The maintenance of telomeres in the budding yeast Naumovozyma castellii

Itriago, Humberto

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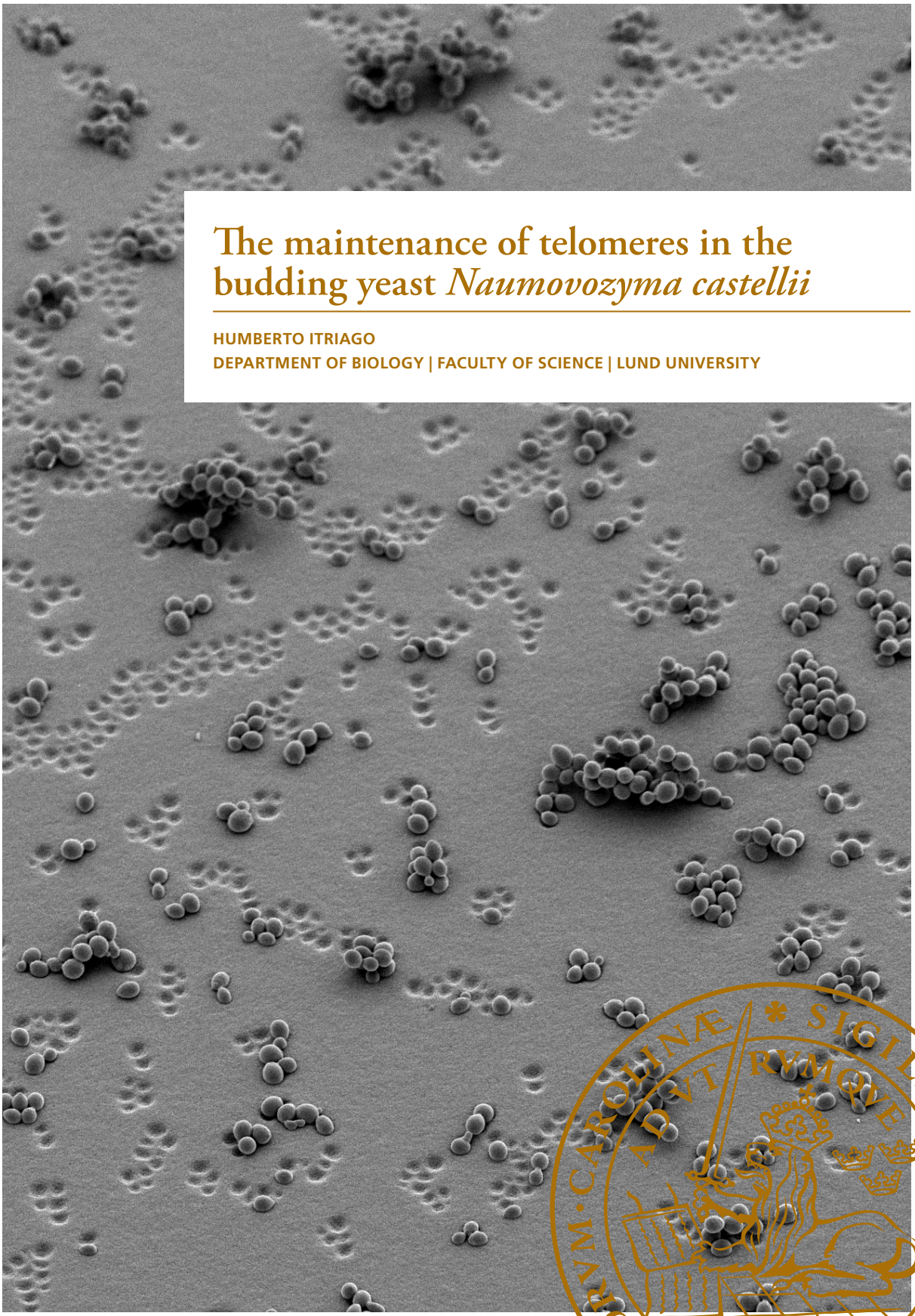

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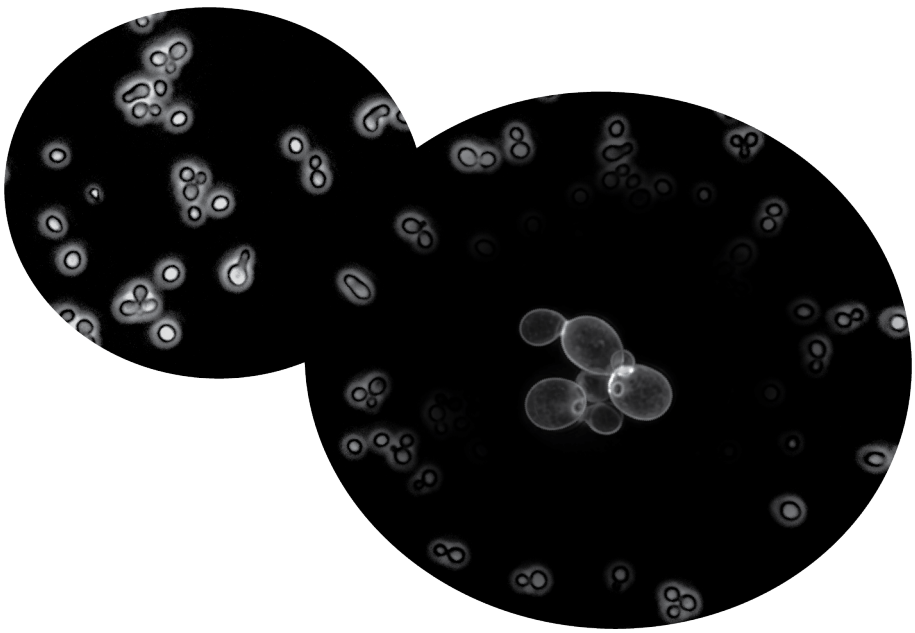

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Science at Lund University to be publicly defended on the 21st of October 2022 at 09.00 in the Lecture Hall A, Department of Biology, Sölvegatan 35, Lund.

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Abstract

Linear chromosomes of eukaryotic cells require the presence of functional nucleoprotein terminal structures, known as telomeres, to protect the integrity of the genome. The telomere is a highly dynamic and regulated structure constituted by short tandem DNA repeats rich in guanine nucleotides that extent as double-stranded DNA ending in a single-stranded 3′ overhang. An abundant number of proteins bind these sequences, like the double-stranded binding protein Rap1 and the single-stranded binding protein Cdc13. Telomeres protect the genomic DNA from end-to-end fusions, degradation and recognition as damaged DNA by the DNA repair machinery of the cell.

