.

Survivor maintenance is different than formation. Survivors need the ALT mechanisms to keep long telomeres to continue rapid proliferation, while senescing cells need to bypass the DNA damage response and activate an ALT mechanism to elongate the telomeres [33]. A challenge lies in unravelling the different mechanisms that are used in formation and maintenance of survivors.

References

1. Lopez-Otin, C., et al., *The hallmarks of aging.* Cell, 2013. **153**(6): p. 1194-217.
2. Shay, J.W. and S. Bacchetti, *A survey of telomerase activity in human cancer.* Eur J Cancer, 1997. **33**(5): p. 787-91.
3. Bryan, T.M., et al., *Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines.* Nat Med, 1997. **3**(11): p. 1271-4.
4. Godin, S., et al., *The Shu complex interacts with Rad51 through the Rad51 paralogues Rad55-Rad57 to mediate error-free recombination.* Nucleic Acids Res, 2013. **41**(8): p. 4525-34.
5. Sasanuma, H., et al., *A new protein complex promoting the assembly of Rad51 filaments.* Nat Commun, 2013. **4**: p. 1676.
6. Gaines, W.A., et al., *Promotion of presynaptic filament assembly by the ensemble of S. cerevisiae Rad51 paralogues with Rad52.* Nat Commun, 2015. **6**: p. 7834.
7. Huang, P., et al., *SGS1 is required for telomere elongation in the absence of telomerase.* Curr Biol, 2001. **11**(2): p. 125-9.
8. Lee, J.Y., et al., *Evidence that a RecQ helicase slows senescence by resolving recombining telomeres.* PLoS Biol, 2007. **5**(6): p. e160.
9. Shor, E., J. Weinstein, and R. Rothstein, *A genetic screen for top3 suppressors in Saccharomyces cerevisiae identifies SHU1, SHU2, PSY3 and CSM2: four genes involved in error-free DNA repair.* Genetics, 2005. **169**(3): p. 1275-89.
10. Tong, A.H. and C. Boone, *Synthetic genetic array analysis in Saccharomyces cerevisiae.* Methods Mol Biol, 2006. **313**: p. 171-92.
11. Zhao, X. and R. Rothstein, *The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3746-51.
12. Huang, M., Z. Zhou, and S.J. Elledge, *The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor.* Cell, 1998. **94**(5): p. 595-605.
13. Lee, Y.D., et al., *Dif1 is a DNA-damage-regulated facilitator of nuclear import for ribonucleotide reductase.* Mol Cell, 2008. **32**(1): p. 70-80.
14. Miller, K.M., O. Rog, and J.P. Cooper, *Semi-conservative DNA replication through telomeres requires Taz1.* Nature, 2006. **440**(7085): p. 824-8.
15. Ohki, R. and F. Ishikawa, *Telomere-bound TRF1 and TRF2 stall the replication fork at telomeric repeats.* Nucleic Acids Res, 2004. **32**(5): p. 1627-37.
16. Romano, G.H., et al., *Environmental stresses disrupt telomere length homeostasis.* PLoS Genet, 2013. **9**(9): p. e1003721.
17. Collart, M.A., *The Ccr4-Not complex is a key regulator of eukaryotic gene expression.* Wiley Interdiscip Rev RNA, 2016. **7**(4): p. 438-54.
18. Woolstencroft, R.N., et al., *Ccr4 contributes to tolerance of replication stress through control of CRT1 mRNA poly(A) tail length.* J Cell Sci, 2006. **119**(Pt 24): p. 5178-92.
19. Cotobal, C., et al., *Role of Ccr4-Not complex in heterochromatin formation at meiotic genes and subtelomeres in fission yeast.* Epigenetics Chromatin, 2015. **8**: p. 28.
20. De Keersmaecker, K., et al., *Exome sequencing identifies mutation in CNOT3 and ribosomal genes RPL5 and RPL10 in T-cell acute lymphoblastic leukemia.* Nat Genet, 2013. **45**(2): p. 186-90.
21. Vicente, C., et al., *Targeted sequencing identifies associations between IL7R-JAK mutations and epigenetic modulators in T-cell acute lymphoblastic leukemia.* Haematologica, 2015. **100**(10): p. 1301-10.
22. Puente, X.S., et al., *Non-coding recurrent mutations in chronic lymphocytic leukaemia.* Nature, 2015. **526**(7574): p. 519-24.
23. Vicente, C., et al., *The CCR4-NOT complex is a tumor suppressor in Drosophila melanogaster eye cancer models.* J Hematol Oncol, 2018. **11**(1): p. 108.

24. Pavlova, N.N. and C.B. Thompson, *The Emerging Hallmarks of Cancer Metabolism.* Cell Metab, 2016. **23**(1): p. 27-47.
25. Kohnken, R., K.M. Kodigepalli, and L. Wu, *Regulation of deoxynucleotide metabolism in cancer: novel mechanisms and therapeutic implications.* Mol Cancer, 2015. **14**: p. 176.
26. Aird, K.M., et al., *ATM couples replication stress and metabolic reprogramming during cellular senescence.* Cell Rep, 2015. **11**(6): p. 893-901.
27. Chabes, A., et al., *Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase.* Cell, 2003. **112**(3): p. 391-401.
28. Le, T.M., et al., *ATR inhibition facilitates targeting of leukemia dependence on convergent nucleotide biosynthetic pathways.* Nat Commun, 2017. **8**(1): p. 241.
29. Elledge, S.J., et al., *DNA damage and cell cycle regulation of ribonucleotide reductase.* Bioessays, 1993. **15**(5): p. 333-9.
30. Aye, Y., et al., *Ribonucleotide reductase and cancer: biological mechanisms and targeted therapies.* Oncogene, 2015. **34**(16): p. 2011-21.
31. Cesare, A.J. and R.R. Reddel, *Alternative lengthening of telomeres: models, mechanisms and implications.* Nat Rev Genet, 2010. **11**(5): p. 319-30.
32. Sobinoff, A.P. and H.A. Pickett, *Alternative Lengthening of Telomeres: DNA Repair Pathways Converge.* Trends Genet, 2017. **33**(12): p. 921-932.
33. Cesare, A.J. and R.R. Reddel, *Telomere uncapping and alternative lengthening of telomeres.* Mech Ageing Dev, 2008. **129**(1-2): p. 99-108.

English summary

In the cell, chromosomes carry the genetic material (DNA) of an organism. Telomeres are repetitive DNA structures that are located at the ends of chromosomes. The telomeres provide chromosomal stability and cell viability. The enzyme telomerase ensures that the telomeres are extended and maintained at a proper length. In most human cells, telomerase is downregulated and telomeres shorten with each round of cell division. The shortening of telomeres is also associated with aging and cancer cell development. Cancer cells use certain mechanisms to prevent telomere shortening. Most cancer cells (85-90%) express telomerase to counteract the shortening of telomeres. Other cancer cells (10-15%) do this by activating the Alternative Lengthening of Telomeres (ALT) mechanism. It is still unclear how this ALT mechanism works in the development of cancer cells and telomere elongation.

In this thesis we use the model organism *Saccharomyces cerevisiae* (baker's yeast) to study this ALT mechanism. By deactivating telomerase in the yeast cell, the telomeres become shorter after each cell division. When the telomeres become very short, cells enter a state that blocks cell division; this is called senescence. Using telomerase-independent mechanisms these cells can overcome senescence and maintain their telomere length and function. These yeast cells are called "survivors". For *S. cerevisiae*, there are two main types of survivors, type I and type II. These types of survivors differ from each other in telomere sequence and the homologous recombination (HR) proteins that are needed to extend the telomeres. Both survivor types are dependent on the Rad52-dependent HR protein and the DNA polymerase δ subunit Pol32, which is required for break-induced replication (BIR). HR and BIR play a role in repairing breaks in the DNA, but are also linked to telomere extension in survivors. Furthermore, type I survivors use the HR proteins Rad51, Rad54, Rad55, Rad57 and involve the expansion of subtelomere sequences to maintain telomeres. The type II survivors use the HR proteins Mre11, Rad50, Xrs2 (known as the MRX complex), Sgs1, Rad59 and involve the expansion of the terminal TG1-3 telomere repeats.

The Shu complex, which consists of Shu1, Shu2, Psy3 and Csm2, promotes HR and may also play a role in BIR. Given the role of the Shu complex in HR and the role of recombination proteins in senescence and survivor formation, it is possible that the Shu complex also plays a role in survivor formation. In **chapter 2** we investigated whether the absence of the Shu complex has an influence on telomere length, senescence and survivor formation. We removed the four genes that encode for the Shu complex proteins in telomerase-deficient cells. We found that these mutant cells showed similar growth and telomere length as the control group (the

telomerase-deficient cells). The absence of the Shu complex also had no influence on type I or type II survivor formation. From these results we concluded that the Shu complex is not important for the formation of survivors.

To determine which proteins and metabolic pathways are involved in survivor formation, we set up large-scale genome-wide screens. During these screens, we examined whether the absence of a certain gene influences survivor formation.

In **chapter 3** we performed a genome-wide screen to identify novel genes that are involved in type II survivor formation. We identified 23 genes, 17 of which were not previously reported as being important in type II survivor formation. A number of the genes that we have identified (*DUN1*, *MOT2*, *CCR4*) are involved in the regulation of intracellular deoxyribonucleoside triphosphate (dNTP) levels. dNTPs are the essential building blocks of nucleic acid molecules, such as DNA and RNA. We have shown that dNTP levels are increased early after inactivation of telomerase, and that this increase is important to generate type II survivors.

In **chapter 4**, a high-throughput genome-wide screen was performed to identify novel genes that are involved in type I survivor formation. We identified 34 genes during the first screening round, of which 29 were not previously reported as being important in type I survivor formation. This list of genes must be validated by additional experimentation.

The aim of this thesis was to identify novel genes and underlying mechanisms involved in the formation of survivors in *S. cerevisiae*. These findings are discussed in **chapter 5**. We have identified new genes that are important for type I and type II survivor formation that have not been mentioned before in the literature. Given the similarities between survivors and ALT cancer cells, these findings can further help us identifying genes and mechanisms that play a role in ALT cancer cell development.

Nederlandse samenvatting

In de cel dragen chromosomen het erfelijk materiaal (DNA) van een organisme. Telomeren zijn repetitieve stukjes DNA met gebonden eiwitten die zich bevinden aan de uiteinden van de chromosomen. De telomeren zorgen voor stabiliteit en beschermen de uiteinden van de chromosomen. Het enzym telomerase zorgt ervoor dat tijdens de celdeling de telomeren worden verlengd. In de meeste cellen bij de mens wordt telomerase niet voldoende tot expressie gebracht wat tot gevolg heeft dat de telomeren bij elke celdeling een stukje korter worden. Ook wordt de verkorting van telomeren geassocieerd met veroudering en het ontstaan van kanker. Kankercellen gebruiken bepaalde mechanismen om telomerenverkorting tegen te gaan. De meeste kankercellen (85-90%) brengen telomerase tot expressie en werken hier de verkorting van telomeren tegen. Overige kankcellen (10-15%) doen dit door het alternatieve verlenging van de telomeren (Alternative Lengthening of Telomeres (ALT)) mechanisme te activeren. Het is nog onduidelijk hoe dit ALT-mechanisme werkt in het ontstaan van kankercellen en het verlengen van de telomeren.

In dit proefschrift gebruiken wij het modelorganisme *Saccharomyces cerevisiae* (bakkersgist) om dit ALT-mechanisme te bestuderen. Door telomerase te deactiveren in de gistcel worden de telomeren korter na elke celdeling. Wanneer de telomeren erg kort worden, komen cellen in een toestand die de celdeling blokkeert, dit wordt senescentie genoemd. Met behulp van telomerase-onafhankelijke mechanismen kunnen deze cellen senescentie overwinnen en hun telomeerlengte en -functie behouden. Deze gistcellen worden 'survivors' genoemd. Voor *S. cerevisiae* zijn er twee types survivors beschreven in the literatuur: type I en type II. Deze types survivors verschillen van elkaar in telomerensequentie en de homologe recombinatie (HR) eiwitten die nodig zijn om de telomeren te verlengen. Beide survivortypes zijn afhankelijk van het Rad52-afhankelijke HR eiwit en de DNA polymerase δ subunit Pol32, dat essentieel is voor het break-induced replication (BIR) mechanisme. HR en BIR spelen een rol bij het repareren van breuken in het DNA, maar ook bij telomerenverlenging in survivors. Verder gebruiken type I survivors de HR eiwitten Rad51, Rad54, Rad55, Rad57 en gebruiken ze de zogenoemde 'subtelomeren' sequentie om de telomeren te verlengen. De type II survivors gebruiken de HR eiwitten Mre11, Rad50, Xrs2 (bekend als het MRX complex), Sgs1, Rad59 en verlengen de telomeren door de $TG_{1–3}$ sequentie te gebruiken.

De Shu-complex, dat bestaat uit vier eiwitten (Shu1, Shu2, Psy3 en Csm2), is een regulator voor HR. Gezien de rol van de Shu-complex in HR en de rol van recombinatie-eiwitten in senescentie en het vormen van survivors is het mogelijk dat de Shu-complex een rol speelt in het vormen van survivors. In **hoofdstuk**

2 hebben we onderzocht of de afwezigheid van de Shu-complex invloed heeft op de lengte van telomeren, senescentie en het ontstaan van survivors. Hiervoor hadden we de vier genen die coderen voor de Shu-complex eiwitten verwijderd in telomerase-deficiënte cellen. We zagen dat deze mutante cellen vergelijkbare groei en telomeerlengte vertoonden als in de controlegroep (de telomerase-deficiënte cellen). De afwezigheid van de Shu-complex had ook geen invloed op het ontstaan van type I en type II survivors. Uit deze resultaten concludeerden we dat de Shu-complex niet belangrijk is voor het vormen van survivors.

Om een antwoord te vinden op de vraag welke eiwitten en metabole routes betrokken zijn bij het ontstaan van een survivor, hebben we grootschalige genetische screenings opgezet. Bij deze screenings werd er gekeken of de afwezigheid van een bepaald gen (dat codeert voor een eiwit) invloed heeft op het ontstaan van een survivor.

In **hoofdstuk 3** hebben we deze screening opgezet om te kijken welke genen invloed hebben op het ontstaan van type II survivors. We hebben 23 genen geïdentificeerd waarvan er 17 niet eerder werden gerapporteerd als zijnde belangrijk bij type II survivor vorming. Een aantal van de genen die we hebben geïdentificeerd (*DUN1*, *MOT2*, *CCR4*) zijn betrokken bij de regulatie van intracellulaire deoxyribonucleotide trifosfaat (dNTP) concentraties. dNTPs zijn zijn de essentiële bouwstenen van nucleïnezuurmoleculen, zoals DNA en RNA. We hebben aangetoond dat dNTP concentraties vroeg na inactivering van telomerase toenemen en dat deze toename belangrijk is om type II survivors te genereren.

In **hoofdstuk 4** hebben we de genetische screening geoptimaliseerd en vervolgens gekeken welke genen invloed hebben op het ontstaan van type I survivors. We hebben 34 genen geïdentificeerd tijdens de eerste screeningronde waarvan er 29 genen nog niet eerder werden gerapporteerd als zijnde belangrijk bij type I survivor vorming. Deze lijst met genen moet worden gevalideerd. Dit wordt gedaan door de geïdentificeerde genen op een grotere schaal te screenen.

Het doel van dit proefschrift was om nieuwe genen en onderliggende mechanismen te identificeren die betrokken zijn bij de vorming van survivors in *S. cerevisiae*. In **hoofdstuk 5** worden de bevindingen bediscussieerd. We hebben nieuwe genen gevonden die essentieel zijn voor type I en type II survivor formatie. Gezien de overeenkomsten tussen survivors en ALT-kankercellen kunnen deze nieuwe bevindingen ons verder helpen met het identificeren van genen en mechanismen die een rol spelen bij het ontstaan van een ALT-kankercel.

Curriculum Vitae
List of publications

Curriculum Vitae

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List of publications

Trappetti, C., E. van der Maten, Z. Amin, A.J. Potter, A.Y. Chen, **P.M. van Mourik**, A.J. Lawrence, A.W. Paton, and J.C. Paton, *Site of isolation determines biofilm formation and virulence phenotypes of serotype 3 Streptococcus pneumoniae clinical isolates*. Infect Immun, 2013. **81**(2): p. 505-13.

van Mourik, P.M., J. de Jong, D. Agpalo, C. Claussin, R. Rothstein, and M. Chang, *Recombination-mediated telomere maintenance in Saccharomyces cerevisiae is not dependent on the Shu complex*. PLoS ONE, 2016. **11**(3): e0151314.

van Mourik, P.M., J. de Jong, S. Sharma, A. Kavšek, A. Chabes, and M. Chang, *Upregulation of dNTP levels after telomerase inactivation influences telomerase-independent telomere maintenance pathway choice in Saccharomyces cerevisiae*. G3 (Bethesda), 2018. **8**(8): p. 2551-8.

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