# THÈSE

**PRESENTÉE À**

# L'UNIVERSITÉ PIERRE ET MARIE CURIE

ÉCOLE DOCTORALE : ED515 complexité du vivant

Par Kamar-al-Zaman SERHAL

POUR OBTENIR LE GRADE DE

DOCTEUR

SPÉCIALITÉ : Biologie moléculaire, Biochimie

## Describing novel pathways involved in the onset of telomere-dependent replicative senescence in *Saccharomyces cerevisiae*

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Soutenue le 21 Novembre 2014

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# Remerciements

There is no such thing as ending what we started!!!

I would like to start by thanking the jury members for accepting to evaluate my work. I would also like to thank Emmanuelle FABRE, Cecile FAIRHEAD and Angela TADDEI, members of the PhD committee for their advices and fruitful discussion.

My big thanks go to the UMR8226.

Stephane, the adventure is ending, thank you for your advices, and your support.

Nothing would have been possible without the Biology of telomere Team directed by Teresa TEIXIERA.

Teresa, thank you for giving me the opportunity of doing my thesis in your lab. I would like to thank you also for your support, advices and discussion during and in order to finish my thesis.

Biology of telomere members, for me you were a family. You were a scaffold that I can lean on.

Pascalina, the amazing thing is that you come in full package, if you are present no need to search somewhere else. You are an amazing and expert experiment manipulator, you teach French (eternoui, se cruter…), you share talks, you are a good medicine for changing the mood, a joke telling (although It was complicated to understand everything), and I can not forget a great PHOTOGRAPHER :P. Thank you for your continuous help, chocolate bars, and the crazy moments.

Jojo! It was just amazing when someone listens our weird, crazy conservations and feels what are these talking about. Thank you for all your critics before the advices, because it's these that have led me to be ending my thesis now.

Zhou, its true that you are becoming "mechant" and hurting my feelings these days :P, but I cant be the same with you. Thank you for correcting my French, my English, and your fruitful discussions. And I promise I will never forget that I had an amazing "friend" that I can annoy anytime with my questions ☺.

Babe, its true that finally am discovering recently that our love is artificial, but I can never admit that you have made my eyes sparkle several time in these few months that we have been together. Simply, you got my motivation back, Babe!!.

Emilie, I promise you that when I stop passing by and asking what do you think about my germination, I will stop saying “ça a l’air pas de tout cava”!!. Thank you for sharing your experience and expertise with 7L ☺ .

I can’t forget also to thank the unbelievable Marcelo, François, Anna and Pierre François for being also a part of this adventure.

Besides, I would like to thank the whole unit for their unlimited support, fruitful conversation, advices, and for being a second family.

Naiima and Zoi I will never forget your phrase “ aller, c bientôt le fin”. You were a big support for me, you were there to share with me sad and celebrate happy moments. For that I would never find enough words to thank you. I would just tell you let’s meet at O’Jules ☺ .

Isabelle, Laetitia, Adeline, you were always there not only for cool moments but also for difficult, deadlines, and stressful ones. Thank you for everything.

In addition, I would like to thanks also the old unit members, especially Esther, Karim and Marjorie.

Without you, nothing would have been possible. From the first day you believed and trusted me. I hope I have made you proud and return you back at least a small portion of all what you have done for me. My family, especially my PARENTS, my sister and my brother, you are the reason for everything, the reason for why I am here right now, because of you I have learned that the first key to success is to smile ☺. Malouka, sisi, celine, for sure I would never forget our lunch on Sundays and shopping moments :p.

My friends, I can just stand speechless. You have watched all the episodes of my life, the happy ones, the stressed ones, the desperate ones, and the crazy ones. And in all of these you were really “partners in crime”.

Azza, 18 years ago you were my guide, my teacher, and my big sister. 18 years later, you are still my guide, my teacher and my big sister from whom I have learned that when we

free ourself from all toxic stuff, it's then we can shine. Thank you for all your support, for believing in me, and remember its with you with whom I enjoy my coffee the best "ya jara ".

Aline and Elie, "without taking in consideration the M2 internship, you know y!:P. I guess you are the person who has shared big adventures with me. Thank you for always being their aloune, for your advices for your support and even for sharing the best overnights ☺. It's true that Paris is not the same without you, but can you imagine, the " khawta" has done it ☺.

Rinno and Imano, its true that distance never separate sisters, thank you for all your support, and for being a reason to never give up.

Sharshar, Hanna, Diala, Wissam, Ghaydaa, Manal, Hanan, Shamaa, Nagham, dado and all my friends thank you a lot for everything, for our moments, your support, your overnights with me to end the manuscript, our craziness, correcting English, sharing conversation, and even partying.

At the end, the adventure is ending, but my smile will never end and its all because of YOU !

Thank you !!....

PhD done in the Biology of Telomere team under the direction of Maria Teresa TEIXEIRA

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# Abstract:

Linear chromosomes end with special regions, the telomeres, which ensure the integrity and the stability of the genome. In eukaryotes, telomeres also determine cell proliferation potential by triggering replicative senescence. This occurs upon telomere shortening in the absence of telomerase. In *Saccharomyces cerevisiae,* it is likely mediated by the first telomere in the cell that reaches a critically short length. This shortened telomere subsequently activates a DNA-damage-like response. How the signaling is modulated in terms of telomeric structure and context is largely unknown. During my thesis, I aimed at understanding the influence of the chromatin environment on the senescence signal starting at the shortest telomere. By comparing two sets of strains in which the shortest telomere either harbors naturally occurring subtelomeric elements or lacks these elements altogether, we show that a subtelomeric region comprising an X element counteracts the establishment of senescence. This effect is likely not due to differential Rad51-mediated homology directed repair activities at both types of telomeres. Furthermore, TERRA transcription is induced at both types of critically short telomeres, although levels are elevated in the absence of natural subtelomeric elements. Together, our results demonstrate that transcription from a telomere-proximal region greatly increases when the shortest telomere reaches a critical length, regardless of the presence of a native subtelomere or a dedicated TERRA promoter. This transcription at short telomere is intriguingly reminiscent of the transcripts found at double-strand breaks in other organisms.

Keyword: telomeres, subtelomeres, telomerase, TERRA, senescence, *Saccharomyces cerevisiae*

# Résumé:

Les chromosomes linéaires se terminent par des régions particulières, les télomères, qui assurent l'intégrité et la stabilité du génome. Chez les eucaryotes, les télomères déterminent également le potentiel de prolifération de cellules en déclenchant la sénescence réplicative. Ce signal se produit lors du raccourcissement des télomères en l'absence de la télomérase. Chez *Saccharomyces cerevisiae*, il est probablement médié par le premier télomère de la cellule qui atteint une taille courte critique. Ce télomère raccourci, active ensuite une réponse de dommage à l'ADN. Comment la signalisation est modulée en termes de structure et du contexte télomérique est largement inconnue. Au cours de ma thèse, j'ai cherché à comprendre l'influence de l'environnement chromatinien sur le signal de la sénescence à partir du télomère le plus court. La comparaison de deux souches dans lesquelles le télomère le plus court contient les éléments sous-télomériques naturels ou non, nous montre qu'une région sous-télomérique comprenant un élément X s'oppose à la mise en place de la sénescence. Cet effet n'est probablement pas dû à des différences de réparation par récombinaison homologue dépendante de Rad51 aux deux types de télomères. De plus, la transcription de TERRA est induite dans les deux types de télomères courts, bien que les niveaux soient plus élevés en l'absence d'éléments sous-télomériques naturels. Ensemble, ces résultats démontrent que la transcription à partir d'une région proximale du télomère augmente considérablement lorsque le télomère le plus court atteint une taille critique, indépendamment de la présence d'un sous-télomère natif ou d'un promoteur TERRA dédié. Cette transcription au télomère court est similaire à la transcription trouvée aux cassures double-brin chez d'autres organismes.

Titre : Identification des nouvelles voies impliquée dans la sénescence réplicative dépendante de télomère chez le *S. cerevisiae*

Mot clés : télomères, sous-télomères, télomérase, TERRA, senescence, *Saccharomyces cerevisiae*

# Abbreviations

| Abbreviation | |
|---|---|
| **ACS** | ARS Consensus Sequence |
| **ALT** | Alternative Lengthening of Telomeres |
| **ARS** | Autonomously Replication Sequence |
| **CTL** | Control |
| **DDR** | DNA Damage Response |
| **DKC** | DysKeratosis Congenital |
| **DNA** | DeoxyriboNucleic Acid |
| **DSBs** | Double Strand Breaks |
| **dsDNA** | Double Stranded DNA |
| **EST** | Ever Shorter Telomeres |
| **FRT** | Flippase Recognition Target |
| **GBD** | Gal4 Binding Domain |
| **HR** | Homologous Recombination |
| **Kb** | Kilo bases |
| **KDa** | Kilo Dalton |
| **LMP** | Low Melting Point |
| **lnRNA** | Long non-coding RNA |
| **NHEJ** | Non-Homologous End Joining |
| **nt** | Nucleotide |
| **OIS** | Oncogene Induced Senescence |
| **ORF** | Open Reading Frame |
| **PCR** | Polymerase Chain Reaction |
| **5'RACE** | 5' Rapid Amplification of cDNA Ends |
| **SAHF** | Senescence Associated Heterochromatin Foci |
| **SASP** | Senescence-Associated Secretory Phenotypes |
| **Scra** | Scrambled |
| **SDF** | Senescence-associated DNA damage Foci |
| **ssDNA** | Single Stranded DNA |
| **STAR** | SubTelomeric Anti-silencing Repeat |
| **STEX** | Single Telomere EXtension Assay |
| **STR** | SubTelomeric Repeated elements |
| **TAS** | Telomere-Associated Sequences |
| **TERRA** | Telomeric Repeat Containing RNA |
| **TPE** | Telomere Position Effect |
| **TRF** | Terminal Restriction Fragments |
| **TSS** | Transcription Starting Site |
| **UAS** | Upstream Activating Sequence |
| **VST** | Very Short Telomere |
| **6R** | Right arm of chromosome 6 |
| **7L** | Left arm of chromosome 7 |

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# Introduction

Replicative senescence is a type of arrest due to checkpoint activation. During my thesis, I was interested in replicative senescence, which is the progressive loss of the cell capacity to proliferate due to telomere attrition. Telomeres are the ends of linear chromosomes, which are essential for stable maintenance of the chromosomes. Telomere shortening, which leads to replicative senescence, occurs in the absence of the reverse transcriptase enzyme, telomerase, and telomere attrition is detected by the DNA damage checkpoint, leading to a G2/M arrest in *Saccharomyces cerevisiae*.

In my introduction, I present the crosstalk between replicative senescence and the ends of chromosomes. I start by distinguishing replicative senescence from other types of cellular senescence. Then I proceed by exploring the ends of eukaryotic chromosomes. Finally, I finish by detailing our knowledge on telomeric transcripts. These transcripts that start from subtelomeres and end at telomeres have been shown to be pivotal in the crosstalk between telomeres and replicative senescence.

Although *S. cerevisiae* was the main model used in my thesis, I briefly address the points described above in other organisms in order to assess the conservation of these phenomena throughout evolution.

# I. Cellular Senescence

Cellular senescence was first described in the 1960s as a process that limits the proliferation of human fibroblasts in cell culture (Hayflick, 1965). The Hayflick limit was described in these cells, as cells that start to divide normally then lose their ability to proliferate with time, while remaining viable in spite of being supplied with nutrients and growth factors. Non-physiological stimuli that stimulate senescent cells to re-enter the cell cycle, makes senescence an irreversible arrest. Cellular senescence contributes to tumour suppression and aging (Campisi and d'Adda di Fagagna, 2007). Its contribution to tumourigenesis occurs through the prevention of aberrant cellular proliferation, while its contribution to aging occurs through the accumulation of senescent cells in aged tissues and in several degenerative age-related pathologies. This accumulation of senescent cells may be dangerous to the organism, due to depletion of cells that might be important for tissue structure and function. Several senescence-associated markers have been used to identify senescent cells, however, these markers are not universal to all senescent cells. In addition to their arrest, human senescent cells in culture contain G1 DNA and adopt a large morphology. They also resist apoptosis and acquire changes in the transcriptional profile. This change in the transcription profile results in the secretion of senescence-associated secretory phenotypes (SASP), such as cytokines and growth factors (Campisi and d'Adda di Fagagna, 2007). In addition, senescent cells express senescence-associate β-galactosidase (SA-βGal) activity. Furthermore, senescence-associated heterochromatin foci (SAHF) that lack the linker histone H1 but contain heterochromatin proteins (HP1) are considered senescence markers and result in the silencing of genes that are needed for cell cycle progression (Narita et al., 2003). Senescence-associated DNA damage foci (SDF) are also used as markers where the phosphorylated histone H2AX is associated with p53 binding protein (53BP1) (Takai et al., 2003). In addition, the lack of DNA replication, and the expression of the tumour suppressor kinase p16 are also used as senescence markers (Campisi and d'Adda di Fagagna, 2007; Serrano et al., 1997). In mammalian cells, cellular senescence is established through the activation of the tumour suppressor; p53-p21 and $p16^{INK4a}$/pRB pathways(Campisi and d'Adda di Fagagna, 2007). The expression of these kinases as a response to DNA damage results in the blockage of cellular progression.

Mitotically active cells may undergo senescence in response to different stimuli. Two different types of senescence exist: premature senescence and replicative senescence. Premature senescence occurs before replicative senescence and is independent of telomere attrition. However, replicative senescence depends on telomere attrition (Figure 1).

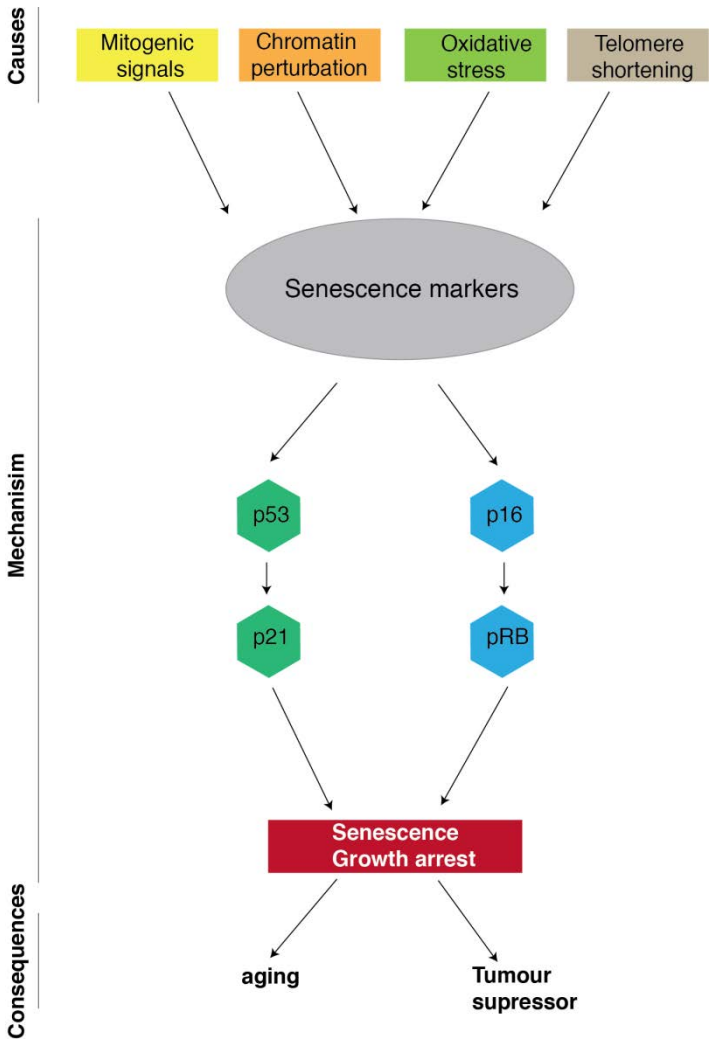



**Figure 1: Causes and consequences of cellular senescence**
In mammalian cells, mitogenic signals, telomere shortening, oxidative stress and chromatin perturbation results in senescence-associated signals that activate the p53/p21 or p16/pRB pathways. This activation results in growth arrest, i.e. cellular senescence. This arrest either contributes to aging or inhibits tumorigenesis. Based on (Campisi and d'Adda di Fagagna, 2007).

# 1. Premature cellular senescence

## 1.1. Oncogene-induced senescence (OIS)

Unbalanced mitogenic signals may promote cellular senescence. An example is the cellular senescence caused by oncogenes. OIS shows an oncogenic form of RAS that stimulates signalling pathways promoting cellular senescence. They were first described in primary fibroblasts after being transformed with *ras* mutations, leading to non-proliferating cells (Serrano et al., 1997). OIS occurs *in vivo*, the human naevi a type of benign tumour, shows the accumulation of senescent cells as a protective physiological response(Michaloglou et al., 2005).

## 1.2. Stress-induced senescence

Senescence can also occur when cells are cultured under inadequate conditions or as a response to severely damaged DNA. Cells explanted from organisms should be provided with normal conditions, such as normal levels of nutrients and growth factors. The absence of one of these conditions may promote culture shock and lead to senescence (Ramirez et al., 2001). Furthermore, oxidative stresses also promote cellular senescence in a p53-dependent manner(Campisi, 2003; Parrinello et al., 2003). To increase life span, cultures should contain a defined number of growth factors and provide physiological oxygen conditions.

## 1.3. Senescence caused by chromatin perturbation

Although SAHF have been considered markers of cellular senescence in some cells, as a result of the activation of the p16/pRB pathway, interestingly, the perturbation of chromatin results in cellular senescence. For example, the absence of a histone deacetylase, such as the Sir2 in yeasts, or treatment with a deacetylase inhibitor that results in chromatin relaxation, promote cellular senescence (Kaeberlein et al., 1999; Ogryzko et al., 1996). As in other types of premature cellular senescence, senescence caused by chromatin perturbation signaling in mammalian cells also depends on the p53 and p16 pathways (Campisi and d'Adda di Fagagna, 2007).

## 2. Replicative senescence

In 1965, Hayflick hypothesized that the loss of proliferation capacity is due to aging or senescence. Later on, this reduced capacity of proliferation was attributed to telomeric loss, in which telomeres shorten progressively (Figure 2A). This shortening is due to the inability of DNA polymerases to replicate the entire genome, the so-called "end replication problem" (Olovnikov, 1973; Watson, 1972). In somatic cells, the absence of telomerase leads to such telomeric shortening, promoting cellular senescence, which is not the case with germ-line cells that express telomerase constitutively (Harley et al., 1990; Wright et al., 1996). The expression of telomerase, a reverse transcriptase enzyme that elongates telomeres, bypasses this replicative senescence in cultured human cells (Bodnar et al., 1998; Harley et al., 1990). Replicative senescence due to short telomeres in mammalian cells results in the accumulation of molecular markers similar to those that accumulate at a double-strand break. Furthermore, this telomere-initiated senescence results in the activation of a DNA damage checkpoint (d'Adda di Fagagna et al., 2003). Furthermore, the presences of five dysfunctional telomeres in mammalian cells are required to trigger senescence (Kaul et al., 2011). This in turn results in the upregulation of the p53 pathway, but not the p16 pathway (Herbig et al., 2004). The arrest observed in OIS is similar to that of replicative senescence but the over-expression of telomerase does not restore cell growth is OIS cells (Wei and Sedivy, 1999).

Although it is still not clear if replicative senescence is linked to organismal aging, a decrease in telomere length has been detected in aged tissue (Kuilman et al., 2010). Furthermore, much evidence suggests a correlation between replicative senescence and aging. Aged primates have been shown to accumulate senescent fibroblasts associated with an increase in telomere dysfunction (Herbig et al., 2006). In humans, several studies have suggested that telomere shortening may be involved in different human disorders. For example, Dyskeratosis congenital (DKC), a rare inherited multisystem disorder that appears later in life, is associated with several mutations in genes involved in telomere maintenance (Garcia et al., 2007). In addition, the over-expression of telomerase in mice leads to an increase in the longevity of the mice accompanied by a delay in the appearance of aging symptoms (Tomas-Loba et al., 2008). Moreover, zebrafish lacking telomerase may develop phenotypes similar to those of aged

phenotypes (Henriques et al., 2013). This suggests that telomeres act as a molecular clock to signal replicative senescence (Harley et al., 1990).

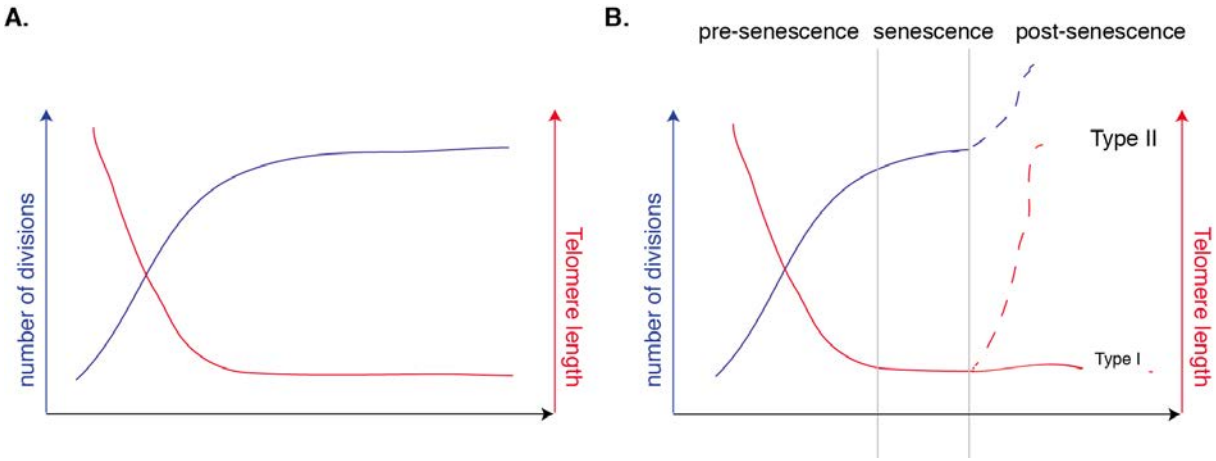



**Figure 2: Telomere and replicative senescence**
The loss of proliferation capacity is coupled with a gradual telomere shortening. Based on (Harley et al., 1990; Hayflick, 1965). (B) In S. cerevisiae, a three-step model of yeast cells behavior in a liquid culture as a response to the loss of telomerase. In the first step there is a gradual loss of viability accompanied by telomere shortening. This step is followed by a senescence stage where cells cease to divide and telomere length reaches a threshold. Finally, some cells escape senescence and become post-senescence survivors. Post-senescence may be Type I or Type II, where type I is the amplification of the subtelomeric Y' and type II is the amplification of telomeric repeats. Based on (Lundblad and Blackburn, 1993; Lundblad and Szostak, 1989).

## 3. Replicative senescence in budding yeast

Although mammalian cells have a cell proliferation limit, the unicellular organism *S. cerevisiae* does not obey this rule. On the contrary, telomerase, which is the main regulatory element of replicative senescence, is constitutively expressed in *S. cerevisiae*, which is not the case with multicellular organisms. Thus, telomere length is maintained. However, the experimental removal of telomerase promotes telomere-shortening, resulting in replicative senescence that is similar to that observed in mammalian cells (Lendvay et al., 1996; Lundblad and Szostak, 1989; Singer and Gottschling, 1994). These mutants exhibit a growth rate that decreases progressively after 60–80 generations (Teixeira, 2013). When cultured on solid media, colonies become smaller in size after several passages and share irregular shapes. Under microscopy, these cells increase in volume, arrest in the G2/M phase of the cell cycle, and are metabolically active (Enomoto et al., 2002; IJpma and Greider, 2003). Furthermore, replicative senescence

provokes a change in gene expression (Nautiyal et al., 2002; Platt et al., 2013). The response of a yeast cell to the loss of telomerase can be defined in three steps (Figure 2B). In the first step, there is a gradual loss of viability accompanied by telomere shortening. This step is followed by a senescence stage where cells cease to divide. Then some cells escape senescence through telomerase-independent mechanisms and re-acquire proliferation capacity. These cells are called post-senescence survivors. How yeast cells signal telomere shortening is discussed later. Before discussing how these cells signal telomere shortening, I will present these special structures that are essential to maintain genomic stability.

# II. Telomere

## 1. Telomere discovery

Telomere biology started in the 1930s by the cytological work of Barbara McClintock and Hermann Muller. Due to the analysis of X-ray-induced products in fruit flies, Muller obtained products such as inversions, translocations. However, rearrangements of terminal regions were absent, suggesting the presence of a protective structure at the ends of chromosomes preventing end-to-end joining. Muller named these natural protected free ends "telomeres" (Blackburn, 2006; Muller, 1938). At the same time, Barbara McClintock, while developing microscopic visualization methods on X-ray-mutagenized maize, also observed that no chromosome piece was joined to natural ends (Blackburn, 2006). Thus, in the 1930s, telomeres were defined as special functional structures that protect against chromosome fusion.

To understand the mechanisms underlying telomere function, it was essential to determine the DNA sequences and the structure of these ends. Elizabeth Blackburn used the ciliate protozoan *Tetrahymena thermophila* to take advantage of the presence of thousands of copies of the 21 Kbp linear minichromosome containing rDNA. The isolation of this minichromosome and the sequencing of its extremity, revealed that the ends are composed of tandem repeats, which vary between 20 to 70 copies of GGGGTT on the strand bearing the 3'OH (Blackburn and Gall, 1978). When a linear plasmid containing *Tetrahymena* sequences at its ends was transformed into *S. cerevisiae*, it was able to replicate. This suggested a conservation of telomere function among different species (Szostak and Blackburn, 1982). Later on, telomeric sequences of different organisms were identified. In the budding yeast *S. cerevisiae*, telomeric repeats are composed of repeats of a degenerated motif of $TG_{1-3}$ repeats (Shampay et al., 1984).

## 2. Telomere structure

### 2.1. Telomeric DNA

Telomere sequence and structure are conserved in eukaryotes. In many organisms, they are composed of tandem repeats of G-rich DNA sequences ending with a 3' overhang

(Figure 3). Their length varies among organisms and might reach several Kbp. Telomere length is heterogeneous within a cell, as terminal restriction fragments (TRF) migrate as a smear after DNA separation by electrophoresis. In *S. cerevisiae*, the average telomere length is 350 bp (Xu et al., 2013). Similar to other organisms, the *S. cerevisiae* G-rich strand possesses a 3' extension, which varies in length throughout the cell cycle. Most of the cell cycle, the length of the 3' tail is around 12 to 14 nucleotides. However, at the late S/G2 phase of the cell cycle, it might reach 20–25 nucleotides (Larrivee et al., 2004; Soudet et al., 2014). This overhang structure is not universal. For example, in *Arabidopsis thaliana*, half of the telomeres are blunt-ended (Kazda et al., 2012).

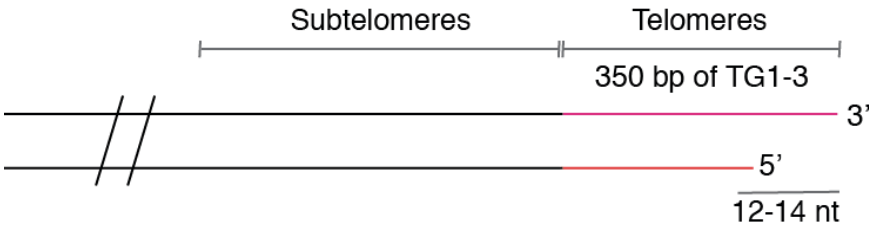



**Figure 3: Telomere DNA structure of the yeast *S. cerevisiae***
Chromosome ends with telomeres that are composed of a double stranded DNA and a 3' overhang in the G-rich strand (pink strand). Centromere-proximal to telomeres exists a subtelomeric region. Based on (Wellinger and Zakian, 2012; Xu et al., 2013).

Telomeres can form structures that are maintained by telomeric proteins. One of these structures is the T-loop, which forms a lariat structure, where the 3' overhang invades the dsDNA telomeric part (Griffith et al., 1999). This T-loop depends on the telomeric protein telomeric repeat binding factor 2 (TRF2) as seen by high-resolution microscopy (Doksani et al., 2013). Another structure is the G-quadruplex (G4), which is formed *in vitro* by telomeric sequences. This telomeric G4 might also occur *in vivo,* but its function remains unclear (Nandakumar and Cech, 2013; Venczel and Sen, 1993).

### *2.2.* Telomeric proteins in *S. cerevisiae*

To protect and stabilize telomere structure, special proteins bind the telomeric region. Some of these proteins are specific to the double-stranded DNA, while others are specific to the 3' overhang single-stranded telomeric part (Figure 4).

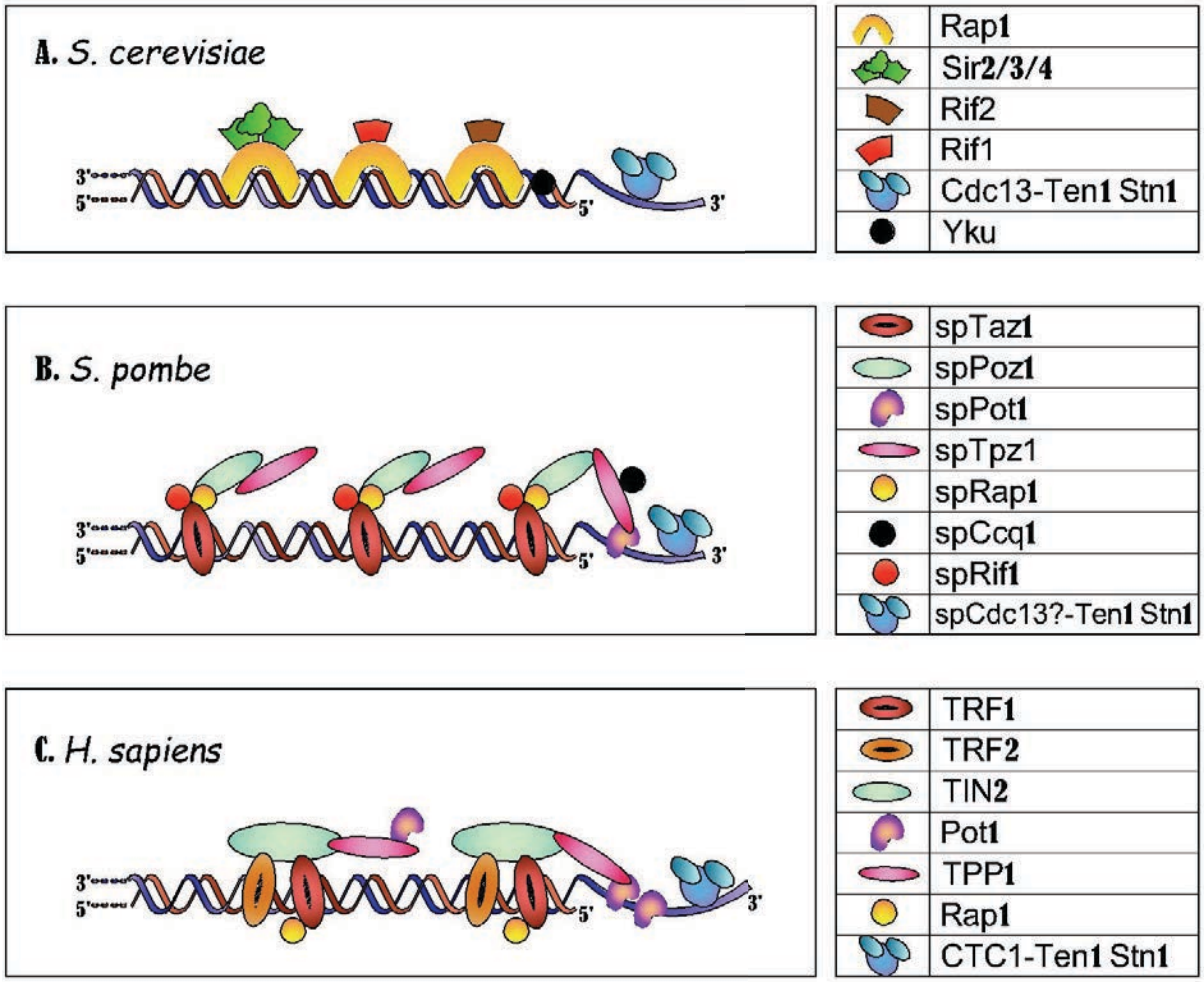



**Figure 4: Telomeric proteins**
Schematic representation of the major proteins at telomeres in (A) *S. cerevisiae* (B) *S. pombe* and (C) *H. sapiens.* Telomeric proteins in S. pombe forms a shelterin-like structure, and in H. sapiens forms a shelterin. Homologous proteins are represented in the same colour. Based on (Pfeiffer and Lingner, 2013).

### 2.2.1. Proteins that bind to telomeric double-stranded DNA

The repressor activator protein 1 (Rap1) is an essential DNA-binding protein in *S. cerevisiae.* It is a cellular transcriptional activator or repressor factor that binds the promoters of several genes (Shore and Nasmyth, 1987). It binds double-stranded DNA through its myb domain and it interacts with other proteins through its carboxyl-terminal (C-terminal) domain (Figure 4A). Rap1p is present at telomeres and possesses high affinity to telomeres (Conrad et al., 1990; Wright et al., 1992; Wright and Zakian,

1995). Rap1 molecules bind telomeric repeats every 18 bp (Gilson et al., 1993). Rap1 functions in telomere protection, telomere silencing, and telomere length regulation.

The C-terminal part of Rap1 interacts with several other telomeric proteins such as Rap1 interaction factors 1 and 2 (Rif1 and Rif2), non-essential proteins (Figure 4A). Mutants of these proteins exhibit long telomeres, suggesting that they participate in telomere length regulation (McGee et al., 2010; Wotton and Shore, 1997). Double mutants possess even longer telomeres, indicating that these proteins participate in independent regulatory pathways (Wotton and Shore, 1997). In addition to the role of Rif1 and Rif2 in telomere protection, Rif1 is also implicated in the control of replication timing in *S. cerevisiae* (Davé et al., 2014; Hiraga et al., 2014; Mattarocci et al., 2014).

In addition to the interaction of Rap1 with Rif1 and Rif2 through its C-terminal domain, through this same domain, Rap1 interacts with the silencing information regulator (SIR) components, Sir3 and Sir4, components of the silencing complex (Figure 2A; see below).

The yKu complex also binds to telomeric DNA (Figure 4A). The yKu complex is composed of the two proteins yKu70 and yKu80. The yKu complex is also essential for telomere function and maintenance. This occurs independently of its NHEJ function (Boulton and Jackson, 1996; Gravel et al., 1998).

Rap1 is conserved among species. A homologue of Rap1 also exists in *S. pombe* and mammalian cells. However, this protein is tethered to telomeres through protein–protein interactions and does not bind directly to telomeric DNA. The tethering to telomeres occurs through the binding of Rap1 to the telomere length regulator Taz1 in *S. pombe*, or telomeric repeat binding factor 1 (TRF1) and TRF2 in mammals (Bilaud et al., 1997; Broccoli et al., 1997; Chong et al., 1995; Cooper et al., 1997).

In mammals and *Schizosaccharomyces. pombe*, six different proteins bind to telomeres. In mammals, these proteins form the so-called shelterin complex. It is composed of TRF1 and TRF2, which interact with TRF1-interacting nuclear factor 2 (TIN2), which in turn interacts with TPP1. TPP1 acts like a bridge between the double-stranded binding proteins and the single-stranded binding protein POT1 (Figure 4C; (de Lange, 2005)). This overall structure is conserved in *S. pombe* in a shelterin-like complex, where spTAZ1 binds the double-stranded telomeric DNA and spRap1. spRap1 binds spPoz1, which bridges the double-stranded telomeric DNA to the telomeric overhang. spPoz1

binds to spTPZ1, which interacts with spCcq1 and spPot1 (Figure 4B; (Dehe and Cooper, 2010).

### 2.2.2. Proteins that bind to telomeric single-stranded DNA

The first single-stranded telomeric protein identified was that of *Oxytrichia nova*, the TEBP alpha/beta. In mammals and in *S. pombe*, protection of telomeres 1 (Pot1) protein binds to the 3' overhang and protects telomeric ends (Nandakumar and Cech, 2013).

At telomeres, proteins also bind the 3' overhang (Figure 4). Cell division cycle 13 protein (Cdc13) is an essential protein that binds single-stranded telomeric DNA in *S. cerevisiae* (Figure 4A; (Bourns et al., 1998; Lin and Zakian, 1996; Taggart et al., 2002)). Cdc13 is capable of binding specifically to single-stranded DNA with a binding site of 11 nt *in vitro* (Lin and Zakian, 1996; Nugent et al., 1996). It is enriched at telomeres during the late S-phase(Taggart et al., 2002), and it functions in both telomere length regulation and telomere protection.

Stn1 and Ten1 interact with Cdc13 to form the CST complex (Grandin et al., 2001; Grandin et al., 1997). The CST complex structure is similar to the replication protein A (RPA) complex that binds to ssDNA during DNA replication and DNA repair, with an additional telomere-specific domain (Gao et al., 2007; Gelinas et al., 2009). Stn1 and Ten1 may also act independently of Cdc13 and their over-expression makes it possible to bypass the absence of the essential Cdc13 in telomere DNA replication (Petreaca et al., 2006).

In mammals, the role of the CST complex is not only limited to the telomere, but it is also implicated in overall DNA replication (Miyake et al., 2009). The functional homologue of Cdc13, Ctc1, is involved in the efficient replication of telomeres and the overall genome replication at stalled forks (Gu et al., 2012; Stewart et al., 2012). *S. pombe* encodes the spStn1p and spTen1p orthologues. However, Cdc13 or Ctc1 homology has not yet been identified (Martin et al., 2007).

# 3. Telomere function

Although telomeres contain a DNA extremity that is similar to those of DSBs, telomeres are not considered DSBs. First, they are unable to interact with breaks resulting from ionizing radiation (Muller, 1938). Second, the experimental removal of telomeres from the ends of chromosomes after digestion with HO endonuclease, which generates a DSB, leads to an Rad9-dependent cell cycle arrest similar to that observed at a DSB. Therefore, the presence of the telomeres protects the ends from a Rad9-dependent cell cycle (Sandell and Zakian, 1993; Weinert and Hartwell, 1988). Finally, the presence of a G-rich sequence near a DSB, seeds *de novo* formation of telomeric repeats, capping the chromosome against its loss, and preventing cell cycle arrest (Diede and Gottschling, 1999; Kramer and Haber, 1993; Michelson et al., 2005).

## 3.1. Telomeres are not considered as double-strand break

### 3.1.1. What is a double-strand break?

Our genome is subjected to a series of exogenous and endogenous stresses that may lead to DNA breaks. The cell rapidly repairs these breaks, to maintain the genome integrity, preventing cells from dying. This repair is triggered by a surveillance mechanism called checkpoints, which inhibit cell cycle progression when damage is detected. Different types of breaks occur in the cell, but DSBs are considered one of the most harmful types of damage that threaten cell viability. Two main pathways exist to repair DSBs: the non-homologous end joining (NHEJ) and the homologous recombination (HR) pathway.

### 3.1.2. Processing of a double-strand break

DSBs in the cell are sensed in a competing manner through the yKu and the MRX complex (Mre11-Rad50-Xrs2) (Figure 5; (Clerici et al., 2008; Shim et al., 2010)). If the break occurs in the G1 phase of the cell cycle, repair occurs by the NHEJ-mediated pathway (Figure 5B-D;(Frank-Vaillant and Marcand, 2002). This pathway involves the yKu complex and leads to end joining between the two ends. For a DSB to be repaired through HR (Figure 5-E-J), a short resection is initiated by the MRX complex and the protein Sae2, generating a 50–100 nt 3' overhang (Lengsfeld et al., 2007; Lobachev et al., 2002). This short resection is followed by a long 5'-3' resection of several Kbp by exonuclease 1 (Exo1), a 5'-3' exonuclease with Flap activity (Tran et al., 2002), and the

Sgs1 protein in complex with Dna2, where Sgs1 is a helicase of the RecQ family and Dna2, is a nuclease. These two pathways operate independently, where one nuclease might compensate the absence of the other. Only cells lacking the three nucleases *exo1Δ sgs1Δ sae2Δ* or *exo1Δ sgs1Δ mre11Δ* are lethal (Gravel et al., 2008; Mimitou and Symington, 2008; Shim et al., 2010; Zhu et al., 2008). Exo1 recruitment is stimulated through the MRX complex (Shim et al., 2010).

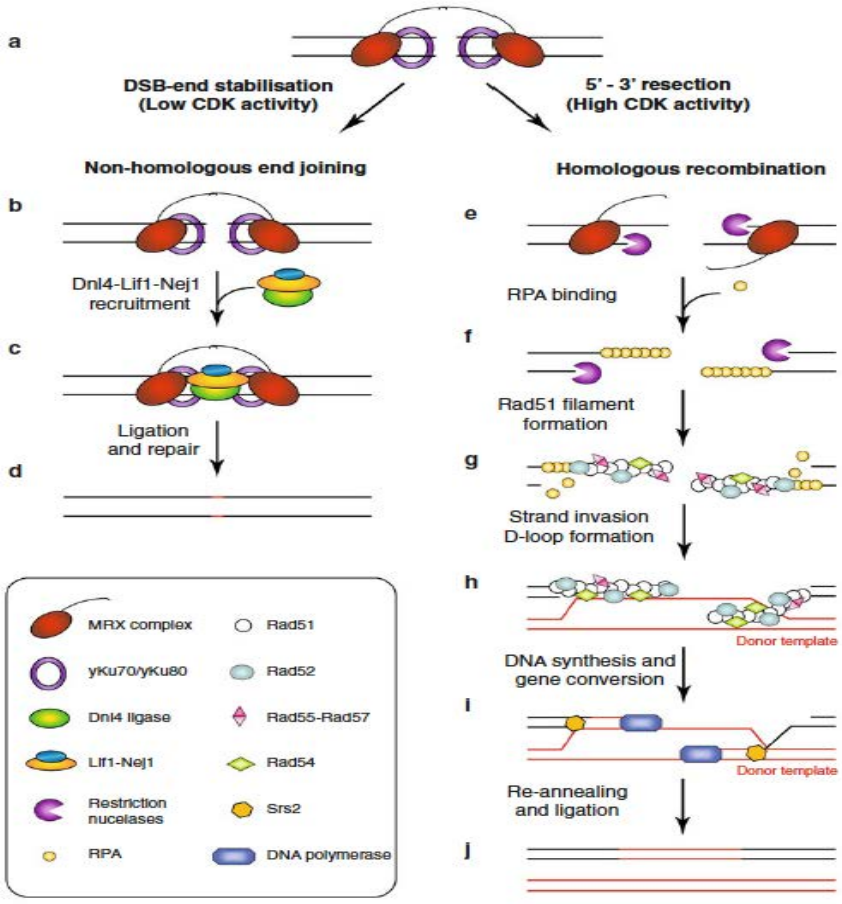



**Figure 5: Illustration of DSB repair by NHEJ and HR pathways in *S. cerevisiae***
After a DSB, MRX and Ku complex binds to DNA ends. (b) During G1 phase of the cell cycle, the CDK activity is low and DSB is repaired by NHEJ. (c) In NHEJ, Dnl4- Lif1-Nej1 ligase complex are recruited (d) The ligase complex mediate the ligation of broken ends and the repair of the lesion. (e) During the S/G2 phase of the cell cycle, where the CDK activity is high, DSB is repaired by HR. DNA ends resection is initiated by the MRX complex and Sae2, the followed by Exo1 and/or Dna2 coupled with the Sgs1 helicase. (f) The ssDNA generated becomes a substrate to RPA (g) Rad51 filament forms around ssDNA. The filament formation involves also Rad52, Rad55-Rad57 and Rad54 (h) the filament formation stimulates strand invasion and the formation of D-loop. (i) The Rad51 filament is the disassembled by Srs2 and this is followed by DNA synthesis. (j) The

Holliday junction is then resolved, and DNA ligation occurs between newly synthesized DNA. Adapted from (Finn et al., 2012).

In mammals, the orthologue of Sgs1 is BLM, and Exo1 exists in mammals. BLM and Exo1 function in a similar way to those of *S. cerevisiae*, but the action of Exo1 is stimulated by BLM (Nimonkar et al., 2011). After resection, single-stranded DNA is covered by RPA, preventing the formation of secondary structures of resected DNA (Chen et al., 2013).

Following RPA loading on ssDNA, Rad51 replaces RPA on ssDNA, forming a filament along this ssDNA by nucleation(Sung and Robberson, 1995; Yang et al., 2001). This binding requires Rad52, which leads to RPA displacement through its domain of interaction with RPA (Krejci et al., 2002; Plate et al., 2008; Seong et al., 2008; Shinohara and Ogawa, 1998; Sugiyama and Kowalczykowski, 2002). In addition to Rad52, Rad55, and Rad57, paralogues of Rad51 are also important for RPA displacement and Rad51 loading (Gasior et al., 2001). Rad51 mediates the search for sequence homology, followed by strand invasion. After strand invasion, new DNA synthesis occurs, using the 3' invading end as a primer. This HR repair pathway occurs in the S/G2 phase of the cell cycle, depending on high cyclin-dependent kinase (CDK) activity (Figure 5G-J; (Ira et al., 2004).

### 3.1.3. A DSB generates a DNA damage checkpoint

The checkpoint response to breaks depends mainly on Tel1 (ATM in mammals) and Mec1 (ATR in mammals) in *S. cerevisiae* (Finn et al., 2012*).* The checkpoint starts with the binding of Tel1 to DNA breaks via already bound MRX (Mre11-Rad50-Xrs2), mainly via its interaction with Xrs2 in *S. cerevisiae* (D'Amours and Jackson, 2001; Grenon et al., 2001; Nakada et al., 2003). The absence of the helicase/nucleases Sgs1 and Exo1 prevents downstream activation of the checkpoint (Gravel et al., 2008). Thus, the long resection step and the coating of ssDNA by RPA act as a substrate for recruitment of Mec1, Ddc2, and the 9-1-1 clamp, which are essential for checkpoint activation (Lee et al., 1998; Sanchez et al., 1999). Mec1 phosphorylates the histone H2A, leading to the binding of the mediator Rad9 (Downs et al., 2000; Hammet et al., 2007). Rad9 activates the Mec1 checkpoint by activating the effector kinases Rad53 and Chk1 (Blankley and Lydall, 2004; Sanchez et al., 1999; Usui et al., 2009). This leads to cell cycle arrest where the cell proceeds to repair the break, preventing chromosome loss in the next mitosis.

In mammalian cells, the ATM and ATR kinases function through the mediators 53BP1 and MDC1, and lead to the phosphorylation of Chk1 and Chk2, respectively (Finn et al., 2012).

## 3.2. Telomeric proteins protect chromosome ends

The main function of telomeres is to protect chromosome ends against degradation, fusion, and checkpoint recognition. This protection involves the telomeric repetitions themselves and a small number of telomeric proteins known to be involved in telomere biology.

Telomere end protection is mediated through different proteins depending on the cell cycle phase. Capping outside of the S-phase occurs through the double-stranded binding proteins Rap1 and Rif2. The absence of Rap1 promotes the formation of telomere-to-telomere fusions in an NHEJ-dependent manner, leading to loss of viability (Bonetti et al., 2010b; Pardo et al., 2006). Furthermore, the artificial targeting of the C-terminal domain of Rap1 at non-telomeric DSBs inhibits repair by NHEJ (Marcand et al., 2008). The inhibition of NHEJ is mediated through Rif2, which inhibits the access of nucleases (Bonetti et al., 2010a; Bonetti et al., 2010b; Marcand et al., 2008). Furthermore, Rif2 plays a role in the recruitment of the histone deacetylase Rpd3L, to maintain telomere looping and therefore protect against nucleases (Poschke et al., 2012).

The yKu complex blocks the single-stranded DNA formation in the G1 phase through inhibiting the action of Exo1 nuclease(Bertuch and Lundblad, 2004; Bonetti et al., 2010a; Bonetti et al., 2010b). In non-dividing quiescent cells, Rap1p protein and the yKu complex mediate the capping (Vodenicharov et al., 2010).

In mammalian cells, the homologue of Rap1, TRF2, functions in protecting telomeres. The absence of this TRF2 protein leads to telomere deprotection, favouring the formation of dicentric chromosomes by eliciting the ATM checkpoint, resulting in NHEJ repair and telomere–telomere fusions (Karlseder et al., 1999; van Steensel et al., 1998). In a similar manner, the loss of spTaz1 in *S. pombe* leads to telomere deprotection and enhanced resection by the MRN complex (Ferreira and Cooper, 2001).

Another capping mechanism occurs with the CST complex, which binds single-stranded telomeric DNA. This capping occurs mainly in the late S-phase and G2/M phase of the

cell cycle, where it prevents the extensive resection that might occur at telomeres (Booth et al., 2001; Grandin et al., 2001; Grandin et al., 1997). Rif1 becomes essential for cell viability in the absence of Cdc13 and Stn1, the telomeric single-stranded binding proteins, therefore assisting in telomere protection (Anbalagan et al., 2011).

# 4. Maintenance of telomeres

To perform their function and protect against genome instability, telomeres should be replicated during each cell cycle and processed to regenerate their special telomeric structure. The presence of the repetitive GC-rich sequence and the presence of the 3' overhang pose problems for the replication machinery that have to be solved to maintain genome integrity and to allow progression through cell division.

## 4.1. Telomere replication

### 4.1.1. Replication of the repeated sequence

Telomeric DNA is replicated by semiconservative DNA replication, where the G-strand serves as a template for lagging strand synthesis and the C-strand serves as a template for leading strand synthesis (Figure 6). This replication occurs late in the S-phase and is mediated by replication origins located in the centromere-proximal region next to telomeric repeats (McCarroll and Fangman, 1988; Wellinger et al., 1993a). However, when telomeres become short, they replicate in the early S-phase (Bianchi and Shore, 2007; Davé et al., 2014; Makovets et al., 2004). Furthermore, the G-rich nature of telomeric DNA increases the possibility of the formation of secondary structures such as G-quadruplex. This was proposed to slow down the progression of the replication fork (Lopes et al., 2011; Paeschke et al., 2011), which has been observed by two-dimensional gel analyses (Ivessa et al., 2002). At telomeres, this pausing is limited by the rDNA recombination mutation helicase Rrm3 (Ivessa et al., 2002).

The progress of the replication fork at non-telomeric loci is bi-directional. Thus the pausing or collapse of the fork can be rescued by another replication fork that comes from the opposite direction. However, at telomeric loci, there is only one replication fork. Therefore, fork-stalking rescue cannot occur the same way as at non-telomeric loci.

### 4.1.2. The DNA end replication problem

The DNA end replication problem was proposed in the early 1970s. As the mechanism of DNA replication was discovered, Olovnikov and Watson realized that the new strand generated after replication was shorter than the starting template (Olovnikov, 1973; Watson, 1972). Indeed, conventional DNA polymerases are unidirectional and synthesize DNA in a 5' to 3' direction. Furthermore, these polymerases lack the ability to start *de novo* synthesis, i.e., they need an RNA primer to initiate DNA synthesis. Thus, a DNA polymerase synthesizing the leading strand is continuous and can extend the RNA primer until the end, theoretically producing a blunt end. On the other hand, the synthesis of the lagging strand is discontinued and needs an RNA primer for each Okazaki fragment. The RNA fragment is then removed by strand displacement, flap formation, and then fill-in occurs. However, the removal of the last RNA primer on the 5' end of the chromosome leaves a gap that cannot be filled by conventional polymerases. This generates a product that is shorter than the template for at least the length of the RNA primer on one strand.

The discovery that chromosome extremities of eukaryotes are not blunt but contain a 3' overhang has challenged the conventional DNA end replication problem by not being only a problem of lagging strand replication (Lingner et al., 1995; Wellinger et al., 1993b). Although the removal of the last RNA primer during lagging strand replication regenerates the 3' overhang, with the synthesized product being shorter than the initial state, unexpectedly, the blunt product of the leading strand also appears to be processed to regenerate a functional telomere with a 3' overhang (Figure 6; (Lingner et al., 1995).

Thus, the DNA end replication problem consists of a continuous loss of information after each replication cycle of the lagging strand, and of the regeneration of the telomeric structure after each cycle of leading strand replication.

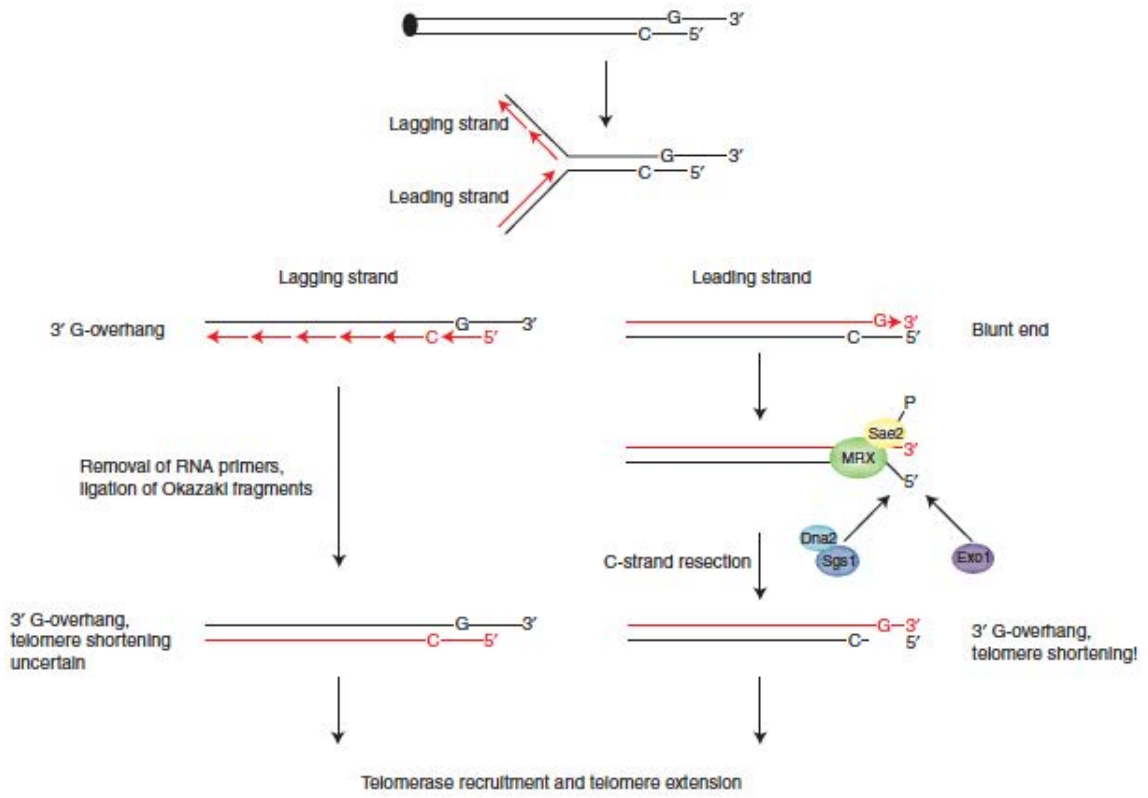



**Figure 6: The DNA-end replication problem**
The G-strand serves as a template for lagging strand synthesis (left) and the C-strand serves as a template for the leading strand synthesis (right). The removal of the last RNA primer at the lagging strand generates a telomere that is shorter than the initial strand and restores the 3' overhang. At the leading strand, 5' resection re-generates the 3' overhang. In the two cases, telomere shortens. Adapted from (Pfeiffer and Lingner, 2013).

### 4.1.3. Regeneration of the 3' overhang

Being important in telomere function, G-overhang regeneration is a must. The 12–14 nt G-overhang regeneration occurs post-replicationally, and consists of the degradation of the C-strand that has just replicated (Dionne and Wellinger, 1996; Larrivee et al., 2004; Soudet et al., 2014). The regeneration of the overhang is only linked to the leading strand, since the G-overhang length of the lagging strand does not change over the cell cycle, and resection activities are found exclusively at leading and not lagging telomeres (Faure et al., 2010; Soudet et al., 2014). The degradation is similar to the degradation that occurs at a DSB with the sole difference that the resection extends several Kbp from the site of a DSB, where the maximum length of the resection at telomeres is around 40

nt (Soudet et al., 2014). The degradation of the C-strand is regulated by the cell cycle and depends on the Cdk1 kinase, and reaches its maximum length at the late S-phase (Dionne and Wellinger, 1996; Larrivee et al., 2004; Soudet et al., 2014). The MRX complex is important for C strand resection, particularly the Mre11 exonuclease (Larrivee et al., 2004). Telomeric resection depends on the MRX-Sae2, Sgs1, or Exo1 in complex with the Dna2 flap endonuclease (Figure 6; (Bonetti et al., 2009; Larrivee et al., 2004)). This resection is limited by Cdc13, which prevents extended resection from occurring in the late S-phase (Booth et al., 2001). This resection is then compensated presumably by fill-in activities. These require RNA priming, as for lagging strand synthesis. Furthermore, it has been suggested that the length of the G-overhang is equivalent to the length of the distal RNA primer, suggesting that the removal of this primer is sufficient to generate the G-overhang at the lagging strand (Soudet et al., 2014).

Despite the similarity of the mechanism used to generate the G-overhang via DSB processing, it is important to note that this limited resection is not enough to trigger checkpoint activation and cell cycle arrest, emphasizing again that the telomere structure is different from that of DSBs.

## 4.2. Telomerase

A consequence of this DNA end replication problem is the generation of a shorter product than the starting template, which then needs to be compensated.

### 4.2.1. Telomerase: a reverse transcriptase

The study of telomerase started with the work of McClintock, in 1939, when she observed that, under certain circumstances, the broken ends of ears of corn can be "healed", resulting in new natural ends (Blackburn, 2006). Then, while many hypotheses were suggested to understand the DNA end replication of telomeres, much evidence of the existence of an enzyme capable of elongating telomeres started to accumulate. For example, the telomeric repeat tract being repeated on a minichromosome in the ciliate *Tetrahymena thermophila* was heterogeneous in length (Blackburn and Gall, 1978) . Furthermore, the sequencing of the linear plasmid containing telomeric *Tetrahymena* extremities that was successfully replicated in yeast showed that yeast telomeric $TG_{1-3}$

repeats were added to ciliate repeats (Shampay et al., 1984). Later on, a telomere addition activity was purified in *Tetrahymena (*Greider and Blackburn, 1985). Then it was found that telomerase consists of protein and RNA subunits. The RNA subunit is complementary to the G-rich strand, and is used as a template to extend the 3' end of the G-rich sequence (Greider and Blackburn, 1987, 1989).

At the same time, screens on yeast cells to identify genes that have defective telomere function were done. These screens led to the identification of the "ever-shorter telomeres" (EST) phenotype (Lendvay et al., 1996; Lundblad and Szostak, 1989; Singer and Gottschling, 1994). This phenotype was characterized by continuous telomere shortening and progressive loss in cell viability. Identified genes were *EST1, EST2, EST3,* and *EST4*. *TLC1* encoding the RNA subunit was found independently (Singer and Gottschling, 1994). Later on, *EST4* was demonstrated to be a point mutation in *CDC13*, then not considered a telomerase subunit, since it did not co-immunoprecipitate with TLC1 (Hughes et al., 2000; Lendvay et al., 1996). Finally, purification of telomerase activity from ciliates led to the identification of the gene and the finding that yeast *EST2* indeed encodes the catalytic activity of the telomerase, and *EST1* and *EST3* encode the accessory subunits. TLC1 is an RNA of 1.157 nt with a 17 nt stretch complementary to the G strand (Singer and Gottschling, 1994).

The four subunits Est1, Est2, Est3, and TlC1 are present at low levels and have been shown to be essential *in vivo* to form the telomerase holoenzyme and perform telomerase activity. However, Est1 and Est3 are not essential *in vitro* (Lingner et al., 1997).

Telomerase composition and structure is conserved in other organisms. In humans it is composed of the hTERT catalytic subunit and the hTR RNA subunit (Nandakumar and Cech, 2013).

### 4.2.2. Telomerase activity

The main function of telomerase is to re-elongate telomeres. Although different telomerase subunits associate with telomeres at different time points of the cell cycle, different studies have demonstrated that this activity occurs at the late S/G2 phase and not at the G1 phase of the cell cycle (Diede and Gottschling, 1999; Gallardo et al., 2011; Marcand et al., 2000; Taggart et al., 2002). In previous studies, the presence of a short

telomere demonstrated that telomere lengthening occurs at the late S/G2 phase (Diede and Gottschling, 1999; Marcand et al., 2000). Furthermore, at artificially induced short telomeres, Est1, Est2, and Cdc13 are enriched at the late S/G2 phase(Bianchi and Shore, 2007; Sabourin et al., 2007). Furthermore, single cell analysis of labeled Tlc1 shows that it associates with telomeres throughout the cell cycle. However, its mobility decreases at the late S phase, and telomere–telomerase complexes increase, forming telomerase recruitment structures (T-Recs) (Gallardo et al., 2011). Telomerase recruitment to telomeres has been shown to be regulated by the cell cycle and to occur in two steps. The first step consists of the recruitment of Est2 to telomeres via the interaction between Tlc1 and Yku occurring in the G1 phase (Fisher et al., 2004; Peterson et al., 2001; Taggart et al., 2002). This step is essential to recruit TLC1 to the nucleus, because in the absence of y*ku80*, TLC1 fails to accumulate telomerase in the nucleus (Gallardo et al., 2008). However, the second step depends on the recruitment of Est2 to telomeres. Est2 recruitment to extremities occurs through Tlc1–Est1 and Est1–Cdc13 interactions, which occur at the late S phase. Thus Est1 bridges the connection between Cdc13 and telomerase (Evans and Lundblad, 1999). Furthermore, Est2 tethering to Cdc13 eliminates the requirement of Est1 (Evans and Lundblad, 1999). These results suggest that telomerase activity is coordinated with DNA replication of telomeres occurring at the late S-phase.

## 5. Telomere length regulation in the presence of telomerase

In a given population, telomeres are always dynamic and are subjected to shortening and lengthening activities. This results in heterogeneity in telomere length among telomeres of a given cell. The distribution of telomere length is asymmetrical, and the shortest telomere of the cell is well distinguishable from other telomeres in the cell (Shampay et al., 1984; Xu et al., 2013).

The artificial generation of short telomeres (Marcand et al., 1999) or the presence of short telomeric tracts near an HO cut (Diede and Gottschling, 1999), demonstrate that telomerase is more active at short telomeres compared to telomeres of wild-type length (Marcand et al., 1999). Furthermore, single telomere extension assays (STEXs) have demonstrated that only 8% of telomeres undergo lengthening by telomerase. This

lengthening is preferential at short telomeres (Teixeira et al., 2004). This action of telomerase on short telomeres has been explained by the protein counting model involved in the regulation of telomere length (Marcand et al., 1997). This protein counting model is defined as the number of telomeric proteins bound to telomeres, depend on the telomeric length. Long telomeres bound by a higher number of telomeric proteins prevent telomerase recruitment or activity. On the contrary, short telomeres are bound by less telomeric proteins leading to the recruitment of telomerase to these short telomeres. In *S. cerevisiae,* the telomere length regulation depends mainly on Rap1, where the interaction Rap1–Rif1–Rif2 serves to dictate telomere length (Marcand et al., 1997). Telomerase recruitment to short telomeres also depends on Tel1 and the modification that might result from telomeric proteins such as Cdc13 (Wellinger and Zakian, 2012). Indeed, multiple posttranslational modifications are employed in telomere maintenance (Peuscher and Jacobs, 2012). SUMOylation, one of these modifications, is the transfer of a small Ubiquitin-like modifier (SUMO) to a target protein via an enzymatic cascade. Cdc13 is one of the proteins being SUMOylated and its SUMOylation is regulated throughout the cell cycle. SUMOylated Cdc13 restrains telomerase elongation in the early S-phase (Hang et al., 2011).

The association of Tel1 is also essential for telomerase recruitment to short telomeres (Ritchie et al., 1999). Tel1 binds short telomeres and its binding depends on the MRX complex and is inhibited by Rif2 (Bianchi and Shore, 2007). This suggests that telomerase activity is tightly regulated, and depends on telomeric proteins to ensure its recruitment to the shortest telomeres.

## 6. Telomeres in replicative senescence

In the absence of telomerase, cells exhibit the “ever shortening telomere” phenotype marked by telomere shortening and loss of cell viability. This loss of cell viability is rarely rescued by the formation of survivors, which maintain telomere length through an alternative mechanism.

Although telomeres protect chromosome ends and prevent the activation of the DNA damage response (DDR), telomerase knockdown in *S. cerevisiae* leads to telomere shortening and loss of viability after 60–80 generations (Lundblad and Szostak, 1989).

This arrest is due to the length of the shortest telomere in the cell, and not to the average telomere length (Abdallah et al., 2009; Fallet et al., 2014; Xu et al., 2013). This short telomere induces a DDR, leading to the accumulation of cells in the G2/M phase (Figure 7; (Abdallah et al., 2009; Enomoto et al., 2002; IJpma and Greider, 2003)). This arrest depends on Mec1 and not on Tel1. Thus short telomeres lead to checkpoint activation, similar to DSBs (Abdallah et al., 2009; Fallet et al., 2014). The shortening of telomeres decreases the amount of telomeric protein bound to telomeres, and leads to their redistribution. Rap1 is one of the telomeric proteins that are redistributed upon senescence. It re-localizes upstream of core histone-encoding genes, and modifies their expression. This re-localization from telomeres is Mec1-dependent (Platt et al., 2013). Furthermore, the absence of Rif1 and Rif2 leads to senescence acceleration, suggesting that the protection against nucleases is also essential in senescence to maintain telomeres (Chang et al., 2011).

The absence of telomerase results in telomere shortening, leading to a change in telomere identity and resulting in signaling that is similar to that of a DSB. Cell cycle arrest depends on the kinase Mec1, which is enriched at short telomeres, and its absence suppresses senescence (Abdallah et al., 2009; Enomoto et al., 2002; IJpma and Greider, 2003; Fallet et al., 2014). Tel1 also binds short telomeres and is involved in the establishment of senescence. A two-step model has been proposed, where telomeres are maintained in a pre-senescence stage when bound by Tel1 and then switch to a senescence stage when bound by Mec1 (Abdallah et al., 2009; Fallet et al., 2014). Checkpoint activation due to short telomeres does not depend on either Rad9 or Rad53 only, since deletion does not suppress cell cycle arrest completely. However, Rad9 phosphorylates Rad53 in the absence of telomerase. This suggests the presence of an alternative pathway. This is ensured by the Mrc1 checkpoint, involved in stalled replication (Enomoto et al., 2002; IJpma and Greider, 2003(Grandin et al., 2005; Teixeira, 2013). Therefore, short telomeres may trigger not only a DSB-like response but also a replication stress response (Teixeira, 2013).

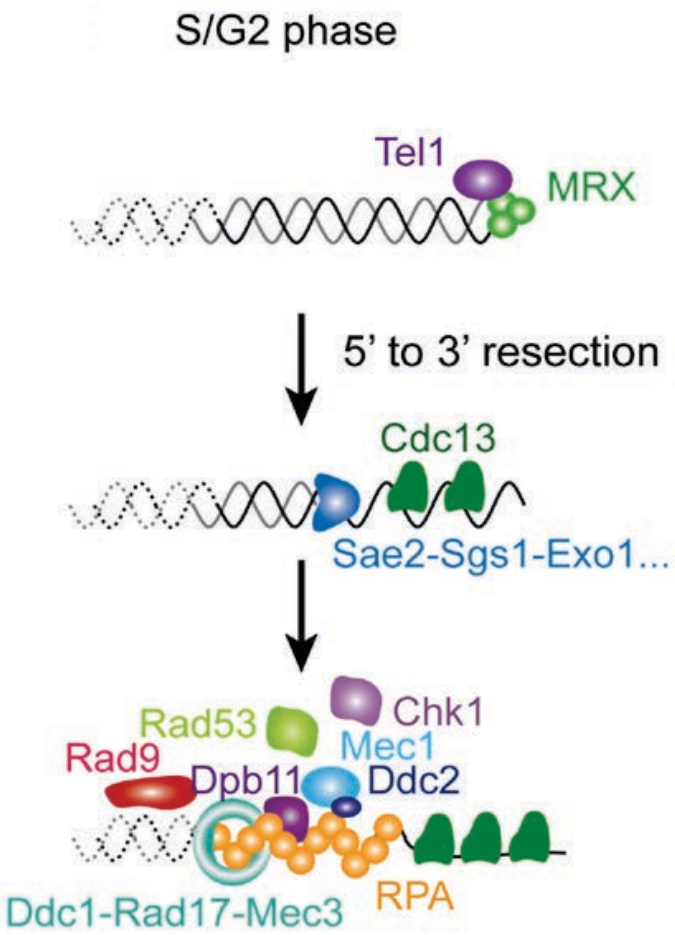



**Figure 7: Activation of DNA damage checkpoints at short telomeres in *S. cerevisiae***
In the absence of telomerase, Tel1 binds short telomeres and maintain telomeres in a pre-senescence phase. Tel1 then recruits the MRX complex. The MRX complex with the Sae2-Sgs1 and Exo1 stimulates extensive resection at short DNA. ssDNA is then coated with RPA, in which then recruits Mec1. Mec1 checkpoint is then activated and leads to a cell cycle arrest. Adapted from (Teixeira, 2013).

One single short telomere reaching a critical length is sufficient to arrest the cell cycle (Abdallah et al., 2009; Fallet et al., 2014; Khadaroo et al., 2009). This short telomere is recognized as a DSB, since it recruits nucleases such as Mre11 and Exo1, and induces resection, therefore becoming Mec1 substrate and signaling checkpoint activation (Abdallah et al., 2009; Fallet et al., 2014; Khadaroo et al., 2009). The absence of these nucleases in telomerase-deficient cells reduces ssDNA at telomeres, and delays replicative senescence (Fallet et al., 2014). Furthermore, HR proteins such as Rad51 and

Rad52 bind to short telomeres in a Tel1-dependent manner, and their presence protects against extended resection. Indeed, the absence of HR proteins leads to an acceleration of senescence accompanied by an increase in single-stranded DNA (Abdallah et al., 2009; Fallet et al., 2014; Khadaroo et al., 2009(Ballew and Lundblad, 2013). In addition, in telomerase deficient cells, SUMOylation is also essential to determine the senescence rate. The absence of the SUMO E3 ligase Mms21 accelerates replicative senescence through the accumulation of recombination intermediates(Peuscher and Jacobs, 2012).

Thus, a short telomere is maintained through a mechanism similar to that of DSB processing. However, in the case of a DSB, the damage is repaired and cells re-enter the cell cycle, while telomere shortening in the absence of telomerase is irreversible and cells remain arrested at the G2/M phase.

## 7. Post-senescence: survivors

Late in cell cultures of senescent yeast cells, some telomerase-deficient cells escape from cell cycle arrest and restart division. These cells are called survivors; they are HR-dependent and have an abnormal telomere length (Lundblad and Blackburn, 1993). Two different types of survivors exist: type I and type II, both being Rad52- and Pol32-dependent (Figure 2A;(Lydeard et al., 2007; Teng and Zakian, 1999)). In a single culture of senescent cells, the two types of survivors might arise but type II outcompetes type I due to better growth. Survival is a reversible mechanism since the re-expression of telomerase allows the re-establishment of telomere equilibrium (Makovets et al., 2008; Teng and Zakian, 1999).

Telomeres in type I survivors consist of massive amplifications of the subtelomeric element Y' of the subtelomere (see below), followed by short telomere sequences ending with a normal G-tail (Larrivee and Wellinger, 2006).

Telomeres in type II survivors consist of large amplifications of telomeric repeats. The long telomeric tracts found in these cells extend over 12 Kbp (Teng et al., 2000; Teng and Zakian, 1999). These long telomeres are subjected to progressive shortening and require constant recombination events to be maintained.

Since HRs such as Rad52 and Pol32 are similarly required for their emergence, telomere maintenance in these cells may occur by a mechanism based on break-induced replication (BIR) (Lydeard et al., 2007). In this model, short telomeres may invade longer telomeres or subtelomeres that can serve as a template for elongation. However, survivors have also been proposed to contain extra-chromosomal circles, i.e., circular Y' elements for type I and TG-repeat circles for type II, which might serve as a substrate for rolling circle recombination (Larrivee and Wellinger, 2006).

Nonetheless, maintenance of chromosome ends in both types may depend on different pathways, as attested by the different genetic requirements for their survival.

Hence, type I survivors require Rad51, Rad54, Rad55, and Rad57 recombination proteins to proliferate (Chen et al., 2001; Le et al., 1999), while type II survivors depend on the MRX complex, Rad59, and Sgs1 (Chen et al., 2001; Le et al., 1999; Teng and Zakian, 1999).

# III. Subtelomeres

Another repetitive element found at the terminal sequences of chromosomes has been identified as telomere-associated sequences (TAS), also known as the subtelomeric region. Despite progress in understanding telomere biology, the exact functions of subtelomeres remain to be identified. Due to their repetitive nature, and their presence at all chromosomes, many technical problems render this region not well explored. This region exists in many organisms. In the organisms where it is present, it can be divided into two regions: a region that is directly adjacent to the telomeric repeats and often lacks genes, and another more internal region usually containing non-essential genes that are expressed only under certain conditions.

## 1. Discovery

To better understand the control mechanism that regulates eukaryotic chromosomal replication in yeasts, it is necessary to identify the regions where replication initiates. Studies that have focused on this have identified two categories of autonomously replicating sequences (ARSs): those that are unique and those that are repetitive in the genome (Chan and Tye, 1980). The first ARS to be identified was in the subtelomeric region (Chan and Tye, 1983a). This region is located adjacent to telomeric repeats and is composed of two elements called the X and Y' elements, plus internal telomeric repeats (Chan and Tye, 1983b; Walmsley et al., 1984). The X element is present at all chromosomes while the Y' element number varies between 0 and 4 copies at chromosome ends (Chan and Tye, 1983b). Later studies identified repetitive sequences near chromosomal termini in different organisms such as humans and different yeasts (Louis, 1995). The subtelomeric region varies in size among different organisms and in some organisms (Figure 8); it is shared among different chromosomes. Comparisons of different subtelomeric structures of eukaryotes have suggested the presence of a mosaic structure of repeated elements, which evolved for adaptation to special environments (Louis, 1995; Mefford and Trask, 2002; Pryde et al., 1997).

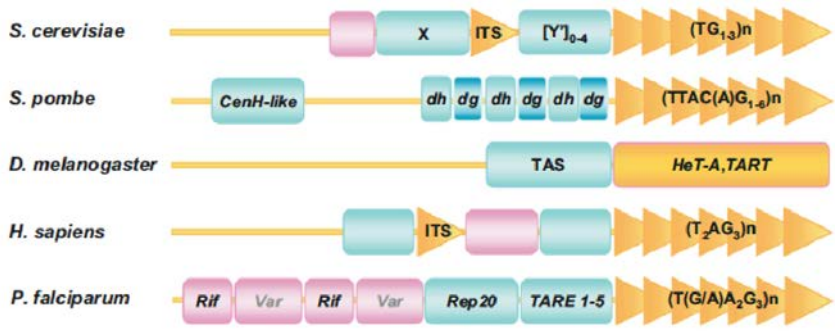



**Figure 8: Chromosome ends of different organisms**
Subtelomeric region is composed of repeated sequences (blue) and genes (pink). The different telomeric ends repeats are represented in yellow. X, X-element; Y', Y'-element; ITS, Interstitial telomeric repeats; dh, dg, *S. pombe* subtelomeric repeats; TAS, Telomere Associated Repeats; Var, Rif, *P. falciparum* subtelomeric genes; Rep20, TARE1-5, subtelomeric repeats. Adapted from (Ottaviani et al., 2007).

## 2. *S. cerevisiae* subtelomeres

Despite their repetitive sequences, the budding yeast subtelomeres and genomes are the best characterized (Goffeau et al., 1996). These repeated sequences exhibit variations in distribution among chromosome ends within the same strain as well as differences among different strains (Figure 9). The sequence of subtelomeres was obtained by inserting a unique URA3 marker at each telomere independently. The integration of URA3 at telomeres was achieved by adding telomeric repeats adjacent to the URA3 marker used for transformation, allowing HR within telomeric sequences. Then Ura3-positive transformants were selected and verified by pulse field gel electrophoresis (PFGE) and southern blots to identify the marker destination and verify its terminal integration. Finally, telomeric ends were digested by restrictive enzymes, cloned, analyzed, and sequenced (Louis and Borts, 1995). *S. cerevisiae* subtelomeres vary in length up to 20 Kbp and many subtelomeric genes have been conserved through evolution among *Saccharomyces* species (Britten, 1998; Fabre et al., 2005). Unlike telomeric repeats, *S. cerevisiae* subtelomeres are organized into nucleosomes (Wright et al., 1992).

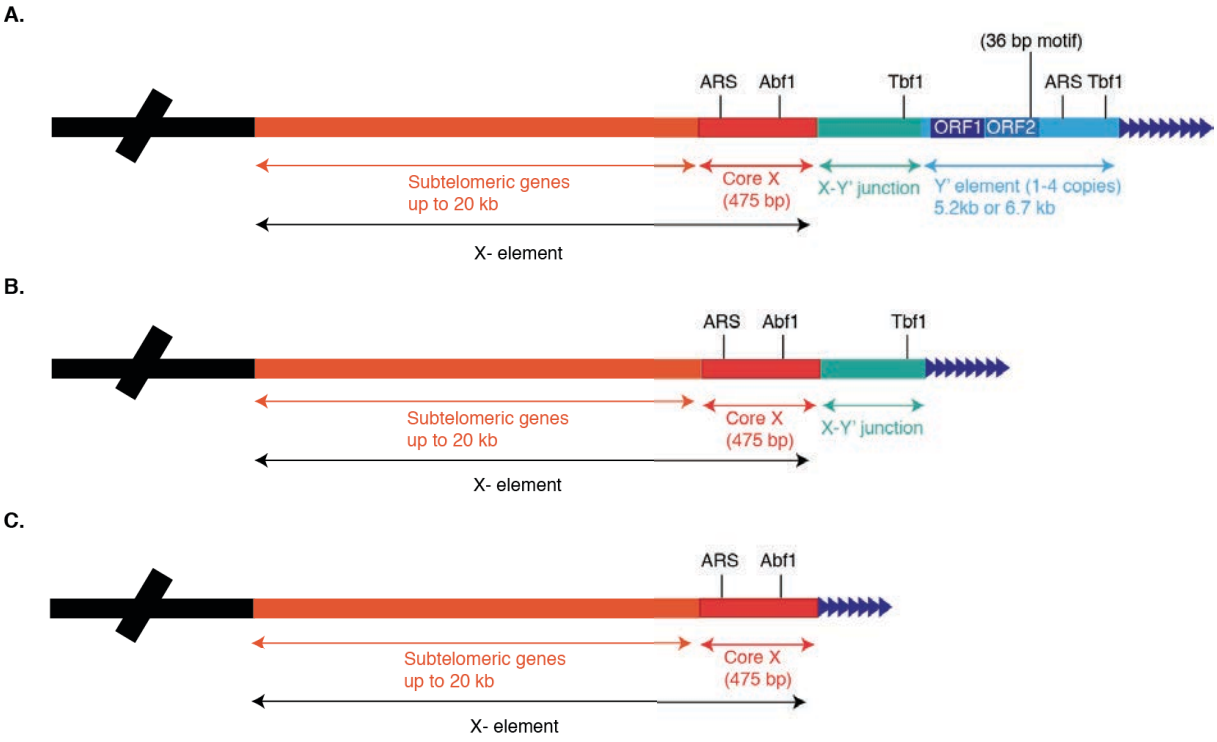



**Figure 9: The different structures of subtelomeric region in *S. cerevisiae***
The end of chromosome is composed of telomeric repeats. Adjacent to these repeats is the subtelomeric region. Different organization of subtelomeres exists (A) The subtelomeric region is composed of the Y' element and the X-element. The number of Y' element when present, varies from one to four copies in tandem. The Y' element contains two open reading frame, 36 bp repeat motif, Autonomously repeating sequence (ARS) and Tbf1-binding sites. All ends have a core X, and a centromere–proximal region containing few subtelomeric genes. The core X contains in addition to and ARS, an Abf1- binding site. Between the Y' element and the core X, exists the X-Y' junction that is composed of subtelomeric repeated elements (Str) Str-A, -B, -C, and -D, Tbf1-binding sites, and /or telomeric repeats. (B) Subtelomeric region lacking the Y' element (C) subtelomeric region may exist of only the X-element. Based on (Louis, 1995).

## 2.1. The Y' element

The Y' element is positioned centromere proximal to telomeric repeats (Figure 9). It is a region that is highly conserved between telomeres with 1% divergence within the same strain and 2% divergence among different strains (Louis and Haber, 1992). The Y' element exists in almost half of the chromosomes and may be present in tandem arrays of up to four copies, separated by short stretches of $TG_{1\text{-}3}$ repeats (Chan and Tye, 1983b; Louis and Haber, 1990b). Two major classes of Y' elements exist in *S. cerevisiae*: the short Y' of 5.2 Kbp and the long Y' of 6.7 Kbp that differ by several insertions and

deletions (Louis and Haber, 1992). Each Y' contains a highly conserved 36 bp motif repeated in tandem that varies in number among strains (Horowitz and Haber, 1984). and 2–3 copies of a 10 bp motif containing the TTAGGG repeat (Louis and Haber, 1992).

Despite variation in the location and copies of the Y' element among strains, several features are conserved (Louis and Haber, 1992). The Y' element contains towards the end an ARS of the repetitive class (Chan and Tye, 1983b; Louis and Haber, 1992). It contains two overlapping open reading frames (ORFs), where one encodes for an RNA helicase, a homologue of the TIF1/2 translation initiation factor in yeast. It also contains all of the features for the expression of these ORFs and contains a sequence for intron splicing. The Y' is only slightly expressed and transcripts are rapidly degraded. Its expression is detected at low levels in vegetative growth, but is increased during meiosis due to changes in the chromatin favoring crossing over (Louis and Haber, 1992).

The Y' element displays high histone occupancy, a positioned nucleosome, and acetylation of the lysine 16 of the histone 4 (H4K16), correlated with transcriptional levels (Pryde and Louis, 1999; Zhu and Gustafsson, 2009). The contribution of the Y' to telomeric maintenance is not fully understood due to the variation in the number of copies present. It has been suggested that helicase may be expressed, but it is not known whether it is functional or not. The Y' element function contributes to cell viability in telomerase-deficient cells, by the formation of type I survivors, which maintain telomere length through the amplification of Y' (Lundblad and Blackburn, 1993).

The evolutionary origin of Y' is not well understood, since it contains features of a virus or a mobile element (Louis and Haber, 1992). Recombination of the Y' element has been demonstrated to occur between Y' elements of the same class at a rate similar to normal recombination events in the genome (Louis and Haber, 1990a). In addition, the Y' element contains a subtelomeric anti-silencing repeat (STAR) that serves as a boundary for the spread of heterochromatin from telomeres into adjacent DNA (Fourel et al., 1999).

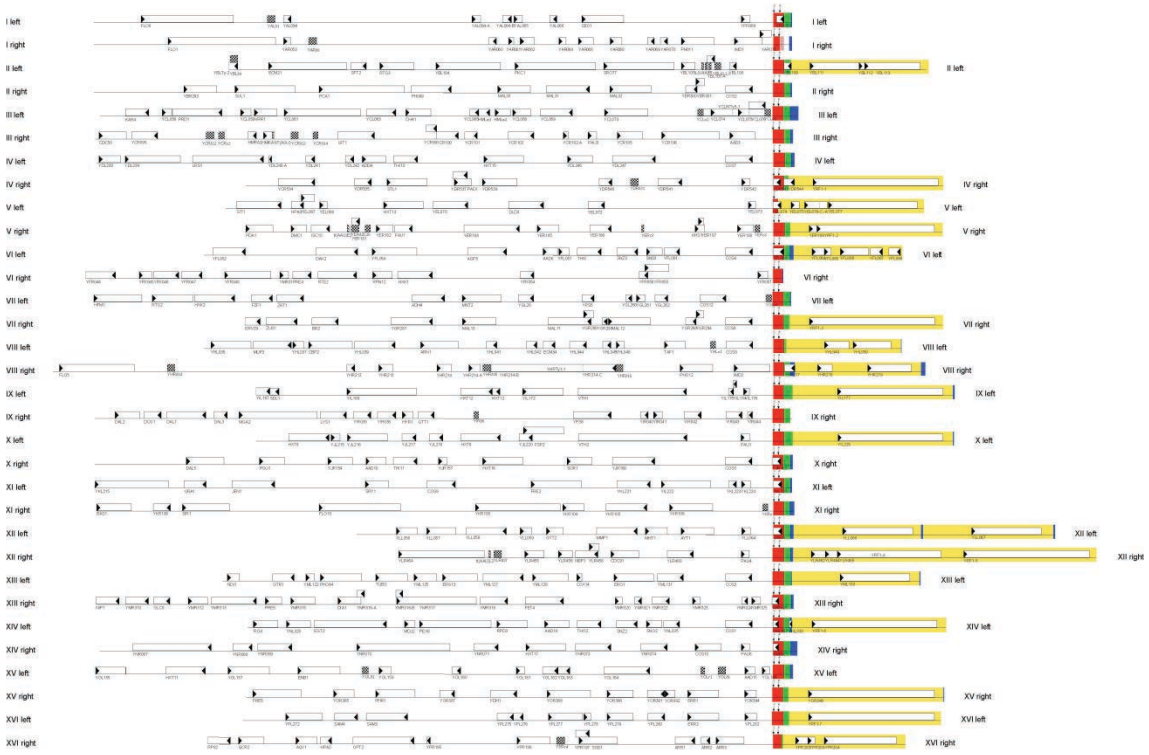


**Figure 10: Map of the 32 chromosomal ends of *S. cerevisiae***
The mapping of the last 30-40 Kb of the two extremities of chromosome ends the mapping is oriented from centromere (left) to the telomere (right). The ORF are represented as white blocks, the core –X is represented in red, STR-elements in green, $TG_{1-3}$ repeats in blue, and the Y' elements in yellow. Alignment of different chromosomal ends is done with respect to the ARS-consensus sequence sequence located in the core X. based on
http://www2.le.ac.uk/colleges/medbiopsych/research/gact/resources/yeast-telomeres/general-structure-of-yeast-chromosome-ends

## 2.2. The X element

Centromere proximal to the Y' element is the X element (Chan and Tye, 1983b). The X element is present at all chromosomes and is known for its mosaic structure with a high divergence percentage of up to 80–90% identity between various Xs in a cell (Louis, 1995). Despite this high degree of divergence, the X elements share a "core X" of 475 bp present at all chromosomes (Louis et al., 1994). Centromere proximal to the core X exists internal repeats of different non-essential genes (Figure 9; (Louis, 1995)). The core X element that is present in all chromosomes contains an ARS consensus sequence (ACS) and an ARS-binding factor 1 (ABF1) binding site (TTAGGG sequences) in-between a GC-rich sequence. Due to the highly mosaic and polymorphic structure of the X

element, the contribution of this region to telomere maintenance has not been fully defined. Nonetheless, the presence of the Abf1 binding sites and origin recognition complex (ORC) in the core X suggests a role in telomere maintenance; the absence of this X element in cells containing a truncated chromosome does not affect cell viability (Sandell and Zakian, 1992). The X element contains a sequence that is homologous to an internal sequence of the Y' element. This homologous sequence is the transposon Ty5 or Ty5 LTR. It has been proposed that the X element may have a potential role in the recombination of the Y' element and in directing the transposition of the Ty5 transposon (Voytas and Boeke, 1992; Zou et al., 1995).

### 2.3. The X-Y' junction

Different types of sequences have been reported to exist between the X element and the telomeric repeats or the Y' element. It has been proposed that internal poly ($C_{1-3}A$) exists at the different junctions (Walmsley et al., 1984). However, it was later shown via sequencing that that is not generally the case (Figure 9; (Louis et al., 1994). The junction is highly variable. It may contain different subtelomeric repeated elements (STR), each varying in size from 35 bp to 150 bp, so-called STR-A, STR-B, STR-C, and STR-D. Each STR group may vary in length between strains, but has around 90% identity between strains (Louis et al., 1994). In addition to STRs, the junction may also contain a part of the mitochondrial cytochrome b intron bi4 (Louis and Haber, 1991; Wright et al., 1992). and it may have nothing in between.

Analyses of human subtelomeres have revealed the presence of a common structure in which telomere-proximal and telomere-distal subtelomeres are separated by degenerated human TTAGGG sequences. The proximal domain seems to be more similar between chromosomes than the distal domain. This structure seems similar to the *S. cerevisiae* subtelomeric organization (Flint et al., 1997; Mefford and Trask, 2002).

## 3. Dynamics of subtelomeric elements

In yeast, recombination between the Y' occurs in meiosis and in mitosis (Horowitz et al., 1984; Louis and Haber, 1990a). Ectopic recombination between Y' long and Y' short might occur. However, this recombination is equivalent to normal recombination events,

which is about $2\times10^{-6}$ events per cell per generation (Louis and Haber, 1990a). Recombination events may increase under certain conditions to amplify genes needed for adaptation (Pryde et al., 1997). Telomere tethering to the periphery plays an important role in preventing recombination. The association of yKu with the core X strengthens the tethering of telomeres to the nuclear periphery and maintains the genome through telomere folding. This tethering protects against recombination. The absence of yKu at subtelomeric regions increases recombination between subtelomeres and interstitial regions, due to the disruption of telomere tethering to the nuclear periphery (Horowitz et al., 1984; Laroche et al., 1998; Marvin et al., 2009a; Marvin et al., 2009b; Pryde et al., 1997).

## 4. Proteins that bind to subtelomeres

TTAGGG repeat-binding factor (TBF1) is an essential transcription factor that binds the telomeric repeat TTAGGG found in many eukaryotes, including vertebrates, and is also present at the subtelomeres of budding yeast and at other sites in the genome (Brigati et al., 1993; Koering et al., 2000). At subtelomeres, the Tbf1 consensus binding sequence is present at the X-Y junction and the Y' element (Brigati et al., 1993). It shares a DNA-binding domain, the "Myb domain", that is similar to the Rap1 and TRF proteins present in yeast and mammals, respectively, and to the Taz1p present in *S. pombe* (Bilaud et al., 1996; Cooper et al., 1997). At telomeres, Tbf1 acts as a negative regulator of telomere length, a pathway masked in the presence of Tel1 (Berthiau et al., 2006). Furthermore, Tbf1 tethering to telomeres leads to telomere displacement from the nuclear envelope (Hediger et al., 2006), and lengthens short telomeres in the absence of Tel1 (Arneric and Lingner, 2007). Moreover, comparisons of short telomeres generated after an HO cut either lacking Tbf1 binding sites or containing Tbf1 sites have shown that Tbf1 associated with long telomeres inhibits the localization of Tel1 and Mre11 at telomeres (Fukunaga et al., 2012). Furthermore, in yeast cells genetically modified to contain TTAGGG repeats at chromosome extremities, instead of $TG_{1\text{-}3}$ repeats, telomerase recruitment is inhibited at long telomeres, suggesting a capping role of Tbf1 at long telomeres (Ribaud et al., 2011). This suggests a backup role for Tbf1 at telomeres that might have experienced chromosomal breakage or telomeric loss, indicating that the subtelomeric TTAGGG repeats may have a telomeric role of chromosome protection. In

addition to its role in telomere capping and telomere length regulation, Tbf1 functions in telomere silencing (Fourel et al., 1999). Furthermore, Tbf1 binding is involved in the regulation of genes involved in ribosomal RNA maturation (Preti et al., 2010).

Another essential protein that binds to subtelomeres and is involved in transcription is RNA polymerase I enhancer binding protein (Reb1). Reb1p is implicated in RNA polymerase I and II transcription. At telomeres, it recognizes its consensus binding sequence present within STRs and Y' elements. Reb1, as Tbf1, is also considered a negative telomere length regulator, which is masked in the presence of Tel1 (Berthiau et al., 2006). Moreover, similar to Tbf1, Reb1 counteracts telomere tethering to the nuclear periphery (Hediger et al., 2006). Reb1 is also considered a boundary factor, which inhibits silencing propagation at telomeres (Fourel et al., 1999).

Abf1 is another essential protein involved in transcription regulation. At subtelomeres, Abf1 binding sites are present in the X element where it is involved in silencing (Pryde and Louis, 1999). Origin recognition complex (ORC) is another subtelomeric binding complex. ORC is the ARS recognition complex that is essential for replication initiation (Bell and Stillman, 1992) and is involved in silencing at telomeres (see below).

In addition to its existence at the mating type locus, the silent information regulator (SIR) complex is also present at subtelomeres, allowing the silencing of the subtelomeric region (Aparicio et al., 1991). At telomeres in the yeast *S. cerevisiae*, the Sir complex consists of the catalytic subunit Sir2, and two structural subunits, Sir3 and Sir4. Sir2 is an NAD-dependent deacetylase of the lysine 16 of the histone H4 (Imai et al., 2000). Furthermore, Sir2 functions as a lifespan regulator, where its absence decreases longevity, and as a regulator of replication (Fabrizio et al., 2005). Sir3 is a member of the ATPase family, but lacks ATPase activity. Sir3 functions in nucleosome binding due to its increased affinity to deacetylated H4. In addition, it is involved in the spreading of silencing and transcriptional repression (Carmen et al., 2002; Cockell et al., 1998; Hecht et al., 1996; Oppikofer et al., 2011; Renauld et al., 1993). Sir4 is recruited to telomeres through its interaction with Rap1 (Moretti et al., 1994; Moretti and Shore, 2001), and then acts as a scaffold to recruit Sir2 and Sir3 (Rudner et al., 2005; Strahl-Bolsinger et al., 1997). Furthermore, Sir4 is involved in the positioning of silenced chromatin to the nuclear periphery through its interaction with Esc1 (Andrulis et al., 2002; Ansari and

Gartenberg, 1997). Sir1 also exists but its silencing role is specific to the mating type locus (Rine and Herskowitz, 1987).

# 5. Heterochromatin state of telomeres

Genomic DNA is organized into heterochromatin and euchromatin regions. Telomeres are part of the heterochromatic DNA and exert transcriptional repression that is spread over a large distance, which is different from the single promoter repression that leads to the silencing of adjacent genes.

## 5.1. Telomere position effect

The telomere position effect (TPE) is not promoter-specific, and was first discovered in *D. melanogaster* and then in other organisms such as budding yeast, fission yeast, and humans (Baur et al., 2001; Gottschling et al., 1990; Levis et al., 1985; Nimmo et al., 1994). The TPE phenotype observed in budding yeast was first reported in an experiment where the subtelomeric region of the left arm of chromosome VII was replaced by a reporter gene. The aim of that experiment was to generate a unique labeled telomere, which could be used for chromatin studies (Gottschling et al., 1990; Tham and Zakian, 2002). The reporter gene is either *URA3* or *ADE2,* whose expression can be measured on an appropriate media. The epigenetic nature of chromatin around these reporter genes influences *URA3* or *ADE2* expression. This repression is reversible and stably transmitted through a number of generations (Gottschling et al., 1990). This TPE phenotype has shown enormous variation at native telomeres modified by the insertion of a reporter gene in the X-ACS of X-only telomeres or in the Y' element of XY' telomeres (Pryde and Louis, 1999). Interestingly, X-only telomeres show higher TPE than XY' telomeres (Figure 11; (Pryde and Louis, 1999)). This can be explained by the fact that X telomeres contain protosilencers, such as ACS and Abf1-binding sites, that are essential for silencing, whereas the XY' telomeres contain subtelomeric anti-silencing sequences (STARs) that act as a boundary for silencing nucleation at the Y' element (Buck and Shore, 1995; Fourel et al., 1999; Lebrun et al., 2001; Pryde and Louis, 1999). This suggests that silencing is discontinued at natural telomeres. In addition to the difference in sequences and proteins bound to this region, telomeric length may be another factor affecting TPE. In general, high TPE is observed in genes inserted near

long telomeres, and reduced TPE is observed in genes inserted near short telomeres (Figure 11;(Buck and Shore, 1995; Kyrion et al., 1993).

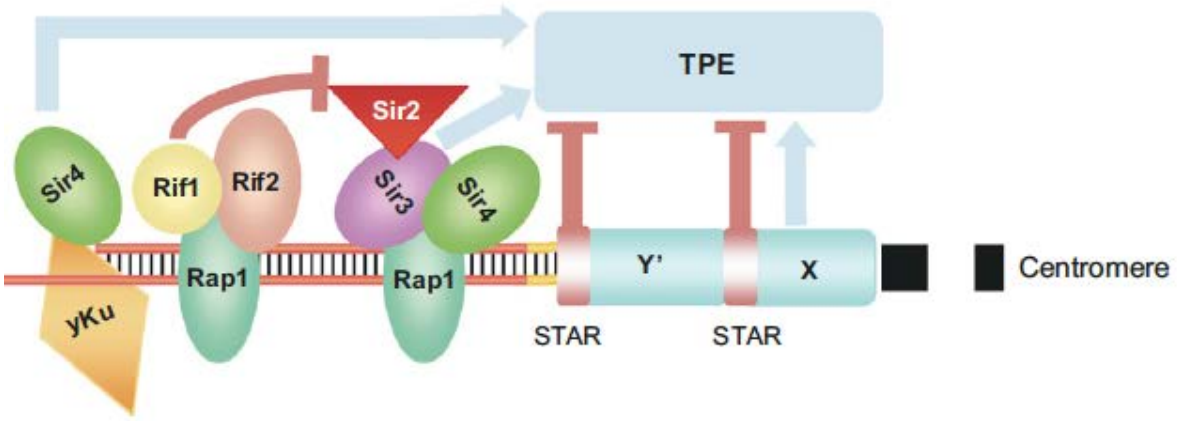



Figure 11: Regulation of TPE at natural telomeres in *S. cerevisiae*

The telomeric proteins Rif1 and Rif2 compete the Sir complex to bind Rap1, therefore affecting TPE. However, the yku recruits Sir4 to telomeres and promote TPE. At natural ends, the subtelomeric anti-silencing sequences (STAR) acts as a boundary for the silencing spreading through the Y' element for therefore preventing TPE in this element, while the X-element-containing protosilencers reinforces the silencing therefore promoting TPE. Adapted from (Ottaviani et al., 2007).

## 5.2. TPE mechanism

Silencing at telomeres occurs through the spreading of the silencing complex along a certain region after its initiation at a specific site (Figure 12). Silencing and TPE at telomeres are mainly regulated by the SIR complex, since Sir mutants abolish TPE, and over-expression of the three subunits enhances silencing (Aparicio et al., 1991; Maillet et al., 1996). Sir complex recruitment to telomeres is Rap1-dependent. This recruitment is mediated by the mediator Sir4, through the interaction of Sir4 with the C-terminal domain of Rap1 (Moretti et al., 1994; Moretti and Shore, 2001). Sir4 stimulates the NAD-dependent deacetylase activity of Sir2, generating high-affinity binding sites for Sir3 and Sir4 proteins with hypo-acetylated histone 3 (H3) and histone 4 (H4) N-terminal tails, allowing the spreading of the Sir complex (Hecht et al., 1995; Strahl-Bolsinger et al.,

1997). The interaction of Sir4 with the C-terminus of Rap1 competes with the interaction of Rif1 and Rif2 with the same domain of Rap1p. In *rif1Δ rif2Δ* cells, which contain long telomeres, TPE increases (Kyrion et al., 1993). In addition, Sir4 interacts with the yKu complex (Boulton and Jackson, 1998; Luo et al., 2002), providing a Rap1-independent pathway to recruit the silencing complex to telomeres. This yKu requirement for silencing can be bypassed by the absence of Rif1 (Mishra and Shore, 1999).

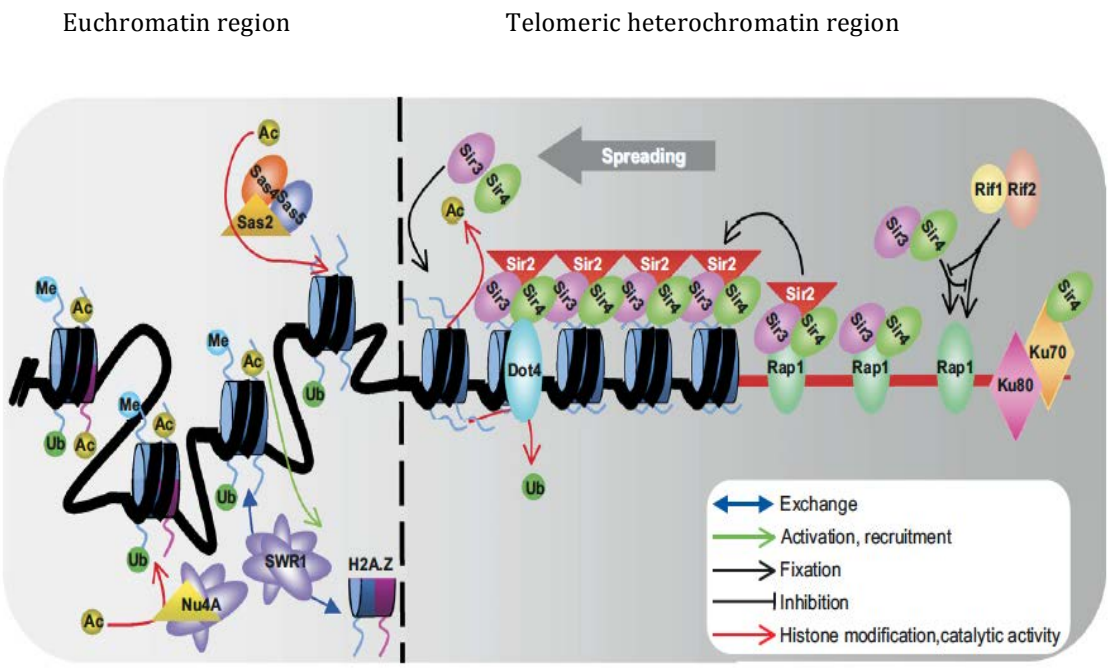



**Figure 12: Regulation of the spreading of Silencing at telomeres in *S. cerevisiae***
The Sir complex is recruited to telomeres through the interaction of Sir 3 and Sir 4 with Rap1. The silencing of telomeres occurs through the deacytelation of the lysine of histone 4 (H4) by the Sir2 deacetylase. The spreading of the repressive chromatin is accomplished by the propagation of the Sir3 and Sir4 all along the chromatin. This propagation depends on the removal of ubiquitin from the histones 2B (H2B) by Dot4. Silencing spreading to euchromatin region is limited by the opposing action of the Sas complex that acetylates the lysine of histone 4. This acetylation prevents the Sir3 and Sir4 binding and the spreading of telomeric silencing. Adapted from (Ottaviani et al., 2007).

The spread of silencing at a truncated telomere is continuous and may occur for several Kbp (Renauld et al., 1993). However, this spreading is discontinuous at natural telomeres, correlating with the discontinuous TPE detected at these natural telomeres (Fourel et al., 1999; Pryde and Louis, 1999; Radman-Livaja et al., 2011). Spreading

occurs through the binding of Sir4 to ubiquitin-specific protease 10 (Ubp10), which reduces the level of the ubiquitination of lysine 123 of histone H2B (H2BK123ub) at telomeres, then by decreasing the methylation of lysine 70 of histone 3 H3K79, leaving histones with high affinity to Sir3 binding (Armache et al., 2011; Oppikofer et al., 2011). The spread of silencing and propagation are limited by the opposing activity of the histone acetyltransferase Sas2, by the boundary effect of the histone deacetylase Rpd3, and by the boundary role of the histone lysine methyl transferase disrupter of telomeric silencing 1 (Dot1), which methylates H3K79 of the adjacent euchromatin, resulting in less affinity of Sir3 to the H4 tail (Figure 12; (Ehrentraut et al., 2010; Fingerman et al., 2007; Grunstein and Gasser, 2013; Kimura et al., 2002)). In addition to histone modifications being markers for boundary spreading, transcription factors such as Reb1 and Tbf1 may also affect this spreading (Fourel et al., 2001; Fourel et al., 1999). Interestingly, recent data challenge the point of view that the Sir complex is the critical step in determining the ON/OFF silent state of chromatin and suggests that the methylation of H3K79 by Dot1 seems to be the critical step (Kitada et al., 2012).

Telomeres are thought to form a loop, due to the presence of Rap1 and yKu 3 Kbp away from TG repeats at truncated ends, which is not the case when silencing is disturbed (de Bruin et al., 2000; Hecht et al., 1996; Poschke et al., 2012; Strahl-Bolsinger et al., 1997). This suggests that silencing favors telomere looping.

TPE also regulates the genes in the subtelomeric region, which are within 20 Kbp from yeast telomeres. A Sir-independent pathway through the histone deacetylase 1 (HDA1) has also been shown to silence genes from 10 Kbp to 25 Kbp from subtelomeres (Robyr et al., 2002; Wellinger and Zakian, 2012). This silencing is responsible for a low level of expression of these genes when compared to other genomic loci. However, under certain environmental stresses, transcription factors have been shown to bind to subtelomeric regions (Mak et al., 2009), leading to the expression of these genes (Wellinger and Zakian, 2012).

In *S. pombe*, silencing depends on the chromatin-associated protein Swi6. The DNA nucleation step depends on spTaz1 and spRap1 (Kanoh et al., 2005; Park et al., 2002). In mammalian cells, silencing and TPE are regulated by the HP1 protein (Koering et al., 2002). Histone modifications also affect telomere silencing in mammalian cells (Baur et

al., 2001). Moreover, the histone deacetylase sirtuin-6 (SIRT6) is also implicated in telomeric silencing at telomeres (Michishita et al., 2008).

In conclusion, the heterochromatin state of DNA, determines the transcription profile of the subtelomeric and the telomeric region.

## 6. Telomere localization

Telomeres forms clusters and are located at the nuclear periphery. Their localization at the nuclear periphery depends on the SIR proteins and on the Ku complex (Khadaroo et al., 2009; Laroche et al., 1998; Palladino et al., 1993). The tethering of telomeres to the nuclear periphery depends on two different nuclear envelope proteins: the Esc1 and the Msp3 proteins (Figure 13; (Andrulis et al., 2002; Bupp et al., 2007). This LacO at the nuclear periphery is linked to TPE, where silent chromatin is anchored to the nuclear periphery (Hediger and Gasser, 2002). Besides, TPE is reduced upon experimental abolishment of telomere clustering (Taddei et al., 2009). Thus, the function of telomeres is tightly connected to their LacO within the nuclear space. In telomerase deficient cells, short telomeres localize to the nuclear pore complex (Khadaroo et al., 2009). This localization to the nuclear pore complex evokes the localization of DNA breaks to the NPC to be repaired. This repair is dependent on the Slx5/Slx8 SUMO-dependent ubiquitin ligase that is found at the nuclear pore complex in interaction with the Nup84 complex (Fabre and Spichal, 2014).

Telomere tethering in the S phase depends on the interaction between Msp3 and the telomerase depending on the Ku complex (Wellinger and Zakian, 2012). The telomeric anchoring strength is also regulated by the structural composition of the subtelomeric region, i.e. Y′ elements or the STR repeats (Fabre and Spichal, 2014).

The role of SUMOylation in telomere maintenance is also through affecting telomere anchoring to the nuclear envelope, then acting as a negative regulator of telomere length. The SUMOylation of the SIR and Ku proteins by PIAS-like SUMO E3 ligase Siz2 is essential for telomere tethering, since in siz2Δ telomeres are randomly positioned in the G1 and S phases (Ferreira et al., 2011). This release of anchoring leads to telomere elongation (Ferreira et al., 2011).

Then, telomere localization inside a cell is essential for telomere processing.

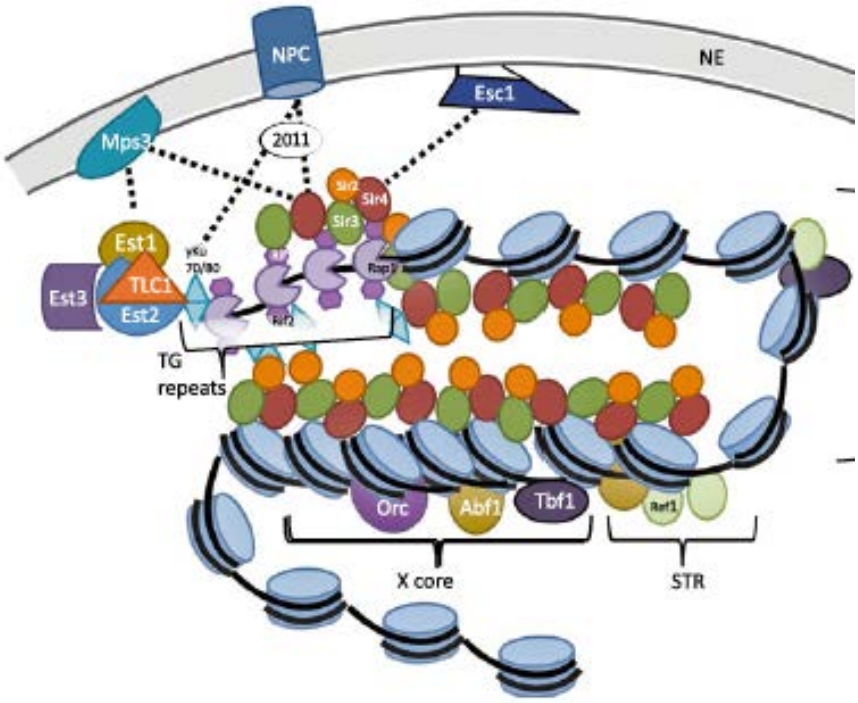



**Figure 13: Model showing the telomere and subtelomere localization.**
Telomeres are anchored to the nuclear periphery through the interaction of Sir4 and Ku proteins with the nuclear envelope proteins Esc1 and Msp3. Telomerase also anchors telomeres to the nuclear envelope through the interaction of Est1 with Msp3 in a Ku-dependent manner. Adapted from (Fabre and Spichal, 2014).

# IV. Telomeric transcripts: TERRA

## 1. TERRA: a long coding RNA

Due to the heterochromatic structure of telomeres and the different silencing profiles detected at telomeres, telomeres were thought to be transcriptionally repressed. However, it has been shown in mammalian cell lines that telomeres are transcribed into long non-coding RNA (lnRNA) telomeric repeat-containing RNA (TERRA; Figure 14;(Azzalin et al., 2007)). Since then, TERRA has been detected in different organisms including humans, mice, zebrafish, and yeasts, emphasizing the conserved nature of TERRA through evolution (Azzalin et al., 1997; Azzalin et al., 2007; Bah et al., 2012; Luke et al., 2008; Schoeftner and Blasco, 2008). TERRA transcripts have been detected by northern blots on total RNAs, using probes against telomeric and subtelomeric repeats, and have been shown to be sensitive to RNase. Therefore, TERRA has been defined as transcripts that start in the subtelomeric region and proceed into telomeric repeats of the C-strand and are synthesized by RNA polymerase II (Azzalin et al., 1997; Bah et al., 2012; Luke et al., 2008; Schoeftner and Blasco, 2008).

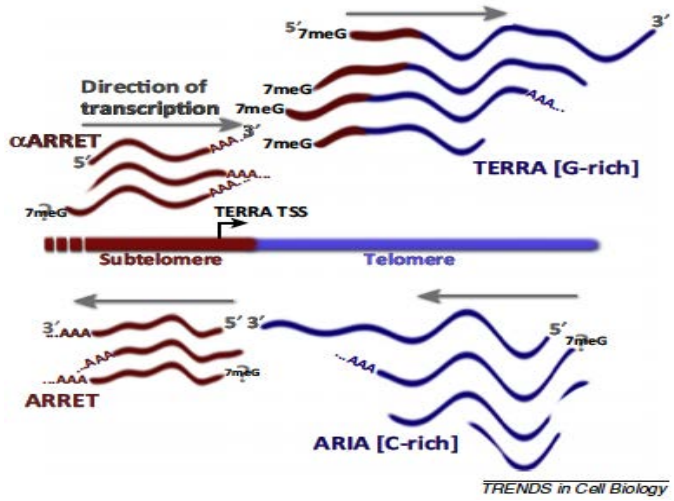



**Figure 14: The different transcripts generated at telomeric ends**
TERRA are transcripts where the transcription starting site (TSS) is in the subtelomeric region (red) and then transcription proceeds into the telomeric repeats (blue). ARIA being an antisense of the TERRA. αARRET are transcripts that correspond only to subtelomeric region, and ARRET being the complementary of. The AAA tail corresponds to transcripts that are poly-adenylated at the 3' ends. The 7meG represent the transcripts that contain a 7-methyl guanosine at 5' ends. TERRA is present in *S. cerevisiae, S. pombe*, mammals, while ARIA, ARRET, and αARRET exists only *in S. pombe.* Adapted from (Azzalin and Lingner, 2014).

In different mammalian cells, TERRA transcripts are restricted to the nucleus, heterogeneous, and vary in length from 100 bp to 9 Kbp. TERRA modifications detected by either northern blots, or by the fractionation of chromatin-associated, nucleoplasmic, and cytoplasmic TERRA have revealed that transcripts are partially poly-adenylated (poly (A)) in mammalian cells, where 40% of poly $(A)^-$ cells are found to be bound to chromatin while poly $(A)^+$ exist in the nucleoplasm. Furthermore, TERRA poly $(A)^+$ are more stable than poly $(A)^-$ (Porro et al., 2010; Schoeftner and Blasco, 2008). In *S. cerevisiae*, detection of TERRA transcripts by northern blots necessitates the thermo-sensitive *rat1-1* 5'-3'exonuclease mutant. Live cell assays in which a single telomere is followed have shown that in wild-type cells only 10% of a population expresses TERRA from a specific telomere at a certain time (Cusanelli et al., 2013; Luke et al., 2008). TERRA in *S. cerevisiae* also varies in length and might range from 100 bp to 1,2 Kb (Luke et al., 2008). Furthermore, in *S. cerevisiae*, most TERRA harbour a poly $(A)^+$ tail, promoting TERRA stability (Luke et al., 2008). In a similar fashion, *S. pombe* TERRA transcripts are also heterogeneous and vary in length from 100 bp to 2 Kbp. They are also partially poly-adenylated as shown by northern blots (Bah et al., 2012).

In addition to TERRA, *S. cerevisiae* exhibits minor ARRET transcripts (Luke et al., 2008). *S. pombe* exhibits other forms of telomeric transcripts such as alpha ARRET and ARIA (Figure 13; (Bah et al., 2012).

## 2. TERRA expression

TERRA expression is tightly regulated throughout the cell cycle. In human cells, TERRA levels have been measured after cell synchronization, demonstrating that TERRA increases in the G1-phase, then decreases as the cells proceed into the S-phase and finally increases again after mitosis (Arnoult et al., 2012; Porro et al., 2010). In *S. cerevisiae*, TERRA expression does not change in a given cell cycle, indicating that its level is maintained (Cusanelli et al., 2013). Knowing that TERRA transcription starts in the subtelomeres, and because of the homology between subtelomeres, the mapping of TERRA promoters has been challenging in different organisms. In humans, the TERRA promoter has been identified at CpG islands present in the subtelomeric region of 20

different human telomeres, but not found at the other 26 telomeres (Nergadze et al., 2009). Recently, it was shown that TERRA transcripts mainly arise from chromosome 18 in mouse cells, and that heterogeneity is due to the regulation of TERRA expression through two different promoters located in the subtelomeric region. These identified promoters contain multiple transcription factor binding sites but lack CpG islands (de Silanes et al., 2014). In *S. cerevisiae*, TERRA promoters have not yet been determined. However, the transcription starting site (TSS) using the 5' rapid amplification of cDNA ends (5'RACE) technique has been mapped in the X-core of the left arm of chromosome I and in 6 different XY' subtelomeric regions of different chromosomes (Pfeiffer and Lingner, 2012). This suggests that TERRA regulation may depend on different types of promoters present at different ends.

Despite the absence of a clear view of TERRA promoter mapping, much evidence supports the notion that heterochromatin markers present at both subtelomeres and telomeres modulate TERRA expression in mammalian cells. In human cell lines, hypomethylation of CpG correlates with high TERRA expression when the DNA methyl transferase (DNMT) *DNMT1* or *DNMT3b* is absent (Arnoult et al., 2012; Nergadze et al., 2009; Ng et al., 2009). However, in mouse embryonic stem cells, the absence of *DNMT1* or *DNMT3a* or *DNMT3b* leads to a decrease in TERRA levels, despite the hypomethylation of CpG islands (Schoeftner and Blasco, 2008). On the other hand, in these mouse cell lines, the absence of the histone methyltransferases *Suv39h* and *Suv4-20h* leads to an increase in TERRA levels (Schoeftner and Blasco, 2008). This suggests that methylation of the CpGs islands may be positive or negative regulators of TERRA, depending on the chromosome and on the cells being examined (Nergadze et al., 2009; Schoeftner and Blasco, 2008).

The acetylation of telomeric nucleosomes are also involved in the regulation of TERRA. TERRA levels become elevated when human cell lines are treated with histone deacetylase inhibitor (Azzalin et al., 2007). Furthermore, the presence of a high density of lysine trimethylation at histone H3 (H3K0me3) and HP1 recruitment at telomeres results in low expression of TERRA (Arnoult et al., 2012). TERRA expression is one of the consequences of facial anomaly syndrome (ICF), which is characterized by the presence of mutations in the DNA methyl transferase DNMT3b, therefore leading to hypomethylation of the CpG island and increases in TERRA levels (Yehezkel et al., 2008).

In *S. cerevisiae,* TERRA expression is regulated by telomeric protein Rap1 (Iglesias et al., 2011). Rap1 regulates TERRA expression and degradation. The regulation of expression differs among the XY' telomeres and the X-only telomeres. At XY' telomeres, Rap1 represses TERRA transcription through the recruitment of Rif1 and Rif2 (Iglesias et al., 2011), whereas on the X-only telomere, Rap1 promotes repression of TERRA expression through Sir2 and Sir3, in addition to Rif1 and Rif2 (Iglesias et al., 2011). Although the regulation of TERRA at XY' telomeres is not clearly understood, the regulation at the X-only telomeres might be explained by the deacytelation of H3 and H4 mediated by Sir2. Furthermore, Rap1 also regulates TERRA degradation through the recruitment of nuclear Rat1 exonuclease (Iglesias et al., 2011; Luke et al., 2008).

TERRA expression is also regulated through its crosstalk with telomere length. In mammalian cell lines, TERRA levels decrease upon telomere elongation (Arnoult et al., 2012). This effect on TERRA regulation might be explained by the change in heterochromatin state between long and short telomeres (Benetti et al., 2007). Although an inverse correlation between TERRA expression and TERRA length has been shown, other groups, using different cell lines in addition to different techniques for TERRA detection, have shown the absence of this inverse correlation in different cell lines (Farnung et al., 2012; Smirnova et al., 2013). For example, no difference in TERRA levels was observed when HeLa cell telomeres were elongated after transfection with the catalytic subunit of telomerase (Farnung et al., 2012). However, in the budding yeast *S. cerevisiae*, it has been demonstrated that the generation of a short telomere induces an increase in TERRA expression, and this expression decreases after telomere elongation by telomerase (Cusanelli et al., 2013). This might be explained by the loss of transcription repression by the telomeric protein as telomeres shorten.

## 3. TERRA localization

TERRA has been demonstrated to localize in the nucleus in mammalian cells (Azzalin et al., 2007; Schoeftner and Blasco, 2008). It forms discrete nuclear foci, some of which associate with telomeres (Azzalin et al., 2007; Porro et al., 2010; Schoeftner and Blasco, 2008). In mouse cells, the association of TERRA with telomeres is not limited to its telomere of origin (de Silanes et al., 2014). In the budding yeast *S. cerevisiae*, time-lapse experiments have shown that TERRA localizes at the nuclear periphery and is then

recruited to its telomere of origin in the late S phase (Cusanelli et al., 2013). TERRA has also been proposed to associate with telomeres, based on immunoprecipitation of DNA-RNA hybrids using an antibody that recognizes these hybrids (Balk et al., 2013; Pfeiffer et al., 2013). Altogether, TERRA expression regulation, localization, and association at telomeres might implicate TERRA in the regulation and maintenance of telomeres.

# 4. TERRA function

## 4.1. TERRA and heterochromatin formation

As described above, the expression of TERRA is sensitive to the heterochromatin state of telomeres. Conversely, the interaction of TERRA with different heterochromatic components in telomerase-proficient cells suggests a role of TERRA in the establishment of heterochromatin in mammalian cells. TERRA transcripts have been shown to interact with the ORC, which binds to the subtelomeric region and is involved in transcriptional silencing; some components of the Swi/Snf complex; and components of histone deacetylase (Deng et al., 2009; Scheibe et al., 2013). Accordingly, partial down-regulation of TERRA by siRNA leads to loss of H3K9me3 and the ORC association at telomeres and induces TIF formation (Deng et al., 2009). Furthermore, TIP5, a component of the nucleolar-remodeling complex (NORC), has been shown to associate with TERRA and regulate heterochromatin formation (Postepska-Igielska et al., 2013). In addition, HP1 and H3K9me3 follow TERRA profile expression, with high expression in the G1 phase, a decrease in the S phase, and then an increase in the G2/M phase (Arnoult et al., 2012). Accordingly, TERRA processed into siRNA in *Arabidopsis thaliana* contributes to telomere chromatin maintenance through the asymmetric methylation of telomeric repeats (Vrbsky et al., 2010). This suggests that TERRA represses its own expression by a negative feedback loop and by promoting the establishment of the heterochromatin state at telomeres after the replication and action of telomerase

## 4.2. TERRA and telomere maintenance

TERRA transcripts emerging from the C-strand contain sequences that are complementary to the telomerase RNA subunit. Because of these complementary sequences, different models of TERRA in mammalian cells have emerged as negative or

positive regulators of telomerase activity, therefore affecting telomere length (Cusanelli et al., 2013; Farnung et al., 2012; Redon et al., 2010; Schoeftner and Blasco, 2008). In mammals, the role of TERRA as an inhibitor of telomerase activity was proposed by following telomerase activity *in vitro* when incubated with $(UUAGGG)_3$ mimicking TERRA oligonucleotide (Cusanelli et al., 2013; Farnung et al., 2012; Redon et al., 2010; Schoeftner and Blasco, 2008). This suggested that TERRA acts an inhibitor of telomerase activity *in vitro*. However, the presence of the heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), which functions in RNA biogenesis and in telomerase inhibition, alleviated the TERRA inhibitory effect on telomerase *in* vitro (LaBranche et al., 1998; López de Silanes et al., 2010; Redon et al., 2010).

In addition, TERRA levels have been proposed not to interfere with either telomerase activity or telomere elongation. Indeed, in mammalian cells that express high levels of TERRA, i.e., that lack the DNMT1 and DNMT3 methyl transferases, telomerase activity is retained at the same level as in those that express low levels of TERRA (Farnung et al., 2012).

Interestingly, in *S. cerevisiae*, TERRA interacts with the yeast TLC1 RNA subunit of telomerase. This proposed TERRA as a positive regulator of telomerase activity. In turn, TERRA that is transcribed from the shortest telomere nucleates telomerase to its telomere of origin to be re-elongated (Figure 15; (Cusanelli et al., 2013)). Despite the proposed role of TERRA in telomerase recruitment to short telomeres, the over-expression of telomeric RNA at a single chromosome, using a strong Gal promoter that induces transcription at telomeres, leads to telomere shortening (Maicher et al., 2012; Pfeiffer and Lingner, 2012). Yet, telomerase recruitment to telomeres is not affected in the presence of TERRA, suggesting that telomere shortening due to TERRA over-expression in *S. cerevisiae* occurs independently of telomerase activity. This shortening is in fact Exo1-dependent (Balk et al., 2014; Pfeiffer and Lingner, 2012). Accordingly, it has been proposed that TERRA transcripts interfere with telomeric end protection proteins, such as the yKu complex, that protect against nucleases (Pfeiffer and Lingner, 2012). While the role of TERRA in regulating telomerase activity is still controversial, TERRA may also play a role in telomere maintenance in the absence of telomerase. TERRA expression is increased in ALT cells, where telomeres are maintained through recombination (Lovejoy et al., 2012; Ng et al., 2009). Accordingly, ALT cells have been

shown to have an open chromatin formation with a decrease in the H3K9me3 and an increase in telomeric transcription (Episkopou et al., 2014), suggesting that this conformation facilitates recombination. In addition, it has been argued that in ALT cells, TERRA transcription might contribute to the formation of DNA–RNA hybrids, thus promoting recombination.

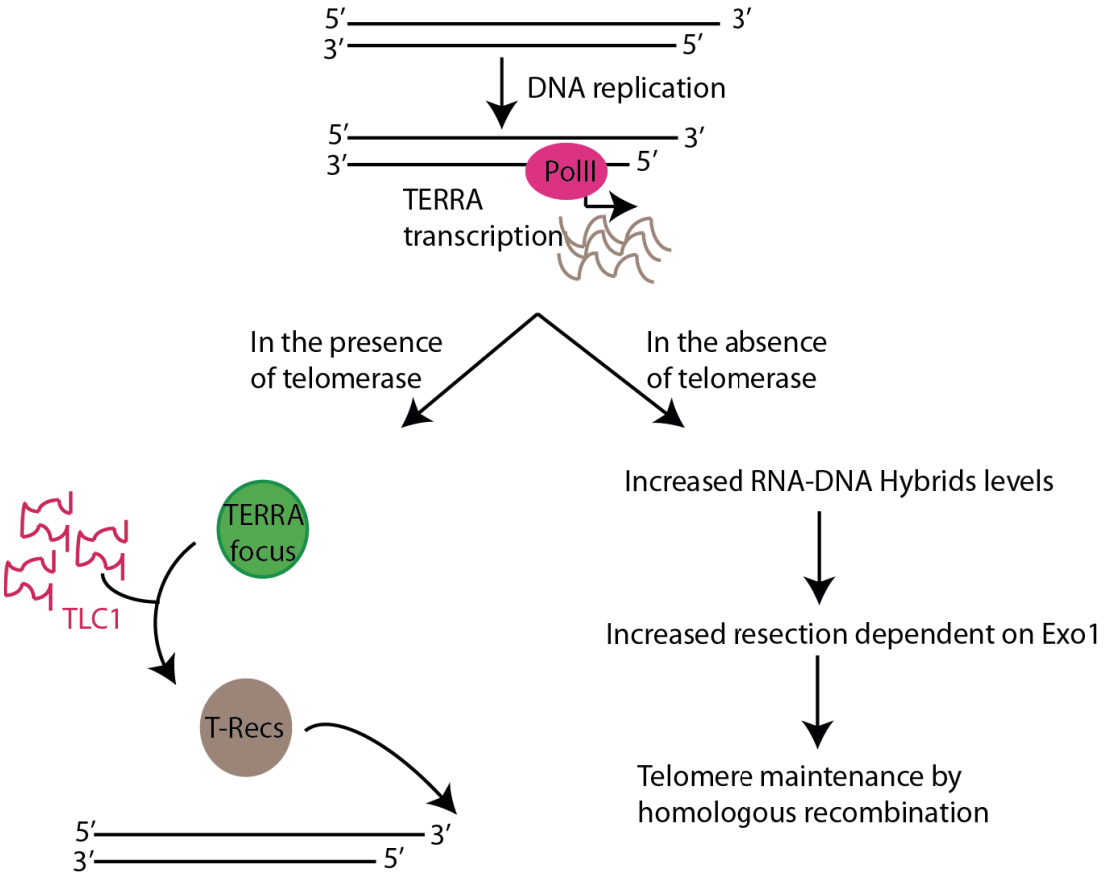



**Figure 15: The role of TERRA in telomere maintenance in *S. cerevisiae***
Short telomeres induce TERRA transcription. In the presence of telomerase, TERRA acts as a scaffold to recruit Tlc1, the RNA subunit of telomerase to the short telomere. However in the absence of telomerase, TERRA forms RNA-DNA hybrids, associated with Exo1 resection. ssDNA generated then induces homologous recombination to maintain telomeres. Based on (Balk et al., 2014; Cusanelli et al., 2013).

In *S. cerevisiae*, and in addition to its possible role in maintaining telomeres in a telomerase-dependent manner, TERRA may also maintain telomeres in a telomerase-independent manner. Indeed, in budding yeast, the shortening of telomeres due to TERRA over-expression in *cis* results in an acceleration of replicative senescence (Balk

et al., 2014; Maicher et al., 2012). This shortening and acceleration of senescence are Exo1-dependent (Balk et al., 2014; Pfeiffer and Lingner, 2012). This suggests that TERRA expression alters the protection of the telomeres from which they are expressed. Moreover, high levels of TERRA transcripts are associated with an increase in DNA–RNA hybrids at telomeres, potentially leading to a stalled replication fork (Pfeiffer et al., 2013). In the absence of telomerase, these DNA–RNA hybrids promote homologous recombination and delay senescence (Figure 15; (Balk et al., 2014; Balk et al., 2013; Pfeiffer et al., 2013; Pfeiffer and Lingner, 2012; Yu et al., 2014)). In addition, experiments using an *RNASEH* mutant or THO/TREX complex mutant, where DNA–RNA hybrids are stabilized, have supported a role of these hybrids in delaying senescence and favoring homologous recombination (Balk et al., 2014; Balk et al., 2013; Pfeiffer et al., 2013; Pfeiffer and Lingner, 2012; Yu et al., 2014). Conversely, the reduction of these hybrids by the over-expression of RNASE H1, or by abolishing TERRA expression *in cis*, results in telomere shortening, a reduction in homologous recombination, and an acceleration of senescence (Balk et al., 2013). Thus, in the case where the DNA–RNA hybrids are stabilized, homologous recombination counteracts telomere shortening and delays senescence in the absence of telomerase.

### 4.3. TERRA and genomic stability

In addition to telomeric proteins that function in maintaining telomere protection, TERRA also helps to preserve genome integrity. UPF1 a central protein in the RNA surveillance non-mediated pathway binds to telomeres at S and G2 phase in an ATR dependent manner. This binding is essential for telomere elongation through its binding to telomerase, and in telomere leading strand replication (Chawla et al., 2011). Indeed, the absence of the UPF1 leads to the accumulation of dysfunctional telomeres. These dysfunctional telomeres are linked to the TERRA molecules bound to telomeres. Therefore, TERRA regulation is important in ensuring telomere replication and maintaining telomere length (Azzalin et al., 2007).

Another role of TERRA in maintaining genome integrity occurs after DNA replication, at which point it functions in displacing RPA from ssDNA with the action of hnRNPA1 to load Pot1 to cap telomeres (Flynn et al., 2011; Maicher et al., 2014). In addition, TERRA expression increases in the absence of TRF2, enhancing the binding of lysine-specific

demethylase 1 (LSD1) at unprotected telomeres. In turn, LSD1 interacts with MRE11, functioning in the processing of uncapped telomeres (Porro et al., 2014).

Therefore, transcription at telomeres is highly regulated and is occurs as a response of different stimuli. TERRA is implicated in different mechanisms at telomeres, and it has been shown to contribute to replicative senescence. However, the relation between TERRA and senescence remains unclear.

# The thesis question

Replicative senescence is the loss of viability due to telomere attrition. This loss of viability occurs progressively, through a Tel1-dependent pre-senescence step and then switches to a Mec1-dependent senescence step, leading to cell cycle arrest (Abdallah et al., 2009). (Abdallah et al., 2009). Several factors have been implicated in replicative senescence, and the processing of the shortest telomere in a cell is a critical step to trigger senescence. Using a system to generate a truncated 7L single short telomere, it was shown that the shortest telomere is processed as a DSB, and several DNA damage proteins regulate replicative senescence (Abdallah et al., 2009; Fallet et al., 2014). Thus, elements that modulate the processing or the signaling state of the shortest telomere are expected to influence the onset of senescence. Therefore, the goal of my thesis was to identify novel pathways implicated in replicative senescence at a short telomere. To do that, three approaches were proposed:

Approach 1: The first approach was to study the effects of subtelomeric elements on replicative senescence. To do that, we took advantage of the 7L VST system, where a truncated version of 7L lacking subtelomeres is generated. We reconstituted the system in a native context, in which the subtelomeric elements were left intact. By comparing the two sets of strains, we were able to investigate the effects of subtelomeric elements in senescence *in cis*.

Approach 2: The second approach was to identify novel factors that delay replicative senescence, by acting specifically at the shortest telomere in a cell. To do this, I have set up a genetic screen.

Approach 3: The third approach was to construct a tool to identify factors that bind to the short telomere. To do so, I developed a system to purify the very short telomere at the 7L and then identify the proteome acting at this short telomere by mass spectrometry.

These approaches complement each other to establish a global view of the molecular mechanisms implicated in replicative senescence.

# Results

## I. Manuscript

### Subtelomeric elements influence senescence rates in *Saccharomyces cerevisiae*

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## Abstract

Linear chromosomes end with special regions, the telomeres, which ensure the integrity and the stability of the genome. In eukaryotes, telomeres also determine cell proliferation potential by triggering replicative senescence. This occurs upon telomere shortening in the absence of telomerase. In *Saccharomyces cerevisiae,* it is likely mediated by the first telomere in the cell that reaches a critically short length. This shortened telomere subsequently activates a DNA-damage-like response. How the signaling is modulated in terms of telomeric structure and context is largely unknown. Here we investigated how subtelomeric elements of the shortest telomere in the cell influence the onset of senescence. By comparing two sets of strains in which the shortest telomere either harbors naturally occurring subtelomeric elements or lacks these elements altogether, we show that a subtelomeric region comprising an X element counteracts the establishment of senescence. This effect is likely not due to differential Rad51-mediated homology directed repair activities at both types of telomeres. Furthermore, TERRA transcription is induced at both types of critically short telomeres, although levels are elevated in the absence of natural subtelomeric elements. Together, our results demonstrate that transcription from a telomere-proximal region greatly increases when the shortest telomere reaches a critical length, regardless of the presence of a native subtelomere or a dedicated TERRA promoter. We propose that subtelomeric elements may however buffer senescence rates, at least partially, by regulating rates of TERRA transcription when telomeres become critically short.

## Introduction

The ends of linear chromosomes, the telomeres, are special nucleoprotein regions that are essential for the stable maintenance of the chromosomes. In eukaryotes, telomeres are composed of TG-rich repeats running from the 5' to the 3' end. Semi-conservative DNA replication of telomeres results in loss of telomeric sequences (Lingner et al., 1995; Soudet et al., 2014). Telomerase, a reverse transcriptase, provides the major specific activity that counteracts this sequence loss (Greider and Blackburn, 1985; Lingner et al., 1997). It extends the 3' protruding ends of chromosomes by reverse transcribing the repeat-containing template region of the tightly associated telomerase RNA moiety in an iterative fashion. In the absence of telomerase, telomeres shorten with each passage of the replication fork leading to replicative senescence, i.e. a permanent cell cycle arrest, despite the continued presence of metabolic viability (Harley et al., 1990). Telomere shortening is considered to be a major tumor-suppressor pathway and consistently telomere maintenance mechanisms are activated in cancer cells and are essential for unlimited proliferation (Shay and Wright, 2010). The current model is that as telomeres shorten progressively, they eventually lose their identity and activate the DNA damage checkpoints kinases ATM and ATR, leading to the cell cycle arrest (d'Adda di Fagagna et al., 2003). *Saccharomyces cerevisiae* is a unicellular eukaryote that depends on telomerase activity for long-term viability (Lundblad and Szostak, 1989). Yeast cells arrest the cell cycle in the G2/M phase 60 to 80 generations after the experimental removal of telomerase activity (Enomoto et al., 2002; Ijpma and Greider, 2003). Similar to mammalian cells, this effect depends on the activation of the DNA damage checkpoint Mec1, the ATR orthologue in *S. cerevisiae* (Teixeira, 2013). In cultured mammalian cells it has been reported that five dysfunctional telomeres are required to trigger senescence, while in budding yeast one critical short one appears to be sufficient to establish the cell cycle arrest (Abdallah et al., 2009; Kaul et al., 2011; Khadaroo et al., 2009; Xu et al., 2013). As shortened telomeres reach a critical length, DNA repairing activities such as 5'-to-3' resection mediated by Sae2, Mre11-Rad50-Xrs2 (MRX) and Exo1 takes place, resulting in the exposure of telomeric and subtelomeric ssDNA and hence activation of Mec1 (Fallet et al., 2014). Thus, the senescence rate should depend on the vulnerability of the telomeric structure at the shortest telomere to both DNA repair activities and/or its signaling capacity. Importantly, telomere signaling capacity

can be modulated by chromatin state in the context of acute telomere dysfunction (Carneiro et al., 2010; Okamoto et al., 2013).

One well described pathway in which way telomeric chromatin influences its own processing in *cis* is through transcription. The non-coding telomeric repeat containing RNA (TERRA) is an RNA polymerase II (RNAPII) –derived transcript comprised of a subtelomeric region as well as the UG-rich repeats. It was first described in human cells, where it localizes to telomeres and has been implicated in telomere protection and heterochromatin establishment (Azzalin et al., 2007; Deng et al., 2009; Flynn et al., 2011; Schoeftner and Blasco, 2008). In *S. cerevisiae*, TERRA expression was shown to be induced at shortened telomere(s), most likely due to relieved repression of telomeric proteins, a proxy of telomere length, combined with rapid degradation by the nuclear ribonuclease Rat1 (Cusanelli et al., 2013; Iglesias et al., 2011; Luke et al., 2008). Thus, it appears that TERRA levels must be tightly controlled in *S. cerevisiae*. Indeed, the experimental induction of very high TERRA levels at a single telomere results in a local increased activity of Exo1. This leads to the shortening of the highly transcribing telomere *in cis* (Balk et al., 2014; Maicher et al., 2012; Pfeiffer et al., 2013; Pfeiffer and Lingner, 2012; Sandell et al., 1994). In the absence of telomerase, such derepression accelerates senescence (Maicher et al., 2012). Conversely, as telomeres naturally shorten in the absence of telomerase, telomeric RNA-DNA (presumably TERRA-DNA) hybrids promote inter-telomeric recombination, counteracting telomere shortening and senescence establishment (Balk et al., 2013). Thus, telomere transcription levels can modulate telomere processing in distinct and eventually opposite ways and the consequences with respect to senescence onset remain to be clarified.

In human and mouse cells TERRA transcription is driven by identified promoters in subtelomeric regions (Lopez de Silanes et al., 2014; Nergadze et al., 2009). In budding yeast, although the identification of a transcription start site (TSS) has been characterized at some chromosome ends (Pfeiffer and Lingner, 2012), the majority of promoters still remain to be defined. *S. cerevisiae* harbors two middle repetitive subtelomeric elements: the X- and the Y' -elements. Known properties of these elements include: maintenance of the silencing of nearby open reading frames, the regulation of DNA replication and differential *cis* effects on telomere length maintenance (Bianchi and

Shore, 2007; Craven and Petes, 1999; Pryde and Louis, 1999). Importantly, eventual *cis* effects on the signaling of senescence when telomeres become very short have never been studied. More specifically, whether these elements are required to regulate TERRA transcription and telomere processing during senescence is not known.

In this work we asked whether the subtelomere of the shortest telomere in the cell affects senescence. To that aim, we compared senescence onset in engineered sets of telomerase-negative strains in which the shortest telomere either harbored natural subtelomeric elements or was devoid of such elements. We observed that cells lacking natural subtelomeric regions at the critically short telomere entered senescence earlier. TERRA was increased at both critically short telomere variants although higher levels of telomeric transcription were seen at short telomeres lacking subtelomeric elements. Importantly, the inhibition of homologous recombination had a negative effect on growth in both sets of strains, as did the removal of TERRA hybrids through the overexpression of RNAse H1. This suggests that these processes can operate in a manner independent of the presence of subtelomeric elements. Finally these results suggest that in budding yeast, telomeric transcription may not dependent on a pre-defined promoter, but rather can be simply triggered by telomere shortening to a critical length. The subtelomere elements may buffer the extent to which TERRA gets transcribed in order to prevent early onset senescence.

## Results and discussion

*15 kb subtelomeric of chromosome 7L promotes viability in the absence of telomerase*

We previously showed that senescence can be initiated by a single, experimentally derived, critically short telomere (Abdallah et al., 2009; Fallet et al., 2014; Khadaroo et al., 2009; Xu et al., 2013). In these studies, we took advantage of a system in which we tracked a defined artificial telomere that can be shortened experimentally in a regulated manner (Abdallah et al., 2009; Marcand et al., 1999). This telomere is a modified version of telomere 7L (left arm of chromosome 7)(Figure 1A) in which the last 15 kb are removed and are thus devoid of subtelomeric elements (7L-VST, Figure 1B-C). The construct contains extra-telomeric repeats flanked by two FRT sites that can be excised

by the action of an inducible site-directed recombinase (Flp1, placed under the control of a galactose-inducible promoter). These extra-telomeric repeats inhibit telomerase action in *cis*, and thus the terminal telomeric tracts are maintained short in *cis* (Figure 1C). Loss of the *URA3* marker allows accurate tracking of the excision reaction. In order to know if the signaling of the shortest telomere depends on subtelomeric elements, we reconstructed the shortening system in a native telomere context, at the 6R telomere, which we called 6R-VST (Figure 1D-F, Supplementary Figure 1). As controls, strains bearing similar constructs in which the shortening is not occurring were also constructed (7L-CTL and 6R-CTL, Figures 1B and E). In the presence of telomerase, the four sets of strains had similar growth rates (Supplementary Figure 1B) and the mean telomere length of Y' telomeres is not affected by our constructs (Supplementary Figure 1C). Upon shortening, we reach a length of about 120 bp in both types of telomeres (Figure 1G, Supplementary Figure 1D-E). Taking into account the distribution of telomere lengths within a cell (Xu et al., 2013), each of these modified telomeres is likely the shortest telomere in their respective strains.

With these strains in hand we then proceeded to test how the presence of subtelomeric elements at the shortest telomere affects senescence. We thus generated independent diploids for the 7L and the 6R telomeric variants, in which the telomeric constructs are homozygous, and one allele of telomerase RNA template-encoding gene, *TLC1*, is deleted. The deleted copy of *TLC1* is replaced by a Nourseothricin resistance gene placed under the control of a promoter which is exclusively expressed in haploid *Mat*α cells (*tlc1::Pr*α*-NatR*) (Decourty et al., 2008). After sporulation, to generate meiotic haploid progeny, we immediately induce the telomere shortening (VST cells) by adding galactose to media and cells are then plated on Nourseothricin-containing media. Many individual telomerase-negative colonies for each set of strains are then tested for loss of the *URA3* marker, telomere length and viability as previously described (Fallet et al., 2014) (Figure 2A-B). Numerical analysis of the spot assays (from Figure 2A) not only takes into account the ability to form colonies, but also the size of colonies, as cells are re-passaged (by spotting) every two days, and lose growth potential (compare passages 1 to 3, Figure 2C). We found that cell division capacity differed depending on the strain. We found that the 6R-CTL colonies (with natural subtelomeres) had a better

proliferation capacity than cells with the modified 7L-CTL telomere lacking subtelomeric elements. This suggests that the 15 kbs at subtelomere 7L *per se* are required for optimal cell growth in the absence of telomerase. In contrast, in the presence of telomerase, we found no effect in equivalent plating conditions, even at different temperatures (Supplementary Figure 1b). We concluded that genetic elements present in this subtelomeric region contribute to viability in senescent cells.

We also found that while 7L-VST strain accelerates senescence compared to 7L-CTL strain, as reported (Abdallah et al., 2009), the 6R-VST cell also accelerates senescence compared to 6R-CTL cell. This demonstrates that even in a native context, the presence of a short telomere accelerates senescence, in accordance with the idea that the length of the shortest telomere in the cell is the major determinant of senescence onset in a context of wild-type telomeres (Xu et al., 2013). However, we still detect a delayed senescence between the 6R-VST and the 7L-VST cells, suggesting that when a critically short telomere arises, subtelomeric elements have the capacity to buffer senescence onset.

*Homologous recombination is similarly active at the shortest telomere regardless of the presence of subtelomeric elements.*

We were intrigued by the fact that cells lacking the 7L subtelomere senesced earlier. A major modulator of senescence is the homologous recombination machinery, that preferentially associates with the shortest telomere in senescent cells even before the generation of survivors (Dewar and Lydall, 2011; Fallet et al., 2014). We thus asked whether the recombination factors Rad51 and Rad52 localized at the 7L- or 6R-VST telomeres to the same extent. Telomerase-negative cell populations of 7L- or 6R-CTL and 7L- or 6R-VST were cultivated and chromatin was immunoprecipitated using primary antibodies against Rad52 and Rad51 (Figure 3A). While Y' telomeres were immunoprecipitated with the same efficiency among strains, the modified telomeres were specifically enriched upon shortening, irrespective of the presence of subtelomeric elements. This indicates that both proteins were similarly recruited to telomeres when they became critically short.

Homologous recombination is proposed to be involved in numerous pathways during telomere shortening-dependent senescence. This repair pathway may promote telomere lengthening or at least limit telomere shortening to sustain cell viability in the absence of telomerase. Possible mechanisms involve sister-chromatid exchanges or inter-telomeric recombination events. These latter are expected to depend not only on partial homology present within the degenerated telomeric repeats, but also on subtelomeric elements, which share homology among various chromosome ends. This could, in theory, account for the increased viability in 6R-VST strains compared to 7L-VST strains in that the former bears a greater homology region with other telomeres (through the subtelomere) than the latter, and hence would have an increased propensity to engage in inter-telomeric homology directed repair. If this were true then impairing HR should eliminate the differences in senescence between the 7L-VST and 6R-VST strains. We thus deleted Rad51 in our system and checked for the effects on proliferation capacity in telomerase-negative clones. Removal of the Rad51 recombinase resulted in a decrease in viability for all strain sets (Figure 3B), and a difference in cell proliferation ability remains between the 7L- and the 6R-VST. Therefore, the 6R-VST cells do not depend more on Rad51-mediated homologous recombination than 7L-VST cells. This indicates that not only Rad51 and Rad52 are recruited to the shortest telomere in senescent cells, but they presumably act with a similar efficacy in the presence or absence of subtelomeric elements. We cannot however rule out the contribution of Rad51 independent HR which may contribute to differences in the absence or presence of natural subtelomeres.

*Transcription at the shortest telomere in senescent cells in the absence of a subtelomeric promoter.*

One consequence of the removal of the subtelomeric element at telomere 7L is the absence of an endogenous TERRA TSS and presumably its native promoter at this chromosome arm. Thus, it is possible that TERRA production at this telomere is altered, preventing the homologous recombination to act *via* the formation of RNA-DNA hybrids as described (Balk et al., 2014; Balk et al., 2013). We thus measured TERRA levels at

telomeres in the four sets of senescing individual clones by qRT-PCR. Total RNAs were thus converted into cDNA using a telomere-specific probe hybridizing to the UG-rich repeats and amplified using a pair of primers targeting unique regions close to the 7L and 6R telomeric tracts (Figure 4A, primer pair -113). As expected, we found that TERRA was moderately up regulated (3-4 fold) at the 6R-VST, compared to 6R-CTL showing that the increase in telomeric transcription in senescent cells, as described previously (Cusanelli et al., 2013), is presumably arising from the shortest telomere(s) (Figure 4B). Importantly, we now demonstrated that this relationship is also true when subtelomeric elements are intact at the induced critically short telomere as is the case for 6R-VST (Figure 4B). Thus, TERRA is up regulated when telomeres shorten both in the presence or absence of telomerase.

Unexpectedly, we detected even higher levels of telomeric transcripts at the 7L-VST, suggesting that transcription can occur from a subtelomere-less chromosome end when it becomes critically short (Figure 4B). Sequencing of the amplicon confirmed the specificity of our RT-PCR (not shown). To have an independent verification that transcripts could be produced from this telomere, we immunoprecipitated the DNA from these cells using the monoclonal antibody S9.6, which specifically recognizes RNA-DNA hybrids as described (Balk et al., 2013; Pfeiffer et al., 2013). In accordance with a modest transcription induction at 6R-VST telomeres, we observed that RNA-DNA hybrids only slightly accumulated at these telomeres, when compared to Y' telomeres in the same cells or the 6R telomere in 6R-CTL cells (Figure 4C). On the other hand, the high increase in 7L-VST transcription correlated with higher levels of RNA-DNA hybrids accumulation. This provided us with an independent indication that TERRA is indeed generated at 7L-VST, which lacks any native subtelomeric element and suggests that subtelomeres may limit TERRA production at critically short telomeres.

We showed previously that TERRA transcription in senescent cells was involved in the maintenance of viability presumably through hybrids accumulation and recombination stimulation (Balk et al., 2013). If 7L-VST transcripts have a similar function, a prediction is that cells bearing the 7L-VST would be sensitive to the

overexpression of RNAse H1, which was previously successfully used to remove telomeric RNA-DNA hybrids (Balk et al., 2013; Pfeiffer et al., 2013). We thus introduced a plasmid encoding the yeast RNAse H1 gene, *RNH1*, under the control of a strong constitutive promoter in our four sets of strains. Upon telomerase removal and shortening of the telomere, we extracted nucleic acids from individual colonies and measured TERRA levels by qRT-PCR and RNA-DNA hybrids by DNA immunoprecipitation as above. To measure senescence, selected telomerase-negative colonies were spotted on solid media as above, in which we added a continuous selection for the *RNH1*-containing plasmid. In all conditions tested, overexpression of *RNH1* leads to accelerated senescence in at least one of the passages (Figure 4D). This result supports the idea that TERRA-like transcripts stemming from 7L-VST have a similar role to 6R-VST TERRA with respect to senescence, i.e. protection from senescence through the formation of pro-recombinogenic RNA-DNA hybrids. Taken altogether, our results suggest that 7L-VST expresses TERRA when a critically short length is obtained. It is possible that the different rates of TERRA production at 7L-VST and 6R-VST are accountable for the differences in senescence rates. In support of this, it is has been shown that strong induction of TERRA at a single telomere leads to Exo1 mediated resection of that telomere both in the absence (Balk et al, 2014) and presence (Pfeiffer et al, 2012) of telomerase. Therefore we speculate that at 7L-VST although pro-recombinogenic RNA-DNA hybrids are formed, they may not be able to compensate for the telomere loss associated with high rates of TERRA transcription.

We then asked how transcripts could be expressed from this region. Such a transcript would correspond to an antisense of the *ADH4* gene (Figure 1A, 4A). We thus searched databases containing all non-coding and cryptic RNAs detected in various conditions (Neil et al., 2009; van Dijk et al., 2011). No trace of *ADH4* antisense RNAs is found that accumulates in wild-type or the absence of a functional exosome, or Xrn1, a major ribonuclease. Furthermore, an RT-PCR using a pair of primers which covers 354 bp upstream of telomeric repeats does not amplify any product at the 7L-VST (Figure 4A, primer pair -354, Supplementary Figure 2A). We thus can map the 7L-VST transcription start site from 113 and 354 bp from the telomeric repeats (Figure 4A). This distance is consistent to that found for TERRA 5' end for the telomere 1L and Y'

(Pfeiffer and Lingner, 2012). Importantly, it supports the notion that this transcript is telomere length-related and not a result of a spurious transcription from an intergenic region at *ADH4* 3' end. Therefore, telomere shortening seems to trigger TERRA expression even in the absence of a pre-defined dedicated promoter.

## Conclusion

By studying senescence of telomerase-negative cells in which we have modified the structure of one of the telomeres in the cell, we show that removal of the last 15 kb of a single subtelomere accelerates senescence. This suggests that elements in this region sustain viability. This is likely unrelated to the ability of homologous recombination to extend telomere length via homology within subtelomeres. Thus, in the absence of telomerase, subtelomeres have a function independent of providing homology for recombination. Unexpectedly, we found that elevated levels of non-coding TERRA-like transcripts arise from the telomere lacking a native subtelomeric region upon critical shortening. Several arguments support the idea that these transcripts are actually TERRA molecules: (i) they generate a cDNA when reverse transcribed with CA-rich-specific primer, (ii) RNA-DNA hybrids accumulate at their encoding telomere, (iii) the transcripts decrease upon RNase H1 overexpression, and (iv) senescence is accelerated in this condition. We concluded that telomere shortening is sufficient to trigger TERRA transcription, which through the formation of RNA-DNA hybrids can stimulate recombination and prevent senescence onset. Subtelomeric elements appear to ensure that TERRA levels are kept in check at critically short telomeres, potentially to avoid Exo1 mediated resection. Since the shortest telomere(s) signals as a DNA damage in a telomere-dependent senescence scenario (Teixeira, 2013), this transcription is intriguingly reminiscent of the transcripts found at double-strand breaks in human and drosophila cells (Francia et al., 2012; Michalik et al., 2012). Thus, transcription at regions of DNA damage is probably also conserved in budding yeast.

## Experimental procedures

*Yeast strains*

All strains are derived from W303 and listed in Supplementary Table 1.

The 6R-CTL and VST strains were obtained by transforming yT360 by plasmids pT26 (6R-CTL) and pT29 (6R-VST) digested with *Sph* I and *Not* I. Transformants were verified by pulse field gel electrophoresis (Biometra Rotaphor according to manufacturer instructions) and Southern blot as described (Fallet et al., 2014). 6R probe was obtained by random priming of a *Sph* I – *Not* I fragment of sp26.

*Plasmid construction*

The *Sph* I-*Hind* III *adh4* region contained in sp225 and sp242 (Marcand et al., 1999) was replaced by a PCR fragment containing 0,5 kb region of the 6R chromosome end next to telomeric repeats. This fragment was obtained by PCR on genomic DNA using the oligonucleotides oT346 and oT347 (Supplementary Table 2).

*Senescence assay*

The senescence assay was performed as described (Fallet et al., 2014). Briefly, diploids (i) carrying one allele of telomerase RNA template gene *TLC1* replaced by the Nourseothricin gene resistance marker under the control of a *Mat* α-specific promoter, (ii) homozygous for the telomeric constructs and (iii) carrying the FLP1 gene under the control of the *GAL10* promoter, are mass sporulated and germinated in the presence of galactose rich medium for 6-9 hours. To control induction, germination of the same sporulation mixture is also performed in glucose –containing media. Telomerase-negative spores are selected by plating the germination mixture on Nourseothricin-containing media. After 2 days, a small portion of the colonies is used to genotype and verify the loss of the *URA3* marker. Remaining cells are then resuspended, adjusted to the same concentration, serially diluted 10 fold and then spotted on solid media, followed by a two days incubation at 30°C. This procedure is repeated twice every two days using mixed cells from the most concentrated spot. Plates are scanned with an

Epson Perfection V750 Pro and analyzed as described (Xu et al., 2013) to obtain the viability plots.

*Chromatin immunoprecipitation*

The protocol described in (Fallet et al., 2014) was slightly modified. Briefly, after germination in galactose-containing media, cells were plated on galactose- and nourseothricine-containing solid media and grown for two days at 30°C. This allowed to reach excision of the *URA3*-containing circle at more than 95% efficiency. Colonies were then pooled and grown to $OD_{600nm}$=0.8 and prepared for chromatin immunoprecipitation as reported using oligonucleotides listed in Supplementary Table 3.

*RNA-DNA hybrid ChIP*

RNA-DNA hybrid ChIPs were performed as previously described in Balk and Maicher et al. (2013)

*TERRA level*

TERRA levels were measured via qRT-PCR. The protocol published in (Luke et al., 2008, Iglesias et al., 2011) was followed. Used primers are listed in Supplementary Table 3.

*Sequencing of 6R and 7L TERRA amplicon*

The qRT-PCR products were purified and sent to GATC Biotech AG to have them sequenced in both forward and reverse direction. The sequencing data were aligned to presumable template sequences with the NCBI Alignment tool.

## Acknowledgements

We wish to thank the Teixeira and Luke lab members for technical advice and fruitful discussions. We also thank all members of the UMR8226 unit for technical support. This work was supported by the European Research Council (ERC- 2010-StG 260906—D-END to MTT), the Mairie de Paris (Programme Emergences), and the ‘‘Initiative d’Excellence’’ program from the French State (grant ‘‘DYNAMO,’’ ANR-11-LABX-0011-01). Research in the Luke lab is supported by the DFG (SFB1036), The Network of Aging Research (NAR) and the BMBF (CancerTelSys consortium–01ZX1302D). MG is supported through a Hartmut Hoffmann-Berling International Graduate School PhD fellowship and KS by the Lebanese National Council for Scientific Research.

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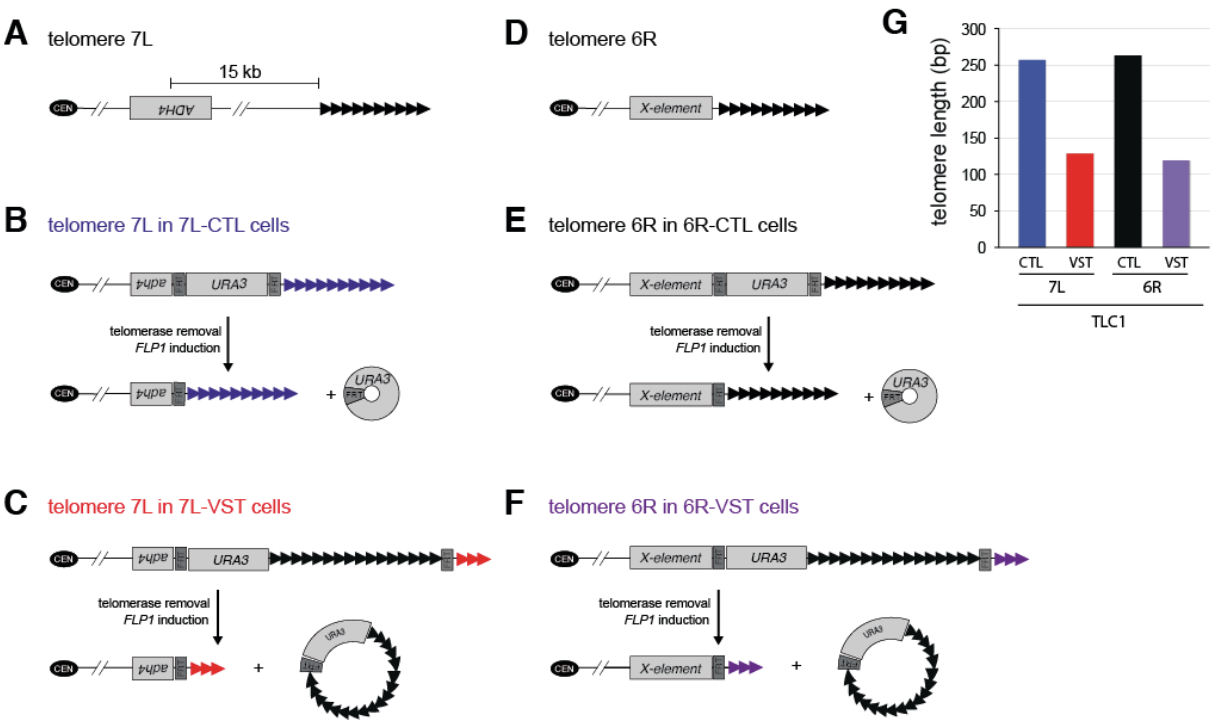



**Figure 1:** Experimental system to shorten a single telomere in the cell. The chromosome end containing the telomere 7L (A) is modified in two ways. In control (CTL) cells (B), the last 15 kb of the telomere end are replaced by a construct in which a *URA3* marker is flanked by two Flippase Recognition Target (FRT) sites and followed by a wild-type length telomeric tract (blue). (C) In cells able to generate a very short telomere (VST), the *URA3* marker is followed by extratelomeric repeats that inhibit the action of telomerase on telomeric repeats *in cis* (red). This results in a short terminal telomeric tract (red). (B-C) Upon telomerase removal and induction of the flippase (encoded by *FLP1*), sequences between the FRT sites are excised in a circle that is diluted out upon cell expansion. Remaining telomeres are wild-type length (B, blue) or very short (C, red). (D) The wild type 6R chromosome end is modified to generate 6R-CTL (E, black) and 6R-VST (F, purple) in a similar manner than for 7L (A-C). (G) Telomere lengths of 7L and 6R telomeres in CTL or VST strains were assessed after *FLP1* induction before telomerase removal by Southern Blot (shown in Supplementary Figure 1C).

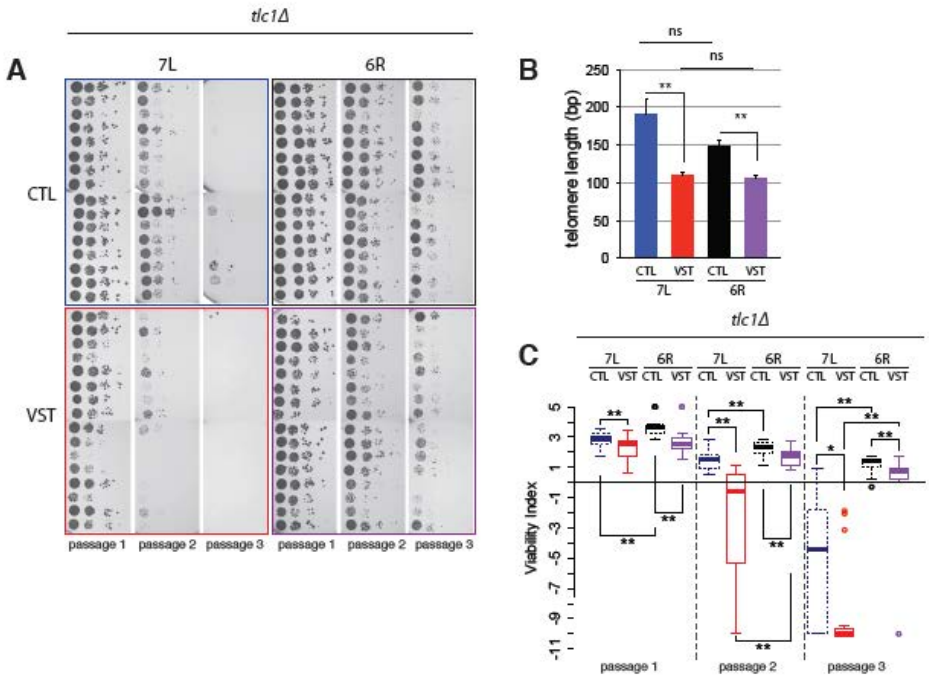



**Figure 2:** Effect of the subtelomeric region on replicative senescence.

(A) 16 Telomerase-negative individual spores carrying the telomere 7L-CTL (blue), 7L-VST (red), 6R-CTL (black) or 6R-VST (purple) as in Figure 1B-C, E-F were germinated for two days on selective media. Colonies grown on selective plates for 2 days are then resuspended to equal concentrations and 10-fold dilutions are spotted on solid media, grown at 30°C for 2 days and scanned at high resolution (passage 1). This is repeated two times (passage 2 and 3). (B) Cells from passage 1 are used to prepare DNA and telomere length measurements by telomere-PCR using specific primers amplifying either the 7L or the 6R-derived telomeres. Mean telomeric length is shown. Error bars correspond to s.e.m. Adjusted P values were obtained by Wilcoxon rank-sum test with a false discovery rate correction ** $P<0.05$ (n=14, 14, 16 and 9, respectively). (C) High resolution scans of plates shown in (A) are analyzed to obtain a numerical value for each serial dilution set that is related to the area of the colonies. Adjusted P values were obtained by Wilcoxon rank-sum test with a false discovery rate correction * $P<0.1$ and ** $P<0.05$. n=16 for 7L-CTL, 6R-CTL and 6R-VST, n=15 for 7L-VST. See Supplementary Table 2 for detailed P-values.

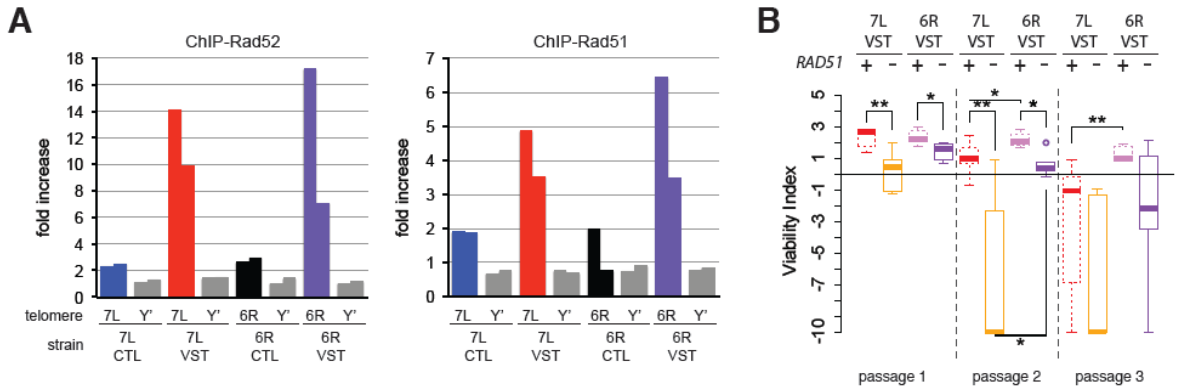



**Figure 3:** Homologous recombination machinery counteracts senescence in both the presence or absence of subtelomeric elements at the VST.

(A) Rad51 and Rad52 associate preferentially with short telomeres in the presence and absence of subtelomere. For each indicated strain, 7L-CTL, 7L-VST, 6R-CTL or 6R-VST, a mixed population of hundreds of independent telomerase-negative clones was grown ~30 population doublings after sporulation. Chromatin was immunoprecipitated using primary antibody against Rad52 (left panel) or Rad51 (right panel). The association of each protein to 7L, 6R or Y' telomeres or *ARO1* locus was quantified by qPCR and the fold increase of telomere enrichment over *ARO1* is represented. Two biological independent experiments are shown. (B) The absence of Rad51 accelerates replicative senescence in the presence or absence of subtelomeric elements at the shortest telomere. Quantitative analysis of senescence serial spot assay of cells carrying the 7L-VST or the 6R-VST as in Figure 2C. Adjusted P values were obtained by Wilcoxon rank-sum test with a false discovery rate correction * P<0.1 and ** P<0.05. n=7, 6, 5, 5 for each strain set respectively. See Supplementary Table 2 for detailed P-values.

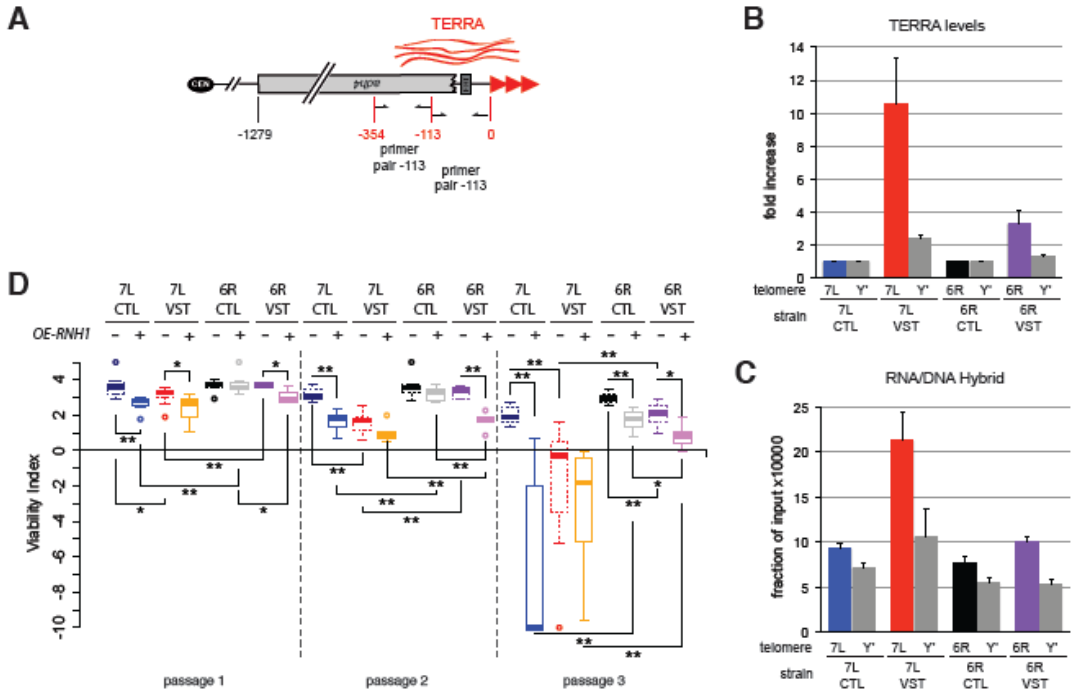
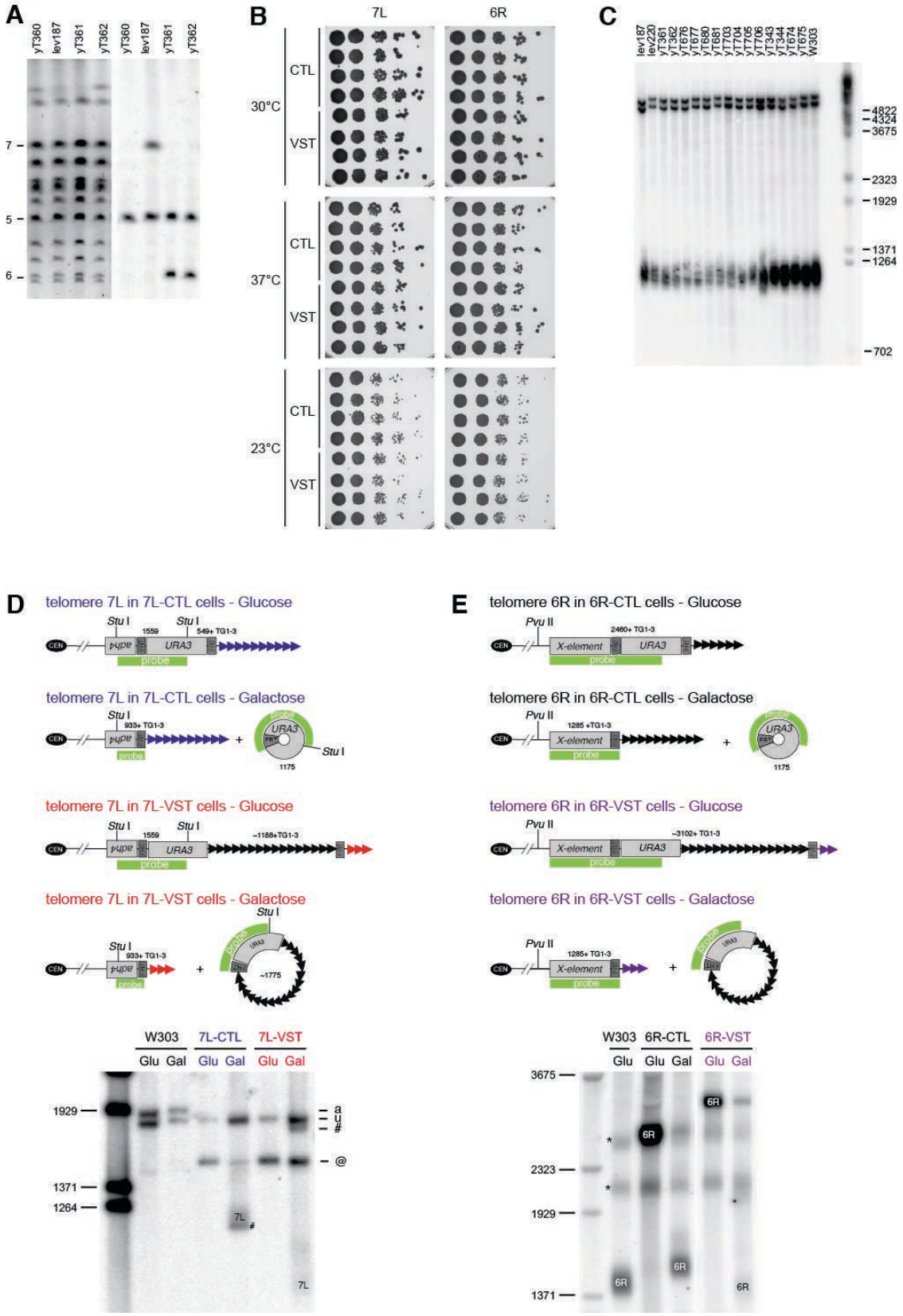



**Figure 4:** Overexpression of RNase H1 accelerates replicative senescence.

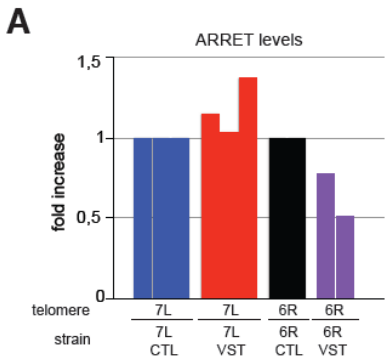
(A) Scheme of the 7L-VST region. "0" indicates the beginning if the telomeric repeats. Distance to "0" of primer pairs to amplify telomeric transcripts is indicated in base pairs. TERRA molecules as defined in this work are represented in red. (B) TERRA levels were measured using primer pair -113 for 7L or as described for the 6R and Y' telomeres (Iglesias et al., 2011) on cDNA prepared with a CA-rich telomeric primer. Mean levels from n=3 (7L-CTL and 7L-VST) or n=6 (6R-CTL and 6R-VST) independent clones are normalized to the corresponding CTL strains. Error bars correspond to s.e.m. (C) RNA-DNA hybrids were measured by DNA immunoprecipitation using the monoclonal S9.6 antibody. Fraction of input DNA of indicated chromosome ends was quantified by qPCR using primers as for TERRA levels in (B). (D) Overexpression of RNase H1 accelerates senescence. Quantitative analysis of senescence serial spot assay in telomerase-negative independent clones cells of indicated strains as in Figure 2C. Adjusted P values were obtained by Wilcoxon rank-sum test with a false discovery rate correction * P<0.1 and ** P<0.05. n=8, 7, 7, 6, 8, 8, 5, 5 for each strain set respectively. See Supplementary Table 2 for detailed P-values.

# Supplementary Information

Serhal et al., Supplementary Figure 1



**Supplementary Figure 1:** (A) Pulse-field gel electrophoresis of indicated strains (left panel) was transferred onto a nylon membrane and hybridized with a *URA3* probe (right panel). (B) Viability of telomerase-positive cells containing the constructs shown in Figure 1B, C, E, F (7L-CTL: lev187; 7L-VST: lev220; 6R-CTL: yT361; 6R-VST: yT362). 10-fold serial dilutions of 4 independent clones were spotted on minimal media and then incubated for 2 days at 30°C, 37°C or 23°C. (C) Lengths of Y' telomeres of indicated telomerase-positive strains were measured by Southern blot of *Xho* I digested genomic DNA hybridized with a Y' specific probe. (D) Schematic representation of Telomere Restricted Fragment (TRF) analysis and corresponding Southern blots at telomere 7L (D) and 6R (E) of strains labeled as in (B). Cells were cultivated for four hours in a rich medium containing either glucose or galactose, to induce shortening, followed by DNA extraction and DNA restrictive digestion by *Stu* I (D) or *Pvu* II (E). u: internal *ADH4* locus; a: internal *ura3* locus ; #: excised URA3-containing circle (linearized); @: fragment of the 7L when excision did not occur; *: corresponds to cross hybridization to other X elements in the genome; 7L: 7L TRF; 6R: 6R TRF.




**Supplementary Figure 2:** ARRET levels were measured using primer pair -113 for 7L or as described for the 6R and Y' telomeres (Iglesias et al., 2011) on cDNA prepared with telomere-specific primers.

**Supplementary Table 1:** Yeast strains used in this study

All strains are in the W303 background. All strains were constructed for this work, except those labelled with *, which were described previously (Abdallah et al., 2009; Fallet et al., 2014; Marcand et al., 1999).

| Strain | Genotype |
|---|---|
| yT130* | Matα cir° ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat telVIIL::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| yT132* | Matα cir° ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat telVIIL::FRT-URA3-[tel600]-FRT-tel pCen-ARS-TRP1-TLC1 |
| yT136* | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 telVIIL::FRT-URA3-FRT-tel/telVIIL::FRT-URA3-FRT-tel |
| yT138* | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 telVIIL::FRT-URA3-[tel600]-FRT-tel/telVIIL::FRT-URA3-[tel600]-FRT-tel |
| yT347* | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-10O/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-Flp1-Leu2/leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat/TLC1 telVIIL::FRT-URA3-FRT/telVIIL::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 rad51::HIS3/RAD51 |
| yT348* | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-10O/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-Flp1-Leu2/leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat/TLC1 telVIIL::FRT-URA3-[tel600]-FRT/telVIIL::FRT-URA3-[tel600]-FRT-tel pCen-ARS-TRP1-TLC1 rad51::HIS3/RAD51 |
| yT360 | Mata cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 |
| yT361 | yT360 telVIRX::FRT-URA3-FRT-tel |
| yT362 | yT360 telVIRX::FRT-URA3-[tel600]-FRT-tel |
| yT483 | Matα cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat telVIRX::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| yT484 | Matα cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat telVIRX::FRT-URA3-[tel600]-FRT-tel pCen-ARS-TRP1-TLC1 |
| yT502 | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 telVIR::FRT-URA3-FRT-tel/telVIR::FRT-URA3-FRT-telMatα cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat telVIR::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| yT503 | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 telVIR::FRT-URA3-[tel600]-FRT-tel/telVIR::FRT-URA3-[tel600]-FRT-tel |
| yT674 | yT361 rad51::HIS3 |
| yT675 | yT362 rad51::HIS3 |
| yT676 | Lev187 pRS413-GPD |
| yT677 | Lev187 pRS413-GPD-RNaseH |
| yT680 | Lev220 pRS413-GPD |
| yT681 | Lev220 pRS413-GPD-RNaseH |
| yT703 | yT361 pRS413-GPD |
| yT704 | yT361 pRS413-GPD-RNaseH |
| yT705 | yT362 pRS413-GPD |

| | |
|---|---|
| yT706 | yT362 pRS413-GPD-RNaseH |
| lev187* | Mata cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 telVIIL::FRT-URA3-FRT-tel |
| lev220* | Mata cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 telVIIL::FRT-URA3-[tel600]-FRT-tel |

**Supplementary Table 2:** p-values for the spot assays determined by pairwise Wilcoxon rank sum test with a false discovery rate adjustment

| Experiment | Genotype 1 | Genotype 2 | Passage | p- value |
|---|---|---|---|---|
| | Control 7L | VST 7L | 1 | 0,036 |
| Figure 2C | Control 7L | VST 7L | 2 | 0,0001 |
| | Control 7L | VST 7L | 3 | 0,08569 |
| | Control 6R | VST 6R | 1 | 0,0013 |
| | Control 6R | VST 6R | 2 | 0,00793 |
| | Control 6R | VST 6R | 3 | 0,01016 |
| | Control 7L | Control 6R | 1 | 0,0013 |
| | Control 7L | Control 6R | 2 | 0,00024 |
| | Control 7L | Control 6R | 3 | 0,000017 |
| | VST 7L | VST 6R | 1 | 0,5936 |
| | VST 7L | VST 6R | 2 | 0,000026 |
| | VST 7L | VST 6R | 3 | 0,000017 |
| | VST 7L RAD51 | VST 7L rad51 | 1 | 0,026 |
| Figure 3B | VST 7L RAD51 | VST 7L rad51 | 2 | 0,034 |
| | VST 7L RAD51 | VST 7L rad51 | 3 | 0,307 |
| | VST 6R RAD51 | VST 6R rad51 | 1 | 0,063 |
| | VST 6R RAD51 | VST 6R rad51 | 2 | 0,063 |
| | VST 6R RAD51 | VST 6R rad51 | 3 | 0,505 |
| | VST 7L RAD51 | VST 6R RAD51 | 1 | 0,755 |
| | VST 7L RAD51 | VST 6R RAD51 | 2 | 0,088 |
| | VST 7L RAD51 | VST 6R RAD51 | 3 | 0,028 |
| | VST 7L rad51 | VST 6R rad51 | 1 | 0,395 |
| | VST 7L rad51 | VST 6R rad51 | 2 | 0,075 |
| | VST 7L rad51 | VST 6R rad51 | 3 | 0,32 |
| | Control 7L/ -RNH1 | Control 7L/ +RNH1 | 1 | 0,007 |
| Figure 4D | Control 7L/ -RNH1 | Control 7L/ +RNH1 | 2 | 0,00097 |
| | Control 7L/ -RNH1 | Control 7L/ +RNH1 | 3 | 0,0033 |
| | Control 7L/ -RNH1 | VST 7L/ -RNH1 | 1 | 0,0891 |
| | Control 7L/ -RNH1 | VST 7L/ -RNH1 | 2 | 0,00097 |
| | Control 7L/ -RNH1 | VST 7L/ -RNH1 | 3 | 0,0033 |
| | VST 7L/ -RNH1 | VST 7L/ +RNH1 | 1 | 0,0891 |
| | VST 7L/ -RNH1 | VST 7L/ +RNH1 | 2 | 0,37462 |
| | VST 7L/ -RNH1 | VST 7L/ +RNH1 | 3 | 0,3278 |
| | Control 6R/ -RNH1 | Control 6R/ +RNH1 | 1 | 0,6432 |
| | Control 6R/ -RNH1 | Control 6R/ +RNH1 | 2 | 0,3283 |
| | Control 6R/ -RNH1 | Control 6R/ +RNH1 | 3 | 0,0011 |
| | Control 6R/ -RNH1 | VST 6R/ -RNH1 | 1 | 0,0841 |
| | Control 6R/ -RNH1 | VST 6R/ -RNH1 | 2 | 0,00335 |
| | Control 6R/ -RNH1 | VST 6R/ -RNH1 | 3 | 0,087 |
| | VST 6R/ -RNH1 | VST 6R/ +RNH1 | 1 | 0,0684 |
| | VST 6R/ -RNH1 | VST 6R/ +RNH1 | 2 | 0,01389 |

| | | | |
|---|---|---|---|
| VST 6R/ -RNH1 | VST 6R/ +RNH1 | 3 | 0,0778 |
| Control 7L/ -RNH1 | Control 6R/ -RNH1 | 1 | 1 |
| Control 7L/ -RNH1 | Control 6R/ -RNH1 | 2 | 0,45094 |
| Control 7L/ -RNH1 | Control 6R/ -RNH1 | 3 | 0,0169 |
| Control 7L/ +RNH1 | Control 6R/ +RNH1 | 1 | 0,0044 |
| Control 7L/ +RNH1 | Control 6R/ +RNH1 | 2 | 0,00097 |
| Control 7L/ +RNH1 | Control 6R/ +RNH1 | 3 | 0,0033 |
| VST 7L/ -RNH1 | VST 6R/ -RNH1 | 1 | 0,0157 |
| VST 7L/ -RNH1 | VST 6R/ -RNH1 | 2 | 0,00471 |
| VST 7L/ -RNH1 | VST 6R/ -RNH1 | 3 | 0,0088 |
| VST 7L/ +RNH1 | VST 6R/ +RNH1 | 1 | 0,2393 |
| VST 7L/ +RNH1 | VST 6R/ +RNH1 | 2 | 0,14504 |
| VST 7L/ +RNH1 | VST 6R/ +RNH1 | 3 | 0,0033 |
| Control 7L/ -RNH1 | Control 7L/ +RNH1 | 1 | 1 |
| Control 7L/ -RNH1 | Control 7L/ +RNH1 | 2 | 0,27894 |
| Control 7L/ -RNH1 | Control 7L/ +RNH1 | 3 | 0,3594 |

**Supplementary Table 3:** Oligonucleotides used in this study

| Name | Use | Sequence 5'-3' |
|---|---|---|
| oT323 | RAD51 F1 | TTCTTCTATCTTCCGTAGTTTCCATATACTAGTAGTTGAGTGTAGCGACAcggatcccgggttaattaa |
| oT324 | RAD51 R1 | GGATGGAAATGAAGATAAAAATGTACGGAACGCAACCTAAGAAAAAGAGGgaattcgagctcgtttaaac |
| oT346 | subtelVIR F | CCCAGATGGAAGCTTTCACTAGCATATTGATATGGCGTACGC |
| oT347 | subtelVIR R | AATCGTGCATGCCGGAGAGTTAACAAGCGGCTGG |
| oT372 | VIR integration R | CCGGGATCTTGAAGTTCCTATTCCGAAGTTCC |
| oT374 | VIR integration F | GCCTTTCAAAGCATACATATGAATATTACAACC |
| oT169 ( ?) | teloPCR | CGGGATCCGGGGGGGGGGGGGGGGG |
| oT182 | teloPCR Y' | TACATGAGGGCTATTTAGGGCTATTTAGGG |
| oT156 | teloPCR VIIL | TTGTTGAAAGCCATACCTGCC |
| oT531 | teloPCR VIR | AGGACTGGGTCATGGGGCGC |
| oT310 | qPCR VIIL F | TGATATGTGTTACGCAGAATAC |
| oT311 | qPCR VIIL R | TGAGAAGCACCGCAATG |
| oT312 | qPCR VIR F | ATCATTGAGGATCTATAATC |
| oT313 | qPCR VIR R | CTTCACTCCATTGCG |
| oT314 | qPCR ARO1 F | TCGTTACAAGGTGATG |
| oT315 | qPCR ARO1 R | AATAGCGGCAACAAC |
| oT570 | qPCR Y' F | GGCTTGGAGGAGACGTACATG |
| oT571 | qPCR Y' R | CTCGCTGTCACTCCTTACCCG |
| oMG20 | qPCR VIR telomere F | GTCATGGGGCGCAATGGAGTG |
| oMG21 | qPCR VIR telomere R | CTAGCATATTGATATGGCGTACGC |

| | | |
|---|---|---|
| oBL258 | qPCR VIR TERRA F | GTGTGTAGTGATCCGAACTCA |
| oBL259 | qPCR VIR TERRA R | GCATATTGATATGGCGTACGCACACGT |
| oMG30 | qPCR VIIL telomere/ TERRA F | ACCTCTACTCTGGGATCTCTCG |
| oMG29 | qPCR VIIL telomere/ TERRA R | CTTTGTATGCGGCGACCAAG |
| oBL292 | qPCR actin F | CCCAGGTATTGCCGAAAGAATGC |
| oBL293 | qPCR actin R and RT-PCR actin | TTTGTTGGAAGGTAGTCAAAGAAGCC |
| oBL207 | RT-PCR TERRA | CACCACACCCACACACCACACCCACA |

# II. Unpublished results

## 1. Genetic screen to identify novel pathways implicated in replicative senescence

Kamar-al-Zaman Serhal, Aurelia Barascu, Maria Teresa Teixeira

Different genome-wide screens were done to obtain a better understanding of telomere biology. Some were performed to identify proteins that affect telomere length, others were done to look for mutants that affect survival pathway in the absence of telomerase (Gatbonton et al., 2006; Hu et al., 2013; Ungar et al., 2009), and still others were conducted to examine senescence kinetics (Chang et al., 2011; Winzeler et al., 1999). In these genetic screens, the authors took advantage of the non-essential mutant collection. This collection contains the mutants corresponding to non-essential ORFs that are deleted (Winzeler et al., 1999). However, none of the genetic screens have yet addressed the mechanisms that occur at a specific telomere. Therefore, we aimed to identify factors that act specifically at a short telomere and interfere in its maintenance and in the signalling of replicative senescence. Through this genetic screen, we aim to investigate the factors that act at the telomeric region and the subtelomeric region, therefore crossing the barriers that render the subtelomeric region a difficult region to study.

In this screen, a library of yeast proteins is artificially recruited to the short telomere in telomerase-negative cells. Yeast proteins are constitutively encoded on a plasmid under a strong promoter and fused to a Gal4-binding domain (GBD). Therefore, the recruitment is based on the interaction between proteins fused to GBD and the upstream activating sequences (UAS) inserted at the very short telomeres. We then select clones in which the binding of a specific protein to the very short telomere allows cells to grow better. The screen is done in *rad52* mutant cells, to take advantage of rapid senescence occurring in this background (Figure 16).

### 1.1. UAS sequences do not affect cell viability in telomerase-negative cells

To identify factors that counteract replicative senescence, we took advantage of the very short telomere system in which a defined telomere can be tracked and shortened experimentally (Abdallah et al., 2009; Fallet et al., 2014; Marcand et al., 1999). In this system, an artificial truncated chromosome 7L is generated and harbors the very short telomere system. The insertion of this system at the left arm of chromosome 7 at the *adh4* locus results in the removal of the non-essential 15 kb corresponding to the subtelomeric region, and located upstream of telomeric repeats. In addition to the *adh4* present in the system, the system telomere harbors extra copies of telomeric repeats flanked by two flippase recognition target (FRT) sequences (Figure 17C). By inducing the site-directed recombinase flippase (Flp1), which is controlled by a galactose inducible promoter, the extra telomeric repeats are excised, leaving an intact telomere end. Since no telomerase activity is present in these cells, a short telomere is generated (7L-VST). Control cells contained a control construct, which lacked the extra-telomeric repeats and in which the VIIL telomere was of nearly wild-type length (CTL) (Figure 17B). With this system, the 7L-VST senesced earlier than the control cells (7L-CTL), and was sufficient to determine the onset of replicative senescence (Abdallah et al., 2009; Fallet et al., 2014).

To perform our genetic screen and identify factors that counteract replicative senescence, we modified the single telomere shortening system VST-7L by inserting 4×UAS binding sites upstream to the first FRT site, allowing the binding of fusion proteins (Figure 17D-E). As a control, we also modified the VST-7L by inserting UAS scrambled (scra) sequences (Figure 17F-G). In a similar way, the UAS scrambled fragment was inserted upstream to the first FRT site. After yeast transformation, transformants were verified by PFGE for the insertion at chromosome 7. Further verification was done by southern blots to verify the telomere shortening.

We then checked whether the insertion of these sites would have an effect on replicative senescence. To do that, we generated a diploid strain, which is heterozygous for the telomerase RNA subunit and homozygous for the 7L-VST-UAS or 7L-CTL-UAS constructs. After sporulation and telomere shortening induction through the addition of galactose, telomerase-negative cells were selected on media containing Nourseothricin.

We verified that several cells had telomere shortened (Material and Methods). Senescence assays were then performed, by assessing cell viability in serial passages performed every 2 days. Despite the presence of the 4×UAS sequences, we also found that 7L-VST-UAS accelerates senescence compared to 7L-UAS CTL (Figure 18A). This demonstrates that the presence of the 4×UAS fragment does not affect replicative senescence signaling. Similar to the 4×UAS sequences, the 7L-VST-UASscra accelerates senescence with respect to the 7L-CTL-UASscra (Figure 18A). This suggests that the UAS scrambled sequence also does not affect the short telomere signaling.

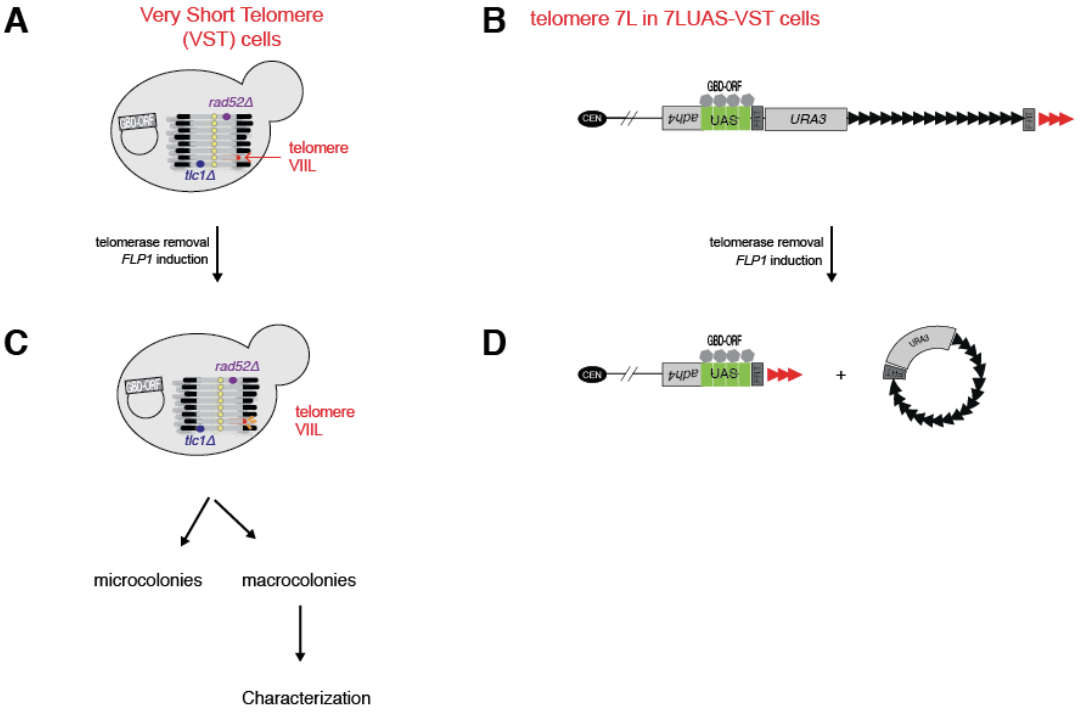



**Figure 16. Schematic representation of the genetic screen**

(A) A *rad52Δ tlc1Δ* yeast cell contains the short telomere at the chromosome 7 and expresses constitutively yeast proteins fused to Gal4 binding domain. (B) The last 15 Kb of chromosome 7L is replaced by a construct by which a *URA3* marker followed by an extra-chromosomal repeats are flanked by two Flippase Recognition Target (FRT) sites. At the extremity of the system, exists a short tract of telomeric repeats. Upstream of the first FRT sites, 4xUAS sequences are present that serves as site of fixation for GBD. (C) The induction of Flippase and the loss of telomerase result in yeast cells bearing a short telomere at the chromosome 7L. (D) The short telomere is generated by the excision of the fragment between the two FRT sites. The generation of a short telomere results in the formation of micro and macro colonies. Macro colonies are then selected and the ORF that lead to the generation of big colonies are characterized.

Accordingly, 7L-VST-UAS did not show a significant difference in senescence with the 7L-VST-UASscr (Figure 18A). Similar results were obtained with the control strains. In addition, UAS and UAS scrambled sequences did not lengthen telomeres, i.e., after telomere shortening, telomere mean length was about 100 bp, in accordance with what is observed before (Figure 18B;( Abdallah et al., 2009)). All together, this suggests that our strains can be used to identify candidates that potentially counteract replicative senescence.

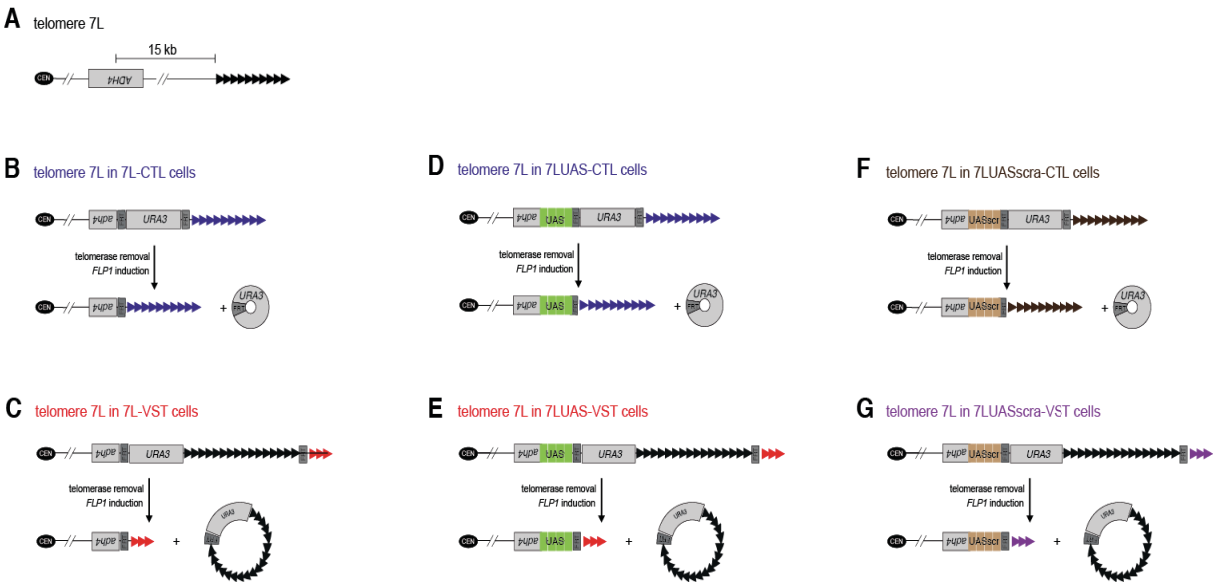



**Figure 17: Experimental system to obtain a single short telomere in the cell.**
(A) The chromosome end of telomere 7L is modified by the removal of the 15 Kb upstream of telomeric repeats. (B-C) Corresponds to the 7L control cells and the 7L VST cells containing the wild type telomere length (blue) and the short telomere repeats (red) respectively (D-E) Modification of (B) and (C) by the insertion of 4xUAS sites upstream of the first FRT site. (F-G) Modification of (B) and (C) by the insertion of UAS scrambled sites upstream of the first FRT site. (B-G) The induction of the Flippase (FLP1) under the galactose promoter will result in the excision of the fragment between the FRT sites, which will be diluted through our cellular divisions.

## 1.2. Gal4-BD binding to short telomeres does not affect cell viability in telomerase-negative cells

The recruitment of yeast proteins to telomeres is based on the binding of GBD to UAS sequences. The protein fused to GBD is expressed constitutively under the strong

promoter ADH1. To eliminate the hypothesis that the delay in senescence that we observed might be due to the effect of the Gal4 expressed from the plasmid binding to UAS, we checked the effect of GBD recruitment to the short telomere on signalling replicative senescence. We aimed to compare senescence between cells that express GBD from the plasmid to cells containing an empty plasmid. Here, we used the pRS313 plasmid as a control, which was used as a backbone to clone the $adh1_p$-GBD-$adh1_t$ fragment. Senescence assay analysis demonstrated that no significant cell viability differences were obtained when comparing cells that encode the GBD to cells that do not. The accelerated senescence phenotype observed is due to the absence of rad52 proteins (Figure 19A; Abdallah et al., 2009)). To validate that GBD is expressed in the cells, western blot using an antibody anti-GBD was conducted, confirming the expression of GBD from the plasmid. The GBD protein is about 16 KDa (Figure 19B).

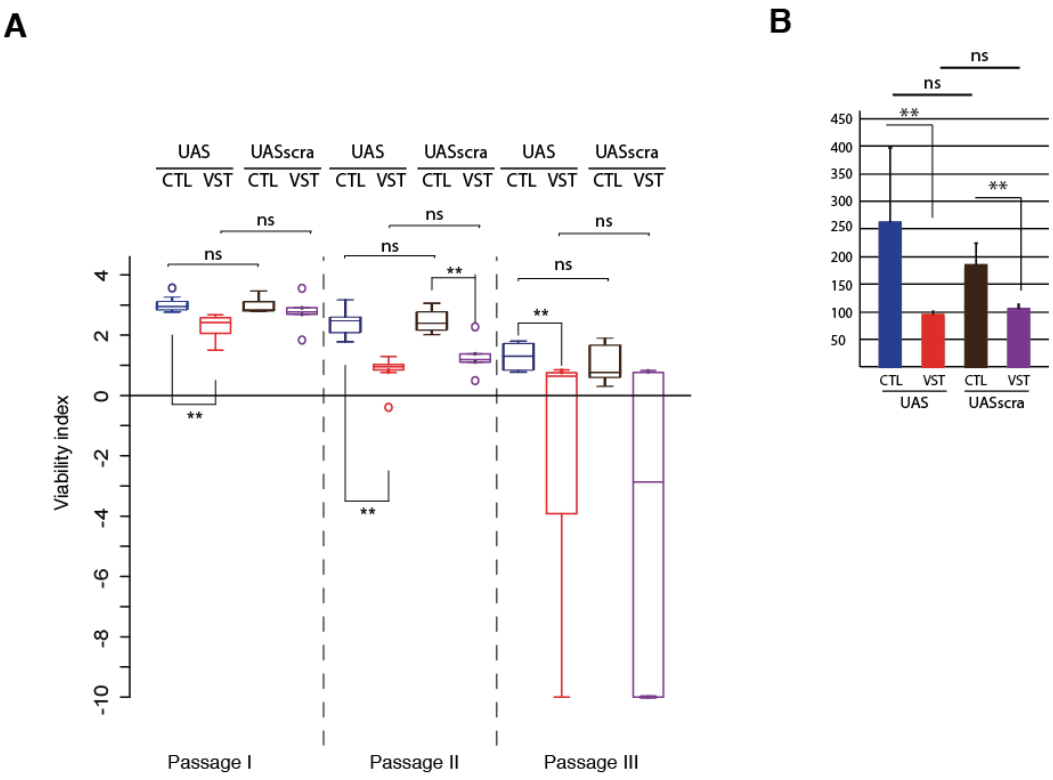



**Figure 18: The effect of UAS sequences on replicative senescence.**
(A) Quantitative analysis of senescence serial spot assay of cells carrying the 7L-UAS CTL, 7L-VST-UAS, 7L-CTL-UASscra, or 7L-VST-UASscra. (B) Cells from passage 1 are used to prepare DNA and telomere length measurements by telomere-PCR using specific primers amplifying the 7L telomeres. Mean telomeric length is shown. Error bars correspond to standard deviation. Adjusted P values were obtained by Wilcoxon rank-sum test with a false discovery rate correction ** $P<0.05$ (n=8, 8, 8 and 8, respectively). See Annex Table 2 and 3 for detailed P-values.

### 1.3. Genetic screen and candidates that delay replicative senescence

To identify factors that act specifically at our short telomere, we generated an *S. cerevisiae* genomic library in a low-copy replicative plasmid. The idea is to recruit yeast proteins that are expressed constitutively in the 7L-VST-UAS strains, and to select for big colonies being formed after telomere shortening. These big colonies could correspond to cells that delay senescence. After sporulation and telomere shortening, cells were plated on plates lacking histidine to select for plasmids encoding the fused proteins, containing Nourseothricin and Genetecin to select for *tlc1Δ* and *rad52Δ* cells, respectively. Plates were then incubated at 30°C for 1.5 days, and colony sizes were analyzed. Plates contained heterogeneous colony sizes. Bigger colonies were selected and checked for telomere shortening, whether they contained the plasmid, and whether they were deficient in *tlc1* and *rad52*. Further analysis was done by PCR on extracted plasmids to check for proteins fused to the GBD. Among the candidates selected, two candidates shared all of the criteria tested. These candidates were sequenced and mapped. Candidate 11 corresponded to two parts of two different genes. The first part corresponds to the C-terminal of the *ILV2* gene located at chromosome 13. This gene encodes acetolactate synthase, which catalyzes the first step of isoleucine and valine biosynthesis. The second part corresponds to the *MYO5* gene, also located at chromosome 13. This gene encodes for one of the two-myosin motors. The second candidate, candidate 13 corresponds to two different parts of two chromosomes that have fused together during the ligation reaction. This corresponds to the c-terminal of the *PEF1* gene located at chromosome 16 and is responsible for polar bud growth. This gene is fused to the X-subtelomeric region of chromosome IV. The theoretical transcription of the GBD followed by the fused proteins under the *ADH1* promoter results in a smaller protein for candidate 11 than the expression of GBD alone (11 KDa). This was explained by the presence of a stop codon after GBD in the fused sequence, while the GBD encoded alone contains a terminator sequence, therefore rendering the GBD alone larger. Candidate 16 expressed a protein larger than GBD alone (38 KDa). These results were then verified on western blots (Figure 19B).

To verify the effects of these candidates on cell viability, the two candidates were transformed independently in the diploid of origin. Senescence assays were then

performed twice on two independent colonies. Different results were obtained in the two experiments and the senescence phenotype obtained was not striking.

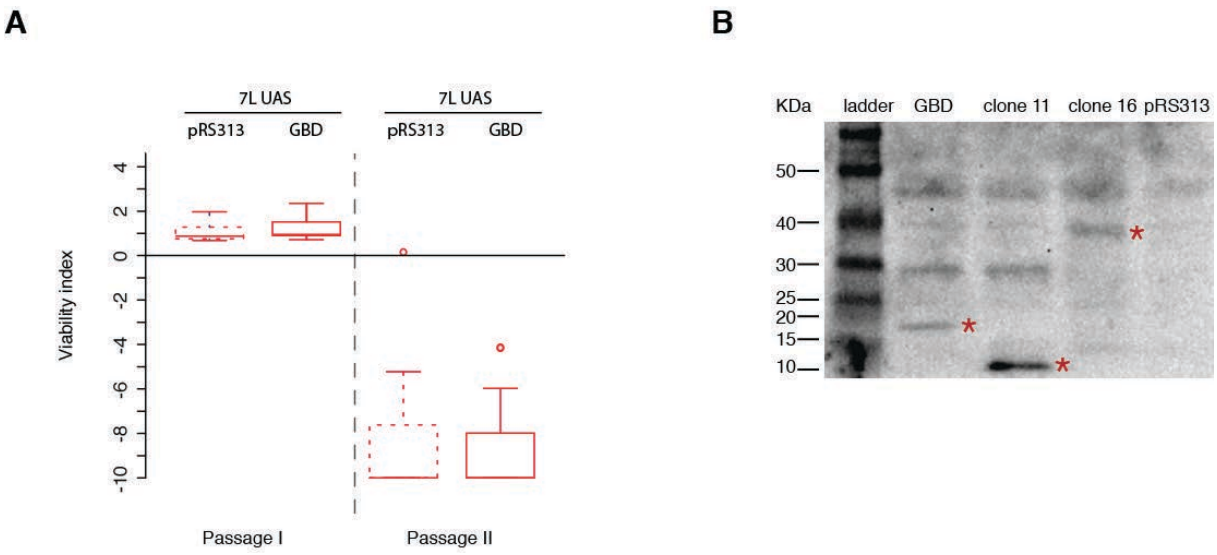



**Figure 19: The effect of GBD recruitment to telomeres on replicative senescence**
(A) Quantitative analysis of senescence serial spot assay of *rad52Δ* cells carrying the 7L-VST-UAS, containing an empty plasmid (pRS313) or either containing plasmid coding for GBD. Adjusted P values were obtained by Wilcoxon rank-sum test with a false discovery rate correction ** P<0.05 (n=8, 7,respectively). See Annex Table 2 for detailed P-values.
(B) Western blot using an antibody ant-GBD. Cells expressing the GBD alone, or candidates selected after the genetic screen were verified for protein expression. pRS313 is a control that does not express GBD. * Corresponds to the expected proteins.

Therefore, the candidates that were identified do not have an effect on the signaling of replicative senescence initiated at the very short telomere. Further experiments and setups should be conducted to identify the factors involved in delaying replicative senescence.

## 2. Purification of the critically short telomere

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Telomere purification has already been attempted in mammalian cells (Dejardin and Kingston, 2009; Grolimund et al., 2013), but never in the context of replicative senescence. Furthermore, several chromatin immunoprecipitation experiments have identified proteins that bind to telomeres in telomerase-deficient cells. However, no genome-wide study has been conducted identify the protein interactome at telomeres. Therefore we aimed to identify the proteins that are differentially associated with a long or a short telomere using a biochemical approach, in telomerase-deficient cells. To identify these proteins, we adopted the DNA sampling method (Butala et al., 2009a).

identify these proteins, we adopted the DNA sampling method (Butala et al., 2009b).

The idea of the DNA sampling method consists of identifying proteins bound to a certain region, after purifying the DNA using an affinity column and analyzing the proteins by mass spectrometry (Figure 20). We will apply this at the VST-7L telomere. To do that, the VST-7L telomere will be purified after telomere shortening, and proteins bound to this region will be identified by mass spectrometry.

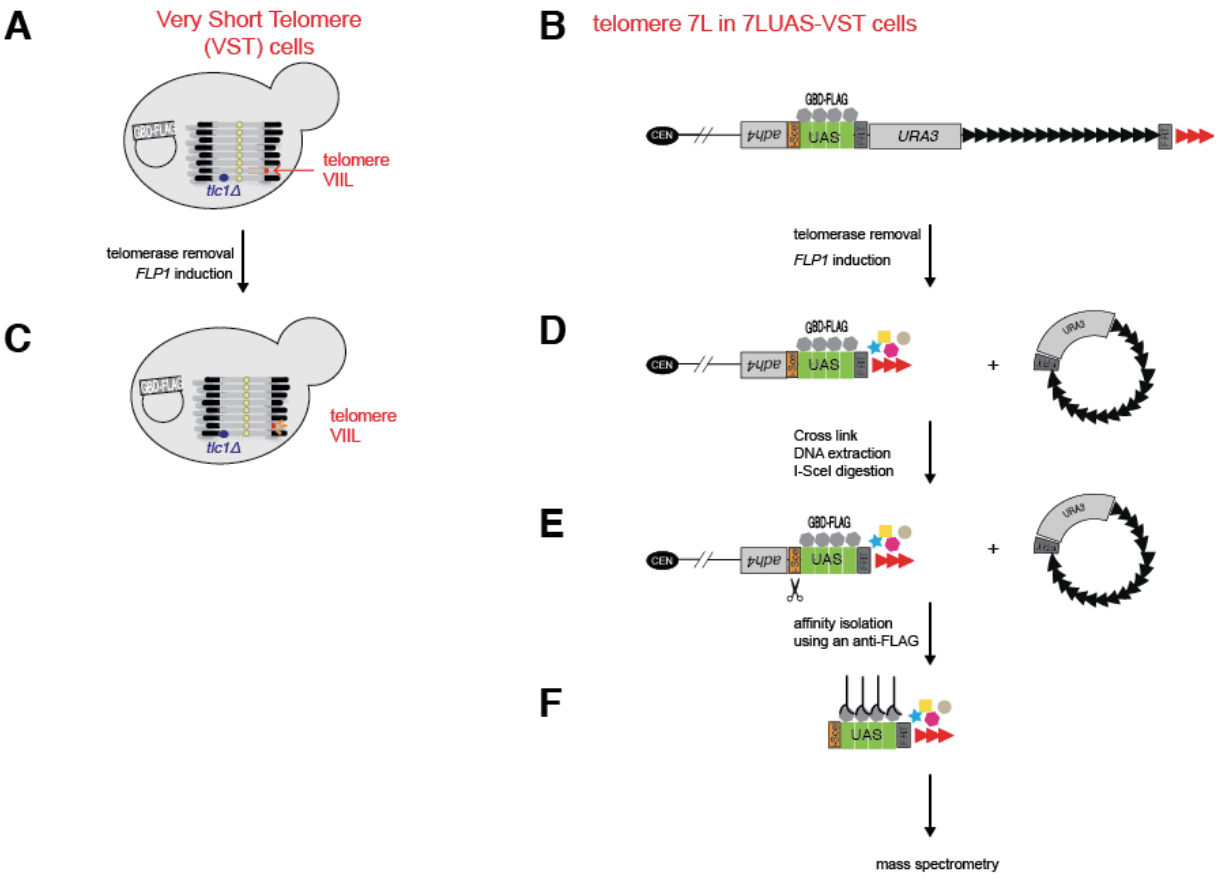



**Figure 20: Purification of the very short telomere**

(A) A *tlc1Δ* yeast cell contains the short telomere at the chromosome 7 and expresses constitutively FLAG protein fused to Gal4 binding domain. (B) The last 15 Kb of chromosome 7L is replaced by a construct by which a *URA3* marker followed by an extra-chromosomal repeats are flanked by two Flippase Recognition Target (FRT) sites. At the extremity of the system, exists a short tract of telomeric repeats. Upstream of the first FRT sites, 4xUAS sequences followed by an I-*Sce* I site are present. (C) The induction of Flippase and the loss of telomerase result in yeast cells bearing a short telomere at the chromosome 7L. (D) The short telomere is generated by the excision of the fragment between the two FRT sites. A consequence of this shortening is the binding of different proteins involved in short telomere signaling. (E) After DNA extraction, DNA is digested with the restrictive enzyme I-*Sce* I. (F) the short telomere with the proteins bound to it is then purified using an antibody anti-FLAG. The proteins bound to the specific short telomere are then identified by mass spectrometry.

## 2.1. I-SceI sequence does not affect cell viability in telomerase-negative cells

To identify proteins that bind to the VST-7L telomere after telomere shortening, we took advantage of the modifications of the VST-7L described above. A unique restriction site for the I-*Sce* I enzyme, otherwise absent throughout the genome of *S. cerevisiae*, was inserted upstream to the 4×UAS-binding sites or the UASscra. This I-*Sce I* site served to separate the VST-7L telomere from the other parts of the genome by restriction digestion after cell lysis. This was followed by a purification step using anti-FLAG beads. These anti-FLAG beads recognize the GBD-FLAG that is bound to telomeres through the interaction between the GBD and the UAS sequences inserted at the telomeres and is expressed constitutively. Bound proteins at the 7L-VST-UAS were identified using mass spectrometry and compared to those obtained in a control strain 7L-VST-UAS. To eliminate all non-specific proteins, purification of the 7L-Ctrl-UASscra and 7L-VST-UASscra was also done.

After transformation and verification with PFGE and southern blots as described above, the accessibility of I-*Sce* I to its restriction site was verified *in vitro.* Extracted genomic DNA was digested with I-*Sce* I, and southern blots were then performed. Analysis of southern blots confirmed the presence of the *I-Sce* I restriction site.

Furthermore, the presence of these I-*Sce* I sequences had no effect on the senescence phenotype. This suggests that these strains can be used to perform the purification and identify the proteins acting at the VST-7L telomere (Figure 15A).

To pursue this project further, the plan is to introduce a GBD-FLAG tag fusion to be expressed in the cells. After telomerase removal and induction of telomere shortening, cells may be cross-linked and cell lysates will be digested using an I-*Sce*I enzyme. The VIIL telomeres will be purified using anti-FLAG beads. Proteins that are bound may be identified using mass spectrometry. Unfortunately, this project was put aside to progress on the other projects.

# Discussion

During my thesis, I aimed to understand telomere-dependent replicative senescence. In the first part, I demonstrated that, in telomerase-negative cells, the subtelomeric region is an essential region that modulates the establishment of replicative senescence. At first sight, this regulation is independent on the maintenance of telomeres through the homologous recombination pathway. Furthermore, I demonstrated that telomere shortening is sufficient to induce telomeric transcript expression independently of the presence of a native promoter in the subtelomeric region. In the second part, I initiated a genome-wide screen to identify factors that counteract replicative senescence in telomerase-negative cells. The aim of this screen was to recruit artificial yeast proteins to the shortest telomere in the cell and then to select for constructs that confer a delay in replicative senescence. In the third part, we aimed for the global identification of proteins that act at the shortest telomere and are involved in replicative senescence signalling.

To accomplish this work, we took advantage of the very short telomere (VST) system that allows the tracking of a specific VST in a cell. The system makes it possible to obtain a single VST in the cell, therefore allowing the study of the effects of the telomeric repeat length. The original version of this system lacked the subtelomeric region, therefore allowing the understanding of the effects of the telomeric repeats themselves on the cell (Abdallah et al., 2009). This system was then modified in the present work in different ways. For the first part, the system was modified to reconstitute a short telomere within a native context, therefore maintaining the subtelomeric region and examining the role of the subtelomeric region in addition to telomeres in replicative senescence. For the second and third parts, the system was modified by inserting upstream activating sites that are Gal4 binding sites. These sites act as a fixation site for proteins fused to the Gal4 binding domain, making it possible to study the effect of these proteins *in cis* on the signaling by the shortest telomere in replicative senescence.

Throughout the discussion, I highlight novel aspects that explain how the subtelomeric region influences the rate of senescence. Furthermore, I discuss the implication of TERRA transcription at short telomeres.

## 1. Telomere length determines the onset of replicative senescence

Telomere structure maintenance is important to maintain genome integrity. This structure is disturbed in the absence of telomerase and is bound by DNA repair proteins. The study of the effects of telomeric repeats in telomerase-negative cells on replicative senescence demonstrated that one single short telomere is sufficient to accelerate replicative senescence. This acceleration was proposed to occur in two steps. In the first, Tel1 binds telomeres and the cells are maintained in a pre-senescence state. This pre-senescence state then switches to a senescence state through the activation of Mec1 checkpoint kinase at the shortest telomere (Abdallah et al., 2009; Fallet et al., 2014). However, in these studies, the subtelomeric region was absent. Thus, we wondered whether the subtelomeric region influenced the signal emitted from the shortest telomere to trigger replicative senescence signaling. Our results demonstrated that even in the presence of a subtelomeric region, the VST still accelerated senescence. This result is in accordance with the idea that the shortest telomere in a wild-type context is the major determinant of replicative senescence as suggested by formal genetic evidence (Xu et al., 2013). We speculate that, similar to the 7L VST that accumulates ssDNA, the native shortest telomere is also resected.

## 2. Homology between subtelomeric elements does not explain the contribution of subtelomeric elements to replicative senescence

Recombination at telomeres maintains telomeres in post-senescence survivors. However, it has been demonstrated that HR proteins bind to the truncated 7L-VST telomere shortly after telomerase removal. This binding does not promote the formation of survivors. Its function at this stage is to protect the short telomeres from excessive resection presumably by exchanges between sister chromatids, therefore delaying senescence (Abdallah et al., 2009; Fallet et al., 2014). Indeed, the lack of subtelomere 7L generates a unique end where no other homologs exist other than the sister chromatid. Therefore, an explanation for the accelerated replicative senescence observed in the absence of subtelomeres might be ineffective recombination events at the truncated telomeres. Thus, we hypothesized that more homologous recombination proteins would

bind to telomeres containing the subtelomeric region. Therefore, we checked for the binding of Rad51 and Rad52 proteins on telomeres that contain the subtelomeres by ChIP. Interestingly, we did not find any striking increase in these proteins at telomeres that contain the subtelomeres when compared with telomeres that lack the 7L subtelomere. Furthermore, the deletion of the Rad51 gene in cells containing intact subtelomeres did not lead to the rapid loss of viability obtained in the cells that lack the 7L subtelomeric region. Therefore, we suspect that the presence of homology on other chromosomes did not stimulate an increase in recombination events that would delay replicative senescence.

## 3. Effect of the core X on replicative senescence

The end of chromosome VIR is composed of an X element adjacent to telomeric repeats. This X element in *S. cerevisiae* can be divided into two main parts: the gene-less core X that is conserved among all chromosomes, and the relatively gene-rich part that varies among chromosomes. The core X is bound through different proteins, such as the transcription factor Abf1 and the ORC. These proteins are involved in telomeric silencing (Fourel et al., 1999; Lebrun et al., 2001; Pryde and Louis, 1999). However, not so much is known about the implication of these proteins in the regulation of telomere length processing and in replicative senescence. Since these proteins are essential proteins, and they are employed in the regulation of gene expression on other loci, it would be interesting to take advantage of the modified 7L-VST system, where UAS sites are inserted. In cells containing this system, we could express construct encoding for components of the ORC or the Abf1 proteins fused to the GBD. Their recruitment to telomeres and effect on senescence would clarify the involvement of these proteins *in cis* in replicative senescence in telomerase-negative cells. Furthermore, it would be interesting to check for the effect of replication timing on senescence. In a wild type cell, ARS located in the X region is dormant. However, this origin becomes active when Sir3 is absent (Stevenson and Gottschling, 1999). Since, transcription occurs at short telomeres, so it is possible that SIR-mediated silencing decreases. This decrease would then lead to the firing of the ARS located in the X-element, therefore, leading to a delay in senescence. In order to test the implication of replication timing in senescence, it would be interesting to delete the ARS located in the X-element, thus forcing replication to start

from an ARS located far from telomeres and then check for the effect of the deletion on senescence. In addition, the employment of the core X in replicative senescence can be addressed, through the insertion of the core X in the 7L VST.

Replicative senescence is known to modulate the expression of different genes throughout the genome. In the following parts, I discuss the effect of replicative senescence in modulating the expression of genes located upstream of a short telomere.

## 4. Replicative senescence modulates the expression of genes located in the subtelomeric region

Subtelomeres contain genes that are silenced but can be expressed under certain environmental conditions (Louis, 1995). For instance, replicative senescence leads to a change in their gene expression profile (Nautiyal et al., 2002). Therefore, the upregulation of certain subtelomeric genes might be essential for survival in replicative senescence. We have demonstrated that the presence of 15 kb of the subtelomeric region of the 7L chromosome is essential for better cell viability in the absence of telomerase, whether or not it is at the shortest telomere. This suggests that the effect on senescence might be exerted in *trans*. We thus checked whether the region lacking in our truncated 7L contained any gene that could influence cell growth in telomerase-negative cells. Although none of the genes present at the 7L subtelomere are essential for cell growth in normal conditions (see figure S1 of the manuscript), we found that many genes were upregulated as a response of telomerase depletion (Figure 21; (Nautiyal et al., 2002)). Many of these genes are unique, supporting the idea that they could have a relevant contribution when upregulated. To test this idea, it would be interesting to re-introduce the upregulated genes of the subtelomere of chromosome 7L in a plasmid, and check the effect on senescence in *trans*. Alternatively, measuring senescence in single mutants of these genes would clarify the implications of these genes in replicative senescence.

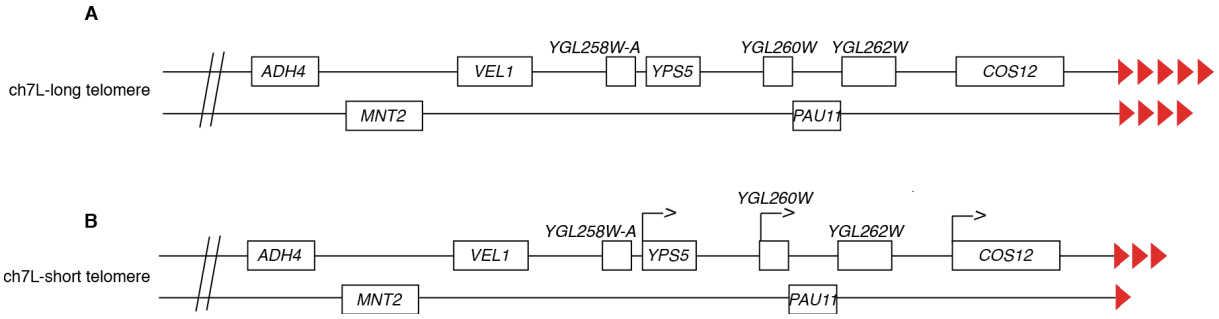



**Figure 21: The effect of telomere shortening on the expression of subtelomeric genes.** (A)The presence of long telomeres at the left arm of chromosome 7 does not influence the expression of the genes located in the subtelomeric region. (B) Telomere shortening leads to the expression of some of the subtelomeric genes such as the *YPS5*, *YGL260W* and *COS12* genes.

## 5. Effect of the signaling of replicative senescence

The TPE is the repression of the adjacent genes located near telomeres. This repression might spread several kbps away from telomeric repeats through the spreading of the Sir complex, leading to the silencing of genes located in the subtelomeric region (Aparicio et al., 1991; Gottschling et al., 1990). Still, the presence of subtelomeric anti-silencing repeats in a native context limits the spreading (Fourel et al., 1999; Lebrun et al., 2001; Pryde and Louis, 1999). In the context of the absence of the subtelomere at the 7L, boundary elements are absent, potentially resulting in the spread of the silencing complex. This spread may lead to gene silencing up to 3.5 Kb away from telomeric repeats (Renauld et al., 1993). While the silencing of the adjacent genes is not essential for cell viability in telomerase-positive cells, telomere shortening in the absence of telomerase may affect their expression in a more deleterious manner. Indeed, short telomeres lose their identity and become recognized and processed as a DSB (Abdallah et al., 2009; Enomoto et al., 2002; Fallet et al., 2014; IJpma and Greider, 2003). This processing leads to a 5'-3' resection, potentially impacting gene function. Moreover, at DSBs, γH2A phosphorylation spreads around 50 kb away from a DSB (Kim et al., 2007). This spreading of γH2A leads to repression of transcription of the surrounding genes. Therefore, the activation of the Tel1 checkpoint and subsequent phosphorylation of H2A could alter gene expression in a much more extensive way than TPE. In this context, it is

possible that a gene essential for cell viability is affected by this response. To check for this possibility, it would be interesting to perform a transcriptome analysis of these genes in a senescence context driven by the truncated 7L-VST.

Furthermore, it would also be interesting to check for heterochromatin markers such as the acetylation of histones and the spread of silencing at the subtelomere to verify that the potential increase in telomeric transcripts observed at short telomeres is due to the acetylation of the core X.

TERRA transcripts are transcribed at short telomeres independently of the presence or absence of subtelomeres. In the following parts, I discuss the implications of these transcripts in replicative senescence.

## 6. Short telomeres induce telomeric transcription

It is not clear whether or not the length of telomeres is stimulating for telomeric transcription in mammals. We have shown that in *S. cerevisiae* the presence of short telomeres in telomerase-negative cells stimulates telomere expression. This expression also occurs specifically at short telomeres in telomerase-positive cells (Cusanelli et al., 2013). When induced artificially at a single telomere, TERRA expression leads to an acceleration of replicative senescence in an Exo1-dependent manner. In turn, this acceleration can be compensated by homologous recombination (Balk et al., 2014). Therefore, the increase in TERRA observed at short telomeres may lead in our experiments to both opposing effects. In our experiments, we saw that 7L-VST generates more TERRA than 6R-VST, together with the observation that the 7L constructs containing strains senesce earlier. Thus, it is possible that the balance between acceleration and delay, i.e., between Exo1 action and recombination, weighs in favour of resection when subtelomeres are lacking. This would provide an additional explanation for part of the difference in senescence rates in the absence or presence of the 7L subtelomere. Accordingly, increased levels of ssDNA were observed at short telomeres in cells lacking subtelomeres (Fallet et al., 2014).

To address whether the subtelomeric region protects against high TERRA expression and exonuclease activity, it would be interesting to also quantify the single-stranded

DNA at telomeres in cells that contain the subtelomeric region, and to test the effect of the deletion of Exo1 on senescence and on TERRA expression.

## 7. TERRA crosstalk with recombination and replication

The absence of the RNA surveillance mechanism in mammals, or the absence of the RNaseH or the THO complex in yeasts, results in the formation of telomeric-induced foci in mammals or in acceleration of replicative senescence in yeasts (Azzalin et al., 2007; Balk et al., 2013). Therefore, two mechanisms triggered by RNA/DNA hybrids potentially operate at telomeres to maintain telomeric integrity. The first mechanism is the recombination stimulated by the DNA/RNA hybrids, and the second mechanism is the prevention of replication fork stalls (Balk et al., 2013; Pfeiffer et al., 2013). Our results show that the over-expression of RNaseH, which removes DNA/RNA hybrids in the cell, leads to an acceleration of replicative senescence, an observation independent of the subtelomeric region. A reason behind this loss of viability is potentially the decreased possibility to trigger HR due to a decrease in DNA/RNA hybrids. To test this hypothesis, it would be interesting to check whether the binding of homologous recombination proteins at short telomeres is altered when RNaseH is over-expressed. If the binding of these HR proteins is reduced after RNaseH overexpression at short telomeres, then the accelerated senescence phenotype observed independently of the subtelomeric region might be explained through TERRA-dependent recombination.

## 8. TERRA transcription occurs independently of subtelomeric region

Telomeric transcripts are transcripts that are initiated in the subtelomeric region and then proceed to the telomeric region. In this study, we demonstrated that telomeric transcripts can be transcribed without the presence of a native promoter. Since TERRA is transcribed by RNA polymerase II, and then poly-adenylated, it would be interesting to check whether these transcripts are also a result of the transcription of RNA pol II or any other RNA polymerase.

In mammalian cells, fish, insects, and plants, a DSB results in the expression of non-coding RNA (ncRNA) near the break (Francia et al., 2012; Michalik et al., 2012; Wei et al.,

2012). These ncRNAs are essential to activate the ATR DNA Damage Response (DDR), in order to repair the damage. DICER/DROSHA, which are two RNase Type III enzymes that process ncRNA hairpin structures, then process RNA, generating small double-stranded RNA. This processing is essential to activate DDR (Francia et al., 2012; Michalik et al., 2012; Wei et al., 2012). We speculate that the increase in telomeric transcripts at short telomeres that lack the subtelomeric region could arise by a similar mechanism. Accordingly, the change in the identity of short telomeres, and their recognition by DSB repairing activities, would promote transcription in the area adjacent to telomeres. To verify this hypothesis, a better characterization of telomere shortening-dependent TERRA is first required. Mainly, the 5' and the 3' ends need to be mapped. Also, any additional processing should be checked, although the RNA-processing DICER and DROSH are absent from *S. cerevisiae*. We should also define which RNA polymerase is responsible for this transcription. In this context, the transcriptome of these cells containing the 7L-VST telomeres we proposed above will definitely serve to distinguish several of our hypotheses. Finally, we should also check whether transcription also occurs near a canonical DSB in budding yeast. The purification of short telomeres described before and then the identification of proteins that bind to the short telomere would reveal new factors that are implicated in the generation of these non-coding RNAs.

# Conclusion

In the absence of telomerase, telomeres shorten progressively, leading to a loss of viability. The first telomere reaching a critical short length is processed similarly to a DSB and triggers the activation of the DNA damage checkpoint and cell cycle arrest. In this work, we investigated the influence of the subtelomeric region on this process. We demonstrated that this region prevents the loss of viability, but its effect is not dependent on the shortest telomere in the cell. This buffering effect may be due to several factors such as the change in expression of genes located in the subtelomeric region and the effect of recombination. These would act *in trans* to sustain cell viability when telomeres become short. We also detected the transcription of TERRA in the absence of a native promoter. This is reminiscent of the RNA found near a DSB in other organisms. This supports the interesting possibility that TERRA is the result of DDR-like activity at chromosome extremities, a feature that would have been conserved throughout evolution.

# Annexes

## I. Materials and methods

### 1. Plasmid construction

**pT32, pT33, pT34, and pT35**

The 7L system (figure 14B-C) was modified to contain 4×UAS sequences. The 4×UAS fragment from the sp126 plasmid was amplified by PCR using the oligonucleotides oT364 and oT371. UAS scrambled fragment oT376 was commercially synthesized (Eurofins), and the second strand was synthesized by primer extension using a *T4* DNA polymerase with the primer oT377. The products obtained were verified on an agarose gel to correspond to dsDNA. The 4×UAS fragment and the UAS scrambled fragment have a *Hind* III and *I-Sce* I restriction site at 3' and *Hind* III restriction site at 5'. These fragments as well as sp225 and sp242 plasmids were then digested with *Hind* III. In addition, sp225 and sp242 were treated with Antarctic phosphatase to remove the 5' phosphate group, and prevent self-ligation. Linearized plasmids were combined with a five-fold molar excess of the PCR-digested product. Each of two digestion products were then ligated together using a *T4* DNA ligase, and 1 ng of the ligation reaction was then used to transform MAX Efficiency® *Stbl2*™ competent bacteria (Invitrogen), which were chosen for their ability to stabilize plasmids containing multiple direct repeats. Transformed bacteria were then selected on ampicillin-containing media cultured at 30°C. Plasmids were then extracted, verified by digestion, and then sequenced. Plasmids pT32 and pT33 corresponded to 4×UAS ligated with sp225 and sp242, respectively, while plasmids pT34 and pT35 constructions corresponded to UAS scrambled ligated with sp225 and sp242.

**pT37**

The 1.4 kb adh1p-GBD-adh1t of the pASDD plasmid (Fromont-Racine et al., 1997) was amplified by PCR using the oligonucleotides oT547 and oT548. The oT547 and pT548 oligonucleotides contained *Not* I and *EcoR* I restriction sites at their 5' ends. The amplicons obtained and the pRS313 plasmid were then digested by *Not* I and *EcoR* I.

Digested plasmids were combined with a five-fold molar excess of PCR-digested product. The two digestion products were then ligated together using a *T4* DNA ligase, and 1 ng of the ligation reaction was then used to transform high-efficiency *DH5α* competent bacteria (NEB). Transformed bacteria were then selected on ampicillin-containing media cultured at 37°C. Plasmids were then extracted, verified by digestion, and then sequenced.

## 2. Genomic Library construction

Yeast genomic DNA was isolated from the W303 yeast using the Qiagen Tip100 kit according to the manufacturer's instructions, and then partially digested with 0.25 U *Sau3AI* restrictive enzyme for 5 min. DNA fragments between 1 kb and 5 kb were purified from the agarose gel. Purified DNA was treated with *T4* PNK for 30 min. pT37 plasmid was digested with *BamH* I restrictive enzyme and then treated with Antarctic phosphatase. Digested pT37 was ligated with purified genomic DNA, and 46.4 ng of ligation mixture was then used to transform *E. coli Neb10-Beta* competent bacteria by electroporation. After growth on ampicillin-containing plates at 37°C, a bacterial complexity of $10^6$ was obtained. Colonies were pooled and plasmids were extracted.

## 3. Yeast library

A yeast library was obtained by transforming the yT628 strain with the genomic DNA library described above, using the LiAc/PEG4000/DMSO high-transformation method. The complexity obtained corresponded to $1.5 \times 10^6$, which covers the entire yeast genome one hundred times (100×). Transformants were then selected on media lacking uracil and histidine. Several transformants were selected and then verified by PCR using oT660 and oT661 for the heterogeneity of transformants. Transformants were then pooled and frozen at $1.7 \times 10^9$ cells.

## 4. Yeast strain

All strains are derived from W303 and are listed in Table 1. The 7L-CTL-UAS (yT368), 7L-VST-UAS (yT369), 7L-CTL-UASscra (yT425), and 7L-CTL-UASscra (yT426) strains were obtained by transforming the yT360 yeast strain with plasmids pT32, pT33, pT34,

and pT35. These plasmids were first digested with *Sph* I and *Not* I, and transformants were then selected on a medium lacking uracil. Transformants were verified by PCR using oT305 and oT153. Further verification was done by pulse field gel electrophoresis and southern blot. Diploids resulting from the cross of yT368, yT369, yT425, and yT426 were then crossed with yT367, sporulated, and $Ura^{+}Trp^{+}Nat^{r}$ spores were selected. These haploids, yT485, yT486, yT487, and yT501, are *TLC1::Pr„NAT* complemented with a plasmid encoding for *Tlc1* and *Trp1*. Back cross of these haploids with yT368, yT369, yT425, and yT426 was finally done to obtain the diploid cells that were used in the study. *Rad52* gene deletion was performed by replacing the *RAD52* gene with *KANMX6* as described previously (Longtine et al., 1998) using the oligonucleotides oT710 and oT343. *Rad52::KanMX6* was obtained in the strains yT368, yT369, yt425, and yT426.

## 5. Southern blots

Cells were pre-cultured overnight. The next day cells were washed and then cultured in media containing either glucose or galactose for 4 hours. Genomic DNA was then extracted through shearing cells with glass beads and phenol chloroform (Amberg et al., 2005). To determine telomere length, extracted DNA was digested with *Stu* I. Fragments were then separated by electrophoresis, transferred to a nylon membrane, and hybridized with an *adh4-URA3* probe. The *URA3* probe was labeled by random priming. Images were obtained with Typhoon Trio and lengths were measured with the MolWt macro in ImageJ 1.46a.

## 6. Pulse field gel electrophoresis

Exponentially growing ($5 \times 10^{7}$) yeast cells were enclosed in a low melting point (LMP) agarose. Cells were washed with 10 mM Tris-HCl pH 8.0, 50 mM EDTA, pH 8.0, and then re-suspended in pre-warmed zymolyase buffer at 42°C to a final concentration of $1 \times 10^{9}$ cells/ml. Agarose plugs using a plug mold about 90 µl (Bio-Rad) were prepared by adding 50 µl of molten LMP agarose 1% to 40 µl pre-warmed cells in the zymolyase buffer. Plugs were then solidified at 4°C for around 30 min and then ejected into tubes containing 0.4 ml of zymolyase buffer per plug. Plugs were incubated overnight at 30°C. Zymolyase buffer was then replaced with proteinase K buffer and incubated for 24 h at 50°C. Finally, the plugs were washed and stored at 4°C. Chromosomal DNA was then

separated by PFGE on 0.9% Agarose Seakem Gold using the 3-1600 kb 24h program of Rotaphor VI (Biometra) according to the manufacturer's manual. Gels were then stained with ethidium bromide for photography. Gels were then transferred to a membrane and hybridized with a *URA3* probe. Images were obtained with Typhoon Trio.

## 7. Senescence assay

Diploids cells, which were homozygous for the construct at the 7L and heterozygous for the RNA subunit of the telomerase *TLC1/tlc1Δ*, were placed on 2% potassium acetate at 30°C for 3–5 days for sporulation. Spores were then germinated in YPGal-Raff (2% galactose; 1% raffinose) for 6 to 9 h at 30°C to induce shortening. To control this induction of shortening, germination of the same sporulation mixture was also performed in media containing glucose. Different cell concentrations of the two germinations were then plated on a media YPD and complemented with 60 mg/ml of Nourseothricin to select telomerase-negative spores. For the genetic screening experiment, cells were plated on an SD-His containing 60 mg/ml and 200 mg of Genetecin (G418), to select for spores that encode the GBD-fused plasmid, are *tlc1Δ* and *rad52Δ*. Plates were incubated for around 1.5 days at 30°C, and then some isolated colonies were verified for their shortening and appropriate genotype. Eight of the verified colonies were selected, re-suspended, adjusted to the same concentration (Abdallah et al., 2009), and then spotted on either YPD+Nat or SD-His+Nat. Spot assays corresponded to a series of 10-fold dilutions with the first spot corresponding to 4000 cells. Plates were then incubated for 2 days at 30°C. Plates were then scanned using the Epson Perfection V750 Pro scanner for further analysis. Loss of cell viability was established by the re-spotting of cells from the most concentrated spot. This step was repeated every 2 days until senescence was observed.

## 8. Quantification of senescence assay

Spot analysis was done by calculating the index of viability for each spot. After background subtraction, spot intensity was first assessed as the area occupied by colonies using the Microarray Profile plugin of ImageJ 1.46a. A threshold value for the intensity was defined and was fixed for all experiments corresponding to exponentially

growing cells. The viability index corresponded to the dilution needed to obtain a spot intensity equivalent to the threshold. Viability indexes were then plotted using R2.15.2, and P-values were determined using the pairwise Wilcoxon rank sum and adjusted with a false discovery rate correction

## 9. Telomere PCR

Telomere PCR was done as described in (Forstemann and Lingner, 2001). Briefly, the different serial dilutions of each of the 8 independent clones of passage I were pooled and genomic DNA were prepared for telomere length determination. The DNA was prepared through shearing cells with glass beads and phenol chloroform. Genomic DNA was denatured and tailed with dCTP and terminal transferase (NEB). Telomeres were then amplified using oligonucleotide oT156 (VIIL telomeres) with oligonucleotide o169 complementary to the dC tail added. PCR products were then separated on a 2% agarose gel. Telomere mean length was calculated using the ImageLab software (Bio-Rad).

## 10. Western blot

Cultured cells (5 ml) at $O.D_{600}$ were collected. Cell shearing was done by adding glass beads and 20% TCA, followed by vortexing at 4°C. The beads were then washed with 5% TCA, and then centrifuged. The liquid was discarded and 2X loading SDS buffer (60mM Tris pH 6.8, 2% SDS, 10% Glycerol, 0.2% bromophenol blue, 100mM DTT) was added with 1M Tris-HCl pH 9.5. Extracts were boiled for 5 min at 65°C, then separated on denaturing gel by SDS-PAGE. The gels were then transferred to a nitrocellulose membrane, and blocked with TBS-Tween 0.05% and 5% milk. The membrane was incubated in the presence of the primary antibody, anti GBD (Santa Cruz), used at 1 µg/ml in TBS-Tween 0.05% and 1% milk. After several washes, the membrane was incubated with the mouse secondary antibody, anti-IgG, coupled with HRP (Sigma) diluted at 1/10000x in TBS-Tween 0.05% and 1% milk. The protein signal was then revealed by the addition of the HRP substrate (as described by the manufacturer, GE Healthcare). Protein signals were then visualized by chemiluminescence using the Image Lab software (Bio-Rad).

# II. Yeast strains

### Table 1: yeast strains used in this study

All strains are in the W303 background. All strains were constructed for this work

| Strain | Genotype |
|---|---|
| **yT360** | Mata cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 |
| **yT367** | Matα cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat pCen-ARS-TRP1-TLC1 |
| **yT368** | yT360 telVIIL4xUAS::FRT-URA3-FRT-tel |
| **yT369** | yT360 telVIIL4xUAS::FRT-URA3-[tel600]-FRT-tel |
| **yT425** | yT360 telVIILUASscr::FRT-URA3-FRT-tel |
| **yT426** | yT360 telVIILUASscr::FRT-URA3-[tel600]-FRT-tel |
| **yT485** | Matα cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat telVIIL4xUAS::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| **yT486** | Matα cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat telVIIL4xUAS::FRT-URA3-[tel600]-FRT-tel pCen-ARS-TRP1-TLC1 |
| **yT487** | Matα cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat telVIILUASscr::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| **yT501** | Matα cir° Ura3-1 trp1-1 his3-11,15 can1-100 ade2-1 leu2-3,112::Gal-Flp1-Leu2 tlc1::PrαNat telVIILUASscr::FRT-URA3-[tel600]-FRT-tel pCen-ARS-TRP1-TLC1 |
| **yT504** | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 telVIIL4xUAS::FRT-URA3-FRT-tel/ telVIIL4xUAS::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| **yT505** | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 telVIIL4xUAS::FRT-URA3-[tel600]-FRT-tel/ telVIIL4xUAS::FRT-URA3-[tel600]-FRT-tel pCen-ARS-TRP1-TLC1 |
| **yT506** | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 telVIILUASscr::FRT-URA3-FRT-tel/ telVIILUASscr::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| **yT507** | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 telVIILUASscr::FRT-URA3-FRT-tel/ telVIILUASscr::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| **yT623** | yT368 rad52::KanMX6 |
| **yT624** | yT369 rad52::KanMX6 |
| **yT625** | yT425 rad52::KanMX6 |
| **yT626** | yT426 rad52::KanMX6 |
| **yT627** | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 rad52::KanMX6 telVIIL4xUAS::FRT-URA3-FRT-tel/ telVIIL4xUAS::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| **yT628** | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 rad52::KanMX6 telVIIL4xUAS::FRT-URA3-[tel600]-FRT-tel/ telVIIL4xUAS::FRT-URA3-[tel600]-FRT-tel |
| **yT629** | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 rad52::KanMX6 telVIILUASscr::FRT-URA3-FRT-tel/ telVIILUASscr::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| **yT630** | Mata/α cir° ura3-1/ura3-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 rad5-535/rad5-535 tlc1::PrαNat/TLC1 rad52::KanMX6 telVIILUASscr::FRT-URA3-FRT-tel/ telVIILUASscr::FRT-URA3-FRT-tel pCen-ARS-TRP1-TLC1 |
| **Yeast library** | yT628 genomic library |

# III. Statistics test

**Table 2: p-values for the spot assays determined by pairwise Wilcoxon rank sum test with a false discovery rate adjustment**

| Experiment | Genotype 1 | Genotype 2 | Passage | p- value |
|---|---|---|---|---|
| | Control 7L-UAS | VST 7L-UAS | 1 | 0,00093 |
| Figure 18 | Control 7L-UAS | VST 7L-UAS | 2 | 0,00093 |
| | Control 7L-UAS | VST 7L-UAS | 3 | 0,022 |
| | Control 7L-UAS | Control 7L-UASscr | 1 | 0,57374 |
| | Control 7L-UAS | Control 7L-UASscr | 2 | 0,87848 |
| | Control 7L-UAS | Control 7L-UASscr | 3 | 0,334 |
| | VST 7L-UAS | VST 7L-UASscr | 1 | 0,14646 |
| | VST 7L-UAS | VST 7L-UASscr | 2 | 0,17879 |
| | VST 7L-UAS | VST 7L-UASscr | 3 | 0,513 |
| | Control 7L-UASscr | VST 7L-UASscr | 1 | 0,42517 |
| | Control 7L-UASscr | VST 7L-UASscr | 2 | 0,01632 |
| | Control 7L-UASscr | VST 7L-UASscr | 3 | 0,248 |
| | PRS313 | GBD | 1 | 0,46 |
| Figure 19 | PRS313 | GBD | 2 | 1 |

**Table 3: p-values for the telomere length determined by pairwise Wilcoxon rank sum test with a false discovery rate adjustment**

| | | | |
|---|---|---|---|
| | Control 7L-UAS | VST 7L-UAS | 0,0035 |
| Figure 18B | Control 7L-UAS | Control 7L-UASscr | 0,4634 |
| | VST 7L-UAS | VST 7L-UASscr | 0,2057 |
| | Control 7L-UASscr | VST 7L/ -RNH1 | 0,0091 |

# IV. Oligonucleotides

**Table 4: Oligonucleotides used in this study**

| Name | Use | Sequence 5'-3' |
|---|---|---|
| oT364 | UAS amplification | GCATAAGCTTAGTTACGCTAGGGATAACAGGGTAATATAGGTTATTACCCTCGACGGATC |
| oT371 | UAS amplification | ATTGAAAGCTTCTCACATCTACCTCTACTCTGGGGATCCGG |
| oT376 | UASscr template | GGTCAAGCTTAGTTACGCTAGGGATAACAGGGTAATATAGACTAAGTCGTCCTGCGTCTGCTGCCA TGCAAGACGTGCGCTGCAGTCTGAGCTCACTCTGCAGCGTCAAGCTTCACG |
| oT377 | UASscr primer | CGTGAAGCTTGACGCTGC |
| oT153 | VIIL integration | CTTCTGCTCGGAGATTACCG |
| oT305 | VIIL integration | GCAATGTACTGATACTTTCTCC |
| 169 | teloPCR | CGGGATCCGGGGGGGGGGGGGGGGG |
| oT156 | teloPCR VIIL | TTGTTGAAAGCCATACCTGCC |
| oT531 | teloPCR VIR | AGGACTGGGTCATGGGGCGC |
| oT547 | Amplification pASΔΔ | ATGTTTGCGGCCGCCTAATTACATGATATCCTTTTGTTGTTTCC |
| oT548 | Amplification pASΔΔ | ATGCGAGAATTCCGCCAAGCTTGCATGCCGGTAGAGG |

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