Many factors contribute to the progressive shortening of telomeres during each replication cycle, including the inability of the canonical DNA replication machinery to fully replicate the telomere, a phenomenon known as the end replication problem. The enzyme telomerase, a DNA polymerase with reverse transcriptase activity, prevents progressive shortening by adding telomeric repeats to the single stranded end of the chromosome using its internal RNA molecule as template. Eukaryotes solve the end replication problem with the use of telomerase but, in its absence, some cells develop telomerase-independent mechanisms for telomere maintenance, like the recombination based alternative lengthening of telomeres (ALT) mechanism that has been observed in both yeast cells and human tumors.

My doctoral thesis focuses on studying the maintenance of telomeres in the budding yeast *Naumovozyma castellii*. I approached the studies from two perspectives: the structural maintenance of the telomeres and the telomerase-independent telomere maintenance. I investigated how Rap1 and Cdc13 provide protection to the 3′ overhang from exonuclease degradation *in vitro* and discovered a previously undescribed function of Rap1: the ability to protect short telomeric overhangs. I investigated the double-stranded and single-stranded junction of the telomeres and determined, for the first time in yeast, that the terminal 5' end nucleotide is regulated to contain primarily an adenine nucleotide in *N. castellii*. With knowledge of the DNA structure and with the implementation of our protection assays, I proposed a model that describes how the binding of Rap1 and Cdc13 protects the telomere from enzymatic degradation. To investigate the genetic requirements for the establishment of the ALT mechanism I first characterized the *RAD52* gene, coding for the main homologous recombination gene in yeast. To investigate if the ALT mechanism of *N. castellii* relies on homologous recombination I designed multiple strains and evaluated their rate of senescence and telomere structure. I found that the establishment of the efficient ALT mechanism of *N. castellii* requires homologous recombination mediated by *RAD52* and *RAD51* gene function. These findings expand the understanding of the mechanistic aspects of telomere maintenance with and without telomerase.

Key words: Telomere, Cdc13, Rap1, telomeric DNA, *RAD52*, *RAD51*, homologous recombination, ALT, BIR, *Naumovozyma castellii*

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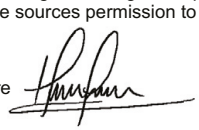
Signature  Date 2022-09-14

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In memory of Carlos Rovira
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List of abbreviations

ss	Single-stranded
ds	Double-stranded
TAS	Telomere associated sequences
MNase	Micrococcal nuclease
TERRA	Telomere repeat containing RNA
DSB	Double-strand break
NHEJ	Non-homologous end joining
HDR	Homology directed repair
HBEJ	Homology-based end joining
DDR	DNA damage response
RPA	Replication protein A
D-loop	Displacement loop
SDSA	Synthesis-dependent strand annealing
BIR	Break-induced recombination
SSA	Single-strand annealing
MMEJ	Microhomology-mediated end joining
G4-DNA	G-quadruplex DNA
TPE	Telomere position effect
TERT	Telomerase reverse transcriptase
TERC	Telomerase RNA component
ALT	Alternative lengthening of telomeres
ITS	Interstitial telomeric sequence
ECTR	Extrachromosomal circle containing telomere repeats

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Popular scientific summary

It is fascinating to think that each cell in our bodies contains the information needed to orchestrate the development of every tissue, organ and organ system that biologically defines us. Inside each cell the information is contained in long linear DNA molecules tightly compacted and meticulously organized to form the chromosomes. Maintaining the integrity of the chromosomes is essential for the cell to read the genetic information, effectuate its orders and perpetuate this information across multiple generations.

Cells have developed mechanisms to repair DNA damage that can potentially disrupt the chromosome functions. For example, to repair DNA breaks, the most toxic type of damage the chromosomes can endure, cells can fuse the broken DNA molecules at the site of the damage or copy the sequence of another similar DNA strand fix the break, through a process called homologous recombination.

A major threat to the genome's integrity is that the two ends of each linear DNA molecule are: 1) constantly exposed to DNA damaging agents that surge from the metabolic reactions of the cells, 2) under risk of degradation by proteins that normally protect the genome from invasions of foreign DNA molecules and, 3) potentially recognized as broken ends by the DNA repair machinery and fused together, generating massive structural rearrangements caused by breakage of the chromosomes during cell division.

Telomeres are the terminal regions of the chromosomes, tasked with preventing all the detrimental consequences of having exposed DNA sequences at the ends. Structurally, telomeres contain repeats of a DNA sequence that strongly associate with a specialized group of proteins to effectively "cap" the ends. However, telomeres shorten progressively with each cell division because the DNA replication machinery is unable to fully replicate the DNA molecule and due to other factors that contribute to telomere attrition.

The symptoms associated with aging, such as increased propensity to heart diseases or diabetes, can be directly correlated to the shortening of telomeres of some of the cells in our body. Indeed, we are born with long telomeres and, as we grow older, our telomeres become shorter. This happens because the cells of our bodies accumulate cells divisions and with each division the telomeres shorten, leading to the failure of the tissues they are part of. In cells that must divide constantly for their functional role, such as stem cells during development, germ-line cells and

hematopoietic cells, we find a specialized protein capable of elongating the telomeres, called telomerase.

Aging research in focused on how to maintain the telomere length so we can age healthier. However, the shortening of the telomeres works to prevent cells from growing uncontrollably by limiting the number of divisions that cells that are not supposed to constantly divide can undergo. Therefore, one of the main requirements for the immortalization of cancer cells is reestablishing the telomere length maintenance and around 80-85% of all human cancer cells reactivate telomerase. Cancer cells that do not reactivate telomerase, utilize mechanisms based on homologous recombination to maintain the ends and become immortal, these are known as the alternative lengthening of telomeres (ALT) mechanisms.

During my PhD studies I was motivated to further the understanding of the mechanisms that govern the maintenance of the telomeres in the hopes that my research would contribute to the development on cancer treatments. Many important discoveries in the telomere biology field have been made utilizing the baker's yeast as a model organism. I investigated the telomeres utilizing the closely related budding yeast *Naumovozyma castellii* as a model organism

The first part of my thesis focuses on how the core telomeric proteins, Rap1 and Cdc13, bind and protect the telomeric DNA. Here, we studied the region where the double-stranded DNA becomes single-stranded, known as the double-stranded and single-stranded junction (ds-ss junction). The double-stranded DNA is bound by the Rap1 protein, and the single-stranded DNA is bound by Cdc13. We found that even though Rap1 cannot fully bind the single-stranded DNA, the protein is capable of protecting it which could be relevant for safeguarding a template for telomerase elongation. We then designed a method to determine the structure of the ds-ss junction and found that there is a mechanism that maintains two specific structures at the chromosomal ends. Thanks to our protection assay we then were able to propose a model on how the telomeric proteins bind and protect the telomeres of our model organism.

The second part of my thesis focused on discovering some of the proteins that are involved in the ALT mechanism of *N. castellii*. Unlike most human cells, yeast cells always have active telomerase to counteract the shortening of the telomeres. In order to study the effects of telomere shortening in yeast, we disrupt the activity of telomerase by deleting the genes involved in its functioning. When *N. castellii* cells lose telomerase, they are able to rapidly activate the ALT mechanism, effectively maintaining the telomeres and guaranteeing the propagation of the cells. Because other known ALT mechanisms depend on homologous recombination, we started by identifying and characterizing genes involved in homologous recombination for our model organism. We then disrupted some of the different genes identified in our telomerase negative cells and found two genes that disrupted the ability to activate ALT, quickly leading the cells to their death.

I believe that telomeres are great therapeutic targets for the treatment of different types of cancer. The structure of the telomere dictates its proper functioning, as it is the special sequence and length of the DNA combined with the binding of the telomeric proteins that protects the genome's integrity. Furthermore, if we aim to effectively target telomerase as a therapeutic agent for the treatment of cancer, it is important to also address the ALT mechanisms as some cancer cells could resort to these mechanisms to survive. Therefore, understanding the establishment of recombination mediated telomere maintenance is fundamental in the fight against cancer.

List of papers

This thesis is based on the following papers:

I. Rikard Runnberg, Saishyam Narayanan, Humberto Itriago and Marita Cohn (2019).

   Either Rap1 or Cdc13 can protect telomeric single-stranded 3′ overhangs from degradation *in vitro*. *Scientific Reports.* 9: 19181.

II. Humberto Itriago, Rishi K. Jaiswal, Susanne Philipp and Marita Cohn (2022).

   The telomeric 5′ end nucleotide is regulated in the budding yeast *Naumovozyma castellii. Nucleic Acids Research.* 50(1):281-92.

III. Humberto Itriago, Zubaida Marufee Islam and Marita Cohn (2022).

   Characterization of the *RAD52* gene in the budding yeast *Naumovozyma castellii. Submitted.*

IV. Humberto Itriago and Marita Cohn (2022).

   Alternative Lengthening of Telomeres is dependent on *RAD52* and *RAD51* gene function in the budding yeast *Naumovozyma castellii. Manuscript.*

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My contributions to the papers

I. I took part in the optimization of the method, adjusting the digestion times to fit the experimental setup. I performed all experiments regarding the protection provided by Cdc13 and, designed and performed the control experiments to validate our Cdc13 results. I took part in performing the Rap1 experiments. I took part in designing and carrying out the data analysis. I took part in the design and creation of figures for the visualization of the results. I took part in the writing of the first draft of the manuscript and the peer-review revision process.

II. I took major part in designing and performing the experiments. I performed all formal analysis of the data. I took major part in the creation of figures and interpretation of the data. I wrote the first draft of the manuscript together with Marita Cohn. I took major part in the peer-review revision process.

III. I took major part in designing and performing the experiments, in the analysis and interpretation of the data, creation of figures and writing the manuscript.

IV. I took major part in designing and performing the experiments, in the analysis and interpretation of the data, creation of figures. I performed all formal analysis of the data and wrote the manuscript.

1 Introduction

The genetic material of eukaryotic organisms is organized as linear chromosomes inside the nucleus of the cell. The end of linear DNA molecules is particularly susceptible to damaging agents and enzymatic degradation. Most notably, the ends can be mistakenly recognized as DNA damage by the cells' DNA repair machinery, which seeks to repair the damage by joining the DNA ends together causing either the loss of linearity or the formation of dicentric chromosomes that can break during cell division, generating massive rearrangements of the genomic material. To prevent these deleterious processes, the ends of eukaryotic chromosomes are capped by specialized structures known as telomeres.

Telomeres are constituted by a repetitive DNA sequence that is bound with high affinity and specificity by a specific group of proteins to form a specialized chromatin cap. Maintaining the DNA structure and orchestrating the protein binding is essential for the proper functioning of telomeres. However, the canonical replication machinery cannot properly synthesize the DNA molecule and telomeres shorten in every replication cycle. To counteract the progressive shortening of telomeres some cells reactivate a telomere-specific DNA polymerase capable of elongating the sequence of the chromosome ends.

In the 1930's the first hypothesis of the existence of telomeres were made individually by Barbara McClintock and Hermann Muller, who observed that chromosomal ends are not fused together in the way that broken chromosome ends are. The description of the structure of the DNA molecule in 1953, was accompanied by the hypothesis of the end replication problem. In the late 1970's the first evidence of repeated sequences at the ends was found by Elizabeth Blackburn in ciliates, and later research in her laboratory described the enzyme telomerase, responsible for extending the ends. The discoveries of telomeres and telomerase in yeast followed shortly after, and by the beginning of the 1990's both were also described in human cells (1, 2).

Quickly telomere biology became relevant in human aging research, because telomerase activity is not sufficient to counteract the natural shortening of human telomeres; and cancer research, because the large number of cell divisions that cancer cells undergo demands a solution to the shortening of telomeres. Indeed, most cancer cells upregulate telomerase expression to reestablish telomere length maintenance and, in term, cell immortalization (3). However, some cancer cells

reestablish end maintenance by a homologous recombination-based mechanism termed the alternative lengthening of telomeres (ALT). The work in yeast as a eukaryotic model organism was important for discovering and understanding the mechanism of maintenance of telomeres in human cells.

The main objective of this thesis was to investigate the molecular mechanisms governing telomere maintenance utilizing the budding yeast *Naumovozyma castellii* as a model organism. We studied the structural maintenance of the telomeres by developing an *in vitro* assay that allowed us to investigate how the telomere binding proteins provide protection to the telomeric DNA. We then determined the primary structure of the telomeres *in vivo* and utilized our findings and our *in vitro* assays to understand how the telomeric proteins bind to the telomeres and provide protection against degradation. We also studied the telomerase-independent telomere maintenance mechanism, first by characterizing the function of the main recombination gene in yeast and then by determining the genetic requirements for genes involved in homologous recombination, and the structural changes that allow the establishment of the ALT mechanism. The research presented here furthers the understanding of the mechanistic aspects of telomere maintenance with and without telomerase, provides molecular methods for telomere research and promotes the use of non-conventional yeast as a model organism.

In the following chapters we will cover the structure of linear chromosomes and how the DNA repair mechanism and telomeres prevent chromosomal instability, based mainly on yeast research. Telomeres are the main topic of interest of this work, and thus we focus on the functional aspects of the telomere DNA structure and the telomeric proteins, the arduous task of replicating the telomeres by the canonical replication machinery, including telomere elongation with or without telomerase, post-replication processing of the telomeres and how all these factors influence telomere length and dynamics.

2 Chromosome organization

2.1 Linear chromosomes

Bacteria and Archaea typically have circular chromosomes, but Eukaryotes commonly have one or more linear chromosomes located inside the cellular nucleus, packed in the chromatin. Chromatin is an association of a DNA molecule with histone and non-histone proteins that, at its highest organization level, forms the structures known as chromosomes. The degree of chromatin condensation and organization is not homogenous along the genome: The less compacted euchromatin is gene-rich, contrary to the highly compacted and gene-poor heterochromatin (4, 5).

Chromosomal organization is important as it is required to maintain the genomic DNA compacted inside the cell. This arrangement also protects the DNA by making it more stable in the cellular environment, allowing for its transmission during cellular replication and allowing for the organization of each DNA molecule that facilitates all cellular events that involve the genome (4).

Genomic stability entails preventing changes in the sequence of the DNA and the structure of the chromosomes, crucial for the survival and propagation of all living organisms. To achieve stability of eukaryotic chromosomes, there are non-coding DNA sequences in the genome that are essential for the correct duplication and segregation of chromosomes during cell division such as the origins of replication and centromeres. Furthermore, linear chromosomes need to be protected from degradation at the ends of the DNA molecule, the loss of genetic material due to limitations of the replication machinery and the processing of the ends as DNA damage sites (6, 7). To face these problems the ends of linear chromosomes are constituted by a specialized nucleoprotein structure known as the telomere (Figure 1, left).

2.2 Subtelomeres

From the centromere towards the end we find the core region of the genome, and just before the telomere there is a region that contains coding and non-coding genetic sequences referred to as the subtelomere. While usually gene-poor,

subtelomeric regions contain several repetitive elements or Telomere Associated Sequences (TAS) (8). The heterogeneity of these repetitive sequences has difficulted the sequencing and mapping of these genetic regions, though with the advancement of long-read sequencing technologies this limitation can be overcome. The subtelomeric region has been described to have multiple functions in telomere maintenance through recombination, chromosome segregation, heterochromatin spreading, among others (reviewed in (9)).

The subtelomeric region of the budding yeast *Saccharomyces cerevisiae* commonly contains X elements, which are composed of a subset of heterogenous repeated sequences. Additionally, the subtelomere can also harbor one or more copies of the Y' element. This element is not as common, being present in at least half of the chromosomes of a given strain after resolution of the chromosomes by Pulsed-Field Gel Electrophoresis (8, 10). While different in structure, both genetic elements share binding sites for different DNA binding proteins and contain sequences that could act as potential replication origins (11).

The subtelomere is organized in nucleosomes and compacted as heterochromatin (12). However, it was recently shown that the region proximal to the telomere remains free of nucleosomal bodies in *Saccharomyces cerevisiae* (11). Instead, there is a 100-200 nt stretch of subtelomeric DNA often bound by Tbf1 and Reb1, DNA binding proteins known to bind subtelomeric regions and promote transcriptional silencing (11, 13). Subtelomeric regions frequently undergo rearrangements by seemingly taking part in meiotic and mitotic recombination events, so it is interesting to see that the non-nucleosomal arrangement seems to be conserved at several subtelomere to telomere junctions (11, 14).

2.3 Telomeres

The telomere is a highly dynamic and regulated structure constituted by short tandem DNA repeats rich in guanine nucleotides and proteins that bind these sequences (15). Telomeres protect the genomic DNA from end-to-end fusions, degradation and recognition as damaged DNA by the DNA repair machinery, preventing genomic instability (16). Furthermore, telomeres have roles in the regulation of the expression of genes proximal to the telomere and aiding the proper segregation of chromosomes during mitosis (17).

In human chromosomes the majority of the telomere is tightly packed by nucleosomes, as determined by digestion of genomic material with the micrococcal nuclease (MNase), an endonuclease that cleaves the linker DNA between nucleosomes (18). By using a similar assay in yeast, a single fragment of telomeric DNA was recovered and shown to be free of histones but highly associated to the dsDNA binding protein Rap1 (12). Therefore, yeast telomeres contain a non-

nucleosomal chromatin organization, sometimes referred to as the telosome, that caps the DNA ends (Figure 1, right).

Telomeres of different chromosomes are clustered and localized to the nuclear periphery of the cells (8). Telomere chromatin has been frequently considered as silent heterochromatin, as transcription of genes placed near the telomere is repressed. However, telomeres are transcribed in yeast and human cells into long non-coding RNAs known as Telomere Repeat containing RNA (TERRA) (19). TERRAs are transcribed by RNA polymerase II and processed as most mRNA. TERRAs associate to the telomeres and seem to influence the regulation of these structures and the protection of the genome (reviewed in (20)).

Maintaining the integrity of the genome is the main task of the telomeres. However, the genome is also threatened by other sources of instability. Before covering the telomeres, which are the central focus of this work, we will study how the DNA repair mechanisms of the cell provide genome stability since the mechanisms and proteins involved in the repair of DNA damage also have roles in the proper functioning and maintenance of the end structures.

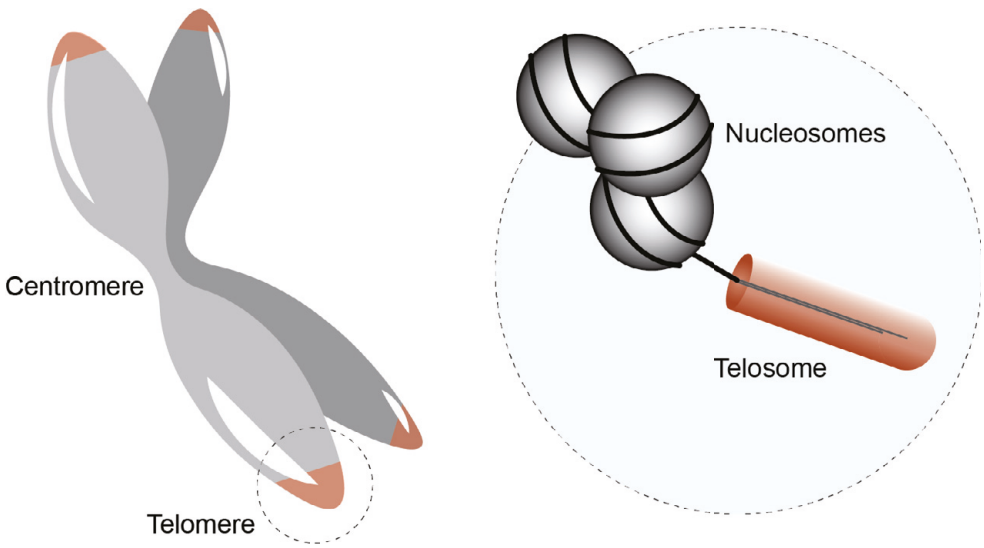

Figure 1. Yeast chromosome organization. Each DNA molecule in the genome is tightly packed as a chromatid, two gene-rich arms extend from the centromere. Telomeres are found at the ends of each chromatid. Here, a specialized non-nucleosomal chromatin structure is formed by the binding of telomeric proteins (Telosome, red).

3 Genome stability: DNA double-strand break repair mechanisms

Maintaining a stable genome is essential for the proper functioning of the cell, its survival, and the transfer of genetic information from one generation to the other. Therefore, cells have developed mechanisms to reduce the incidence of mutations in the DNA sequence, prevent restructuring of DNA by insertions, duplications or translocations, repair different kinds of DNA damage and avoid chromosomal rearrangements, fusions and fragility.

DNA damage and improper DNA repair can lead to a loss of genomic material and chromosomal instability. Single-strand (ss) breaks can be efficiently repaired by utilizing the complementary strand as template to synthesize the gap in the sequence (21). Double strand breaks (DSBs) are the worst kind of DNA damage that can occur in any organism. DSBs can occur spontaneously during the metabolic reactions of the cell or at stalled replication forks. Additionally, external agents such as ionizing radiation and several chemical compounds can cause these DNA lesions (22). Despite being the most toxic form of DNA damage, some DSBs are intermediates of programmed recombination events in the cells, such as the mating type switch of yeast haploid cells or the meiotic recombination during the DNA crossover events (23, 24).

DSBs are repaired by several different mechanisms that involve the end joining of the broken DNA strands or the synthesis of DNA from a sequence with homology. These mechanisms are often depicted as error prone and error-free respectively, but this conception has changed over time (25, 26). Canonical non-homologous end joining (NHEJ) can repair DSBs without introducing deletions or changes to the DNA sequence by ligating ends that are complementary. While homology-directed repair (HDR) mechanism can be error prone when: altering the chromosome structure by resolution of recombination intermediates with crossover events, promoting the loss of heterozygosity during repair or by changing the DNA sequence during DNA synthesis, since recombination is more mutagenic than canonical DNA replication (25). Despite the severity of the consequences of suffering DNA damage, there are mechanisms of DNA damage tolerance that can allow the cells to progress with the cell cycle after DNA replication (reviewed in (27, 28)).

3.1 DNA damage signaling

DNA damage and stalled replication forks are detected by the DNA damage response (DDR) machinery of the cell. DNA damage is signalized by cascade of kinase reactions that lead to the halt of the cell cycle through different effectors. The signaling also recruits the DNA repair machinery to the site of the damage. In the budding yeast *S. cerevisiae*, the Mre11-Rad50-Xrs2 complex (MRX) is responsible for sensing the initial damage and activating the checkpoint signaling, which halts the cell cycle until the damage is repaired (29-31). MRX binds the DSB damage sites (both ends of the break) and recruits the protein kinase Tel1, initiating the DNA damage signaling cascade. The damage site is processed initially by MRX-Sae2, generating ssDNA that is bound by the Replication protein A (RPA) complex. Furthermore, Mec1 is recruited to long ssDNA bound by RPA by the Ddc2 protein to enhance the checkpoint signaling (30, 32).

Chromatin modulation is an important event in the activation of the DDR. Tel1 and Mec1 are both capable of phosphorylating histone H2A in the vicinity of the DSB, to promote the assembly of the DNA repair machinery and aid the repair process by the recruitment of cohesin, histone modifiers and chromatin remodeling complexes (33, 34). Rad9 binds to phosphorylated H2A and is phosphorylated by Tel1 or Mec1. Rad9 then recruits the effectors Chk1 and Rad53 that, when activated by Tel1 or Mec1, arrest the cell cycle and induces transcription of DNA repair proteins (29, 31).

3.1.1 The choice of DNA repair pathway

There are three main pathways for repairing DSBs: HDR, canonical NHEJ and homology-based end joining (HBEJ) (Summarized in Figure 2). Processing of the DSB is one of the determining factors for the selection of the repair pathway, as HDR and HBEJ require ssDNA at the broken ends (23). NHEJ can occur at blunt double-stranded (ds) ends and is more common in G1, because in S/G2 the resection machinery is activated (32). Since HDR involves the use of a homologous duplex template to repair the damage done at the site, these mechanisms preferentially take place during S and G2 phases of the cell cycle (35-38). HBEJ is considered highly mutagenic and more of a backup mechanism if HDR or NHEJ fail to repair the damage (39, 40).

Non-homologous end joining

By directly ligating the broken DNA ends together, NHEJ can rapidly and efficiently repair DSBs. During G1 phase of the cell cycle, the MRX and Ku (Ku70 and Ku80 heterodimer) complexes bind to the site of the DSBs and promote the stabilization and alignment of both DNA strands for successive ligation (30). The

binding of the Ku complex also inhibits resection of the broken ends and recruits the Ligase IV complex (Dnl4/Lif1/Nej1), to ligate the broken ends together, repairing the damage (Figure 2) (40, 41). As mentioned before, NHEJ is an error prone repair mechanism that can sometimes produce small insertions or deletions at the site of damage. Moreover, uncontrolled or incorrect ligation of DNA ends can lead to chromosomal translocations or chromosome fusions.

3.2 Repair of DSBs by homology directed mechanisms

For HDR and HBEJ, the DSB damage site needs to be processed by resection of the 5′ strand to generate ssDNA, necessary for exposing the regions of homology (Figure 2) (41, 42). MRX together with Sae2 are capable of degrading the DNA ends at the site of the damage to generate ssDNA regions (43). The helicase Sgs1 and Dna2 nucleases are responsible for further resection of the damage site. The nuclease Exo1 can also promote extensive resection at the damage site, even in the absence of the MRX complex but this mechanism is inhibited by the binding of the Ku complex (22).

Homologous recombination is inhibited if the mechanisms that regulate the resection of the DSBs ends are mutated (43). After resection, the homology-based DNA repair mechanisms continue by promoting the 3′ strand invasion, generating a displacement loop (D-loop) in the duplex sequence used as template for the repair of the damage (25). If the sequence homology does not involve the end region of the single-stranded DNA, the non-homologous 3′ flaps are digested by the Rad1/Rad10 complex (42).

3.2.1 *RAD52* and the *RAD52* epistasis group of genes

RAD52 is the main recombination gene in yeast and the defining member of the epistasis group of proteins involved in homologous recombination, due to its role in different types of recombination events (37). The *RAD52* epistasis group includes: *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *RH54*, *MRE11, RAD50* and *XRS2* (44). The members of this group have been primarily identified in the budding yeast *Saccharomyces cerevisiae* and characterized as genes that make the cell vulnerable to agents that generate DSBs, especially ionizing radiation (45-47).

As mentioned above, the nucleolytic degradation of the 5′ strand generates a 3′ ssDNA end that is quickly bound by the ssDNA binding protein RPA (48). RPA protects the ssDNA from nucleases and prevents the formation of DNA secondary structures (Figure 2). Rad52, together with Rad55 and Rad57, mediate the displacement of RPA and the binding of Rad51 to the ssDNA to form the DNA-Rad51 filament (Figure 2) (44). Sgs1 has also been proposed to facilitate the

formation of the Rad51 filament (49). The DNA-Rad51 filament can search for the homologous template sequence and promote the strand invasion of the DNA duplex donor sequence (Figure 2). The homology search and strand invasion are facilitated by Rad52 and Rad54 (41). Furthermore, Rad52 can also mediate strand invasion without the formation of a Rad51 filament when in complex with Rad59 (50-52). Because they mediate the homology-mediated strand invasion, *RAD52* and *RAD51* gene function is essential for the majority of the homologous recombination events of the cell.

The Rad52 protein is a highly conserved from yeast to humans (53-55). Rad52 contains three functional domains necessary for its mediator and DNA annealing functions (37). The N-terminal domain of the protein is the most evolutionarily conserved and it contains DNA binding domains, sites of interaction with the paralog Rad59 and two self-association domains which allow for the association of seven or more subunits into a characteristic ring structure (37, 56). The middle region is necessary for the nuclear transport of the protein and the interactions with the RPA components (57). The C-terminal domain can bind DNA but most importantly allows for the association with Rad51 (58).

Rad51 is highly conserved, from bacteria (RecA homolog) to humans, and catalyses the strand exchange between homologous sequences in an ATP-dependent reaction (44, 59). For this, the Rad51 protein contains an essential ATPase domain that culminates at the C-terminus. Rad59 is a homolog of the Rad52 protein found only in yeast (44, 56, 60). Rad59 shares amino acid sequence similarity with the DNA binding domain of Rad52, but lacks the RPA and Rad51 interaction domains.

3.3 Homology directed repair

Homology directed repair is the preferred pathway to repair DSBs in budding yeast. Indeed, cell cycle kinases promote resection of DSBs in the S and G2/M phases by phosphorylating nucleases acting in the resection mechanism such as Sae2, Dna2 and Exo1 (61, 62). Resection prevents NHEJ and creates a substrate for HDR and HBEJ mechanisms. Extensive resection promotes HDR over HBEJ by creating long homology regions for strand annealing. Mec1 depletion causes pronounced failure of the DNA damage repair in yeast, while the effect of Tel1 depletion is less prominent indicating that DSBs are quickly resected and bound by RPA, generating the substrate for Mec1 (33).

Double strand annealing

The DNA-Rad51 filament is capable of promoting complementary strand annealing to the donor duplex. Strand invasion generates a D-loop structure and invading 3′ ssDNA can serve as a primer for DNA synthesis. While new bases are added to the

invading 3′ end, Rad52 captures the second end of the break and anneals it to the D-loop (63). After extension by DNA polymerase the ends are ligated, generating a double Holliday junction that when resolved leads to the repair of the DSB (37, 48). Resolution of the double Holliday junction must occur prior to the anaphase, to guarantee proper chromosome segregation. Resolvases are regulated in a cell cycle-dependent manner and can lead to crossover or non-crossover outcomes (Figure 2). This repair mechanism is also known as double Holliday junction pathway.

Synthesis-dependent strand annealing

For synthesis-dependent strand-annealing (SDSA), after D-loop formation and extension of the invading DNA, the elongated ssDNA can dissociate from the D-loop and complementary anneal to the second end at the site of the break, mediated by the strand annealing function of Rad52 (37, 48). Then gap filling by DNA polymerase and ligation at the break sites concludes the DNA repair (Figure 2). Since there is no junction to resolve, the SDSA mechanism generates exclusively non-crossover products. This mechanism is the preferred pathway of HDR in yeast, since there are mechanisms that prevent the formation of cross-over events that lead the repair of DSBs by double strand annealing to culminate in SDSA.

Break-induced recombination

Break-induced recombination (BIR) is a specialized pathway for the repair of one-ended DSBs and, thus, is a common pathway to rescue eroded telomeres and collapse replication forks (64). BIR can also occur in scenarios where only one of the broken ends finds homology or if the two ends find homology templates at different ectopic positions. Strand invasion during BIR allows copying of the template for hundreds of kilobases, all the way to the end of the chromosome at the telomeres. BIR strand invasion is a complicated event, that characteristically undergoes multiple cycles of strand invasion termed template switching (64). Moreover, DNA synthesis during BIR is carried out by a migrating D-loop leading to conservative replication of the leading strand (41, 64). The synthesis of the lagging strand occurs with a significant delay by an unknown mechanism that fills-in from the leading strand template. All polymerases involved in replication of the DNA participate in BIR, however, the mechanism is dependent on Pol32, a non-essential subunit of Polδ (65). While all HDR mechanism can introduce mutations, BIR is exceptionally prone to cause frameshift mutations, clusters of base substitutions and complex chromosomal rearrangements that generate genome instability (reviewed in (64)).

The mechanisms of telomere maintenance in the absence of telomerase has been shown to be dependent on Rad52 mediated BIR and can occur in a *RAD51*-dependent or *RAD51*-independent mechanism, the latter ocurring at less frequency and requiring the function of Rad59 and Rad50 (66, 67). These mechanisms are covered in chapter 8.

3.4 Homology-based end joining

Homology pairing of sequences can be used by the cells to join the two ends of the DSB damage site together, subsequently after the resection of the DNA, in non-canonical HDR mechanisms known as single-stranded annealing (SSA) and microhomology-mediated end joining (MMEJ) (42). These mechanisms usually require short regions of homology and thus, do not rely on extensive resection of the 5′ ends. Moreover, these end-joining mechanisms are known to be error prone and able to introduce sequence deletions and chromosomal rearrangements.

Single-stranded annealing

The annealing activity of Rad52 in association with Rad59 can mediate the pairing of short homology sites between the broken ends (Figure 2) (48). The non-homologous regions of the single-stranded DNA are removed by Rad1-Rad10 endonuclease complex and the gaps between the paired sequences are then filled-in and ligated to complete the repair of the damage. SSA can also occur without Rad52 but at a much lower rate and it is inhibited by the binding of Rad51 to the single-stranded DNA (42).

Microhomology-mediated end joining

Because it occurs independently of the Ku complex, this mechanism is also known as alternative NHEJ. Unlike SSA, MMEJ is completely independent of Rad52. MMEJ involves the annealing of very short sequences (3-16 bp) after resection of the ends of the DSB site, digestion of the non-homologous 3′ flaps by Rad1-Rad10 complex, error-prone DNA synthesis and subsequent ligation (Figure 2) (25, 42).

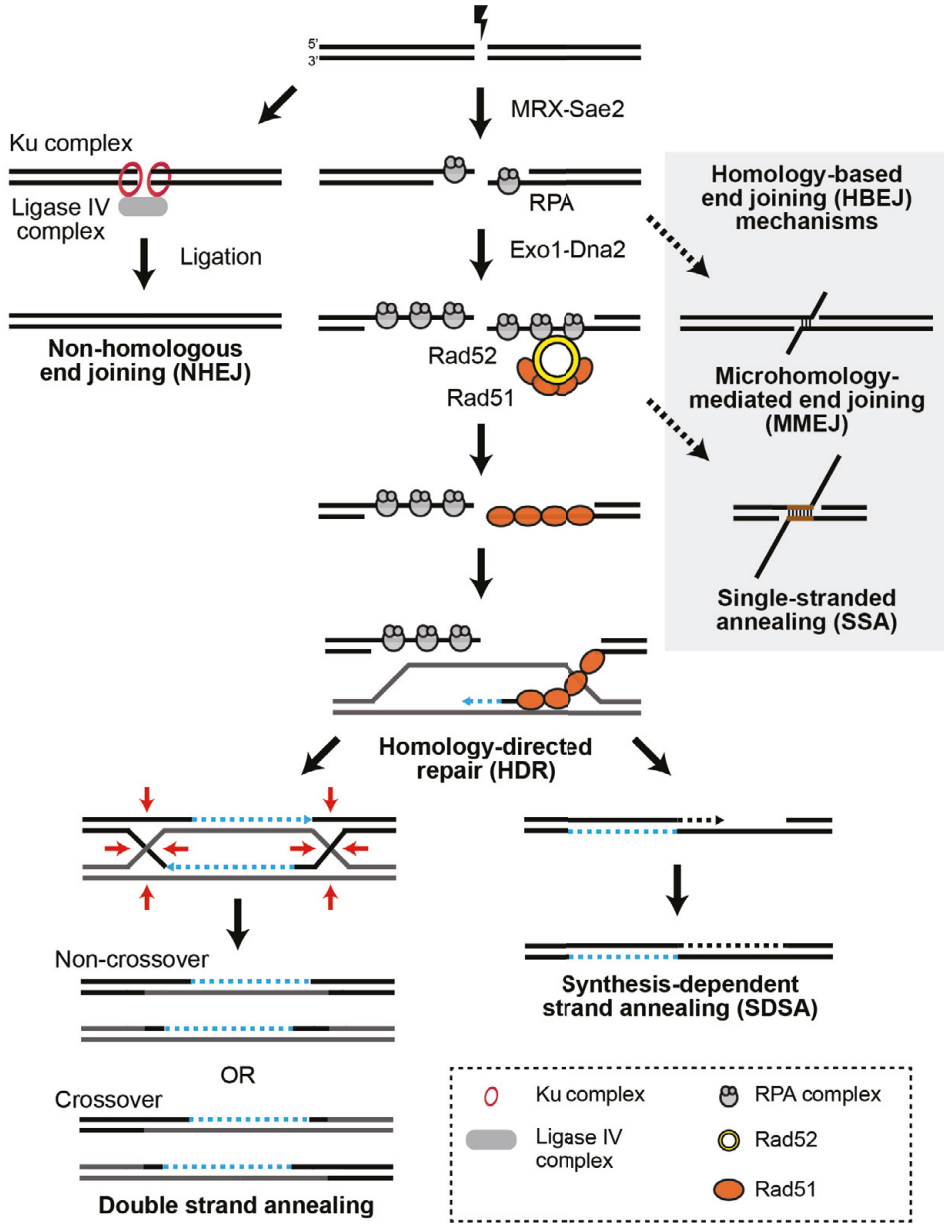

Figure 2. DNA double strand break repair mechanisms. DNA double-strand breaks (DSBs) are repaired by the cell in different ways. During G1 phase, MRX, Ku and Ligase IV complexes bring the ends of the break together and ligate them in a mechanism known as non-homologous end joining (NHEJ). During S and G2 phases, each site of the break is processed by MRX-Sae2 and Exo1-Dna2 nucleases generating ssDNA that is quickly bound by RPA. Thereafter, end joining by microhomology-mediated strand annealing (MMEJ) or single-strand annealing (SSA) may occur, the non-homologous flaps are digested. Homologous directed repair (HDR) is the prefer pathway to repair DSBs. Rad52 facilates the formation of the Rad51 filament at the ssDNA, this filament promotes the homology search and invasion of the donor template, forming a D-loop. The double strand annealing pathway entails the resolution of a double Holliday junction while synthesis-dependent strand annealing (SDSA) only produce non-crossover products. Adapted from Symington, 2016 (22).

4 Telomere DNA structure

Telomeres, the ends of the chromosomes, are protective structures that ensure genome stability. Telomeres are highly vulnerable and exposed to DNA damaging agents, such as ionizing radiation, reactive oxygen species and nucleases, and therefore function as the first barrier of response against environmental treats to the integrity of the genomic DNA (68). To achieve their protective function, the binding of the telomeric proteins cap the chromosomes. In yeast the dsDNA-binding Rap1 and the ssDNA-binding Cdc13 proteins, together with other accessory proteins, constitute the structural cap (covered in Chapter 5). Capped ends are able to protect the DNA ends from being recognized as a DSB by the DNA repair machinery, prevent the fusion of chromosome termini by NHEJ and to stop the degradation of the DNA by the action of exonucleases (Chapter 6) (8, 69, 70). Additionally, capped ends counteract the telomere attrition that surges from the end-replication problem by recruiting telomerase (Chapter 7). Telomeres have also been suggested to act as a biological clock that prevents the accumulation of mutations in multiple organisms by limiting the number of divisions of individual cells.

In the majority of organisms, including yeast, ciliates, plants and vertebrates, the telomeric DNA is composed of short non-coding sequences repeated in tandem (Figure 3). These sequences are rich in G residues along the 5′ to 3′ orientation of the DNA strand (69). While mammalians share a common hexameric sequence, 5′ TTAGGG 3′, other organisms have different telomeric repeats that vary between species (70). Indeed, the telomeric repeats of the members of the Saccharomycetaceae family differ among the different genera and species although, in general, they conserve a similar core to preserve the binding site of the telomeric proteins (71). The telomeres of some species, like *S. cerevisiae*, can host irregular variations of their telomeric repeat along the telomere due to the way their telomeres are processed (72). Other budding yeast species have regularly repeated sequences that can vary greatly in size. For example, *N. castellii* has an octameric telomeric repeat along its structure, while *Kluyveromyces lactis* has a 25-mer repeat (71, 73).

The telomeric repeats are found along the telomeric dsDNA and extends as such for a species-specific length. Yeast usually have telomeres with a length around 300 bp, while higher eukaryotes have rather large telomeric tracts that in human cells extend to around 5-15 kb in length (70). Indeed, *S. cerevisiae* wild type cells have telomeres with a mean length of 300 ± 75 bp and the budding yeast *N. castellii* has telomeres 320 ± 30 bp in length (8, 74). Interestingly, the length of the telomere in each

chromosomal arm is individually regulated in yeast cells, as recently discovered thanks to the advancements in sequencing technologies (75).

Furthermore, the ends of the chromosomes in most eukaryotes are not blunt, but instead the G-rich strand of the telomere protrudes several nucleotides over the dsDNA to form a single-stranded 3′ overhang. The 3′ overhang is highly dynamic, and the length of the overhangs varies depending on the species. *S. cerevisiae* overhangs are generally 12-14 nt long while *N. castellii* has long telomeric 3′ overhangs over its life cycle, ranging from 14 to 200 nt similarly to human telomeres (76, 77), since mammalians overhangs can range from 12 up to several hundred nt (78-81). The 3′ overhang and some intrachromosomal G-rich ssDNA have the characteristic to form quadruplex DNA structures of G-tetrads (G4-DNA), which are four interacting Guanine nucleotides that associate through hydrogen bond base-pairing which then stack on top of each other (82). G4-DNA formation at the 3′overhang has been suggested to provide protection against degradation to the DNA (83). However, G4-DNA is also known to interfere with the progression of replication forks and therefore must be resolved, usually by the helicase Pif1 and Rrm3 (84, 85).

The telomeric DNA mimics the structure of DSBs by having a dsDNA region followed by a ssDNA protrusion. Highly specific telomere binding proteins interact with both the dsDNA and ssDNA in order to protect them from harmful DNA damage responses (86-89), detailed in the next chapter. Moreover, the processing of 3′ overhangs leads to the formation of a dsDNA and ssDNA junction (ds-ss junction) at the terminal end of the complementary C-rich strand (90). This region determines the interaction between the dsDNA and ssDNA binding proteins and thus plays an important role in telomere capping and maintenance (74).

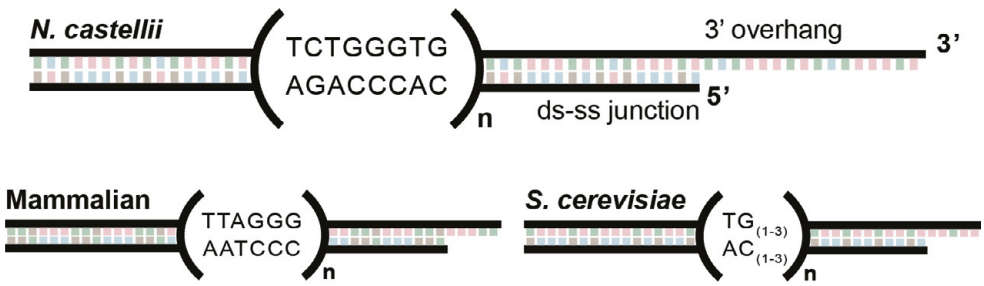

Figure 3. Schematic representation of the telomere DNA structure. Telomeric DNA is constituted by telomeric repeats rich in Guanine nucleotides in the 5′ to 3′ direction. Telomeres are mostly double-stranded but terminate in a single-stranded 3′ overhang, the region of transition between dsDNA and ssDNA is known as the ds-ss junction. Telomere repeats can be regular, such as is in *N. castellii* and mammalian cells, or can be irregular, allowing variants of a repeated element such as in *S. cerevisiae* cells.

5 Telomeric proteins

Telomeres have a specialized chromatin structure that is important for the functional and structural maintenance of the ends, sometimes called the telosome. This structure is constituted by the core telomere binding proteins that bind the dsDNA and ssDNA specifically to the telomeric repeats. These protein in turn associate through protein-protein interactions to a subset of proteins to provide the optimal protection or "capping" of the telomeres. Other proteins do not associate directly to the telomere but play a role on its maintenance and functionality. Here, a description of the main telomeric proteins in yeast is presented (Summarized in Figure 4).

5.1 Telomere dsDNA binding protein: Rap1

Rap1 is the main dsDNA telomere-binding protein. When bound at the telomere it regulates its length, telomere silencing effects, localization of the telomere to the nuclear periphery and has an important role in protection. This abundant protein also acts a transcription factor regulating the activation or repression of gene expression, for what it was first described (Rap1 – *repressor activator protein 1*). *RAP1* gene function is essential for the cells' viability. Rap1 binds telomeric DNA with high affinity (88). Rap1 is conserved as a telomere associated protein across multiple species. Even though Rap1 is not the main duplex DNA binding protein in human cells, hRap1 has the ability to bind specifically to ds-ss junctions and in association with the TRF2 homodimer it increases the affinity of the protein to bind the human telomeric dsDNA (91, 92). Rap1 DNA binding is mediated by two tandemly placed Myb-like domains separated by a linker sequence (Figure 4B) (93). Additionally, the DNA binding domain (DBD) of Rap1 contains a sequence that extends after the Myb-C domain known as the wrapping loop. The wrapping loop interacts with the DNA and latches to the Myb-N domain, locking Rap1 on the DNA (94). The Rap1 N-terminal BRCT domain is responsible for the interactions with phosphorylated proteins and its C-terminal RCT domain is responsible of the interactions with the telomere-associated proteins Rif1, Rif2 and the silencing complexes Sir3 and Sir4 (69, 94, 95).

Multiple Rap1 proteins bind the telomeric tract, spaced out by about 18 base pairs (bp) of distance in *S. cerevisiae* (8). The abundant binding of Rap1 to the telomeres have been observed by Chromatin Immunoprecipitation assays, MNase-Rap1 fusion

proteins and other *in vivo* methods (11, 12, 88). Moreover, *in vitro* experiments have allowed for the characterization of its telomere minimal binding site in multiple organisms of the Saccharomycetaceae family (71). Interestingly, the binding of multiple Rap1-Rif1-Rif2 complexes to the telomere has been suggested to establish a telomere length regulatory mechanism that acts as a counting mechanism in a negative feedback-loop to regulate telomerase activity (96).

5.2 Rif1 and Rif2 proteins

The Rif1 and Rif2 proteins interact with Rap1 at the telomeric dsDNA (Figure 4C). *S. cerevisiae* cells with a knockout of the gene coding for any of the two proteins show extended lengthening of the telomeres as a characteristic phenotype (97). This phenotype is dependent on telomerase activity, and therefore the Rif proteins have been identified as regulators of telomerase activity. The long-telomere phenotype is intensified when both proteins are knocked out in the cell, suggesting that the proteins have independent functions in the regulation of telomerase activity (97). Furthermore, the Rif1 and Rif2 proteins prevent the chromosome ends from being recognized as DSBs. Individually, both proteins seem to have protection functions that prevent the degradation of the telomeric DNA by exonucleases, when associated with Rap1 (98). This last feature is also additive, being stronger when both proteins act together. Moreover, the Rap1-Rif2 complex prevents the access of nucleases at the telomere and prevents NHEJ between the ends of the chromosomes (99). Individually, Rif1 is an evolutionary conserved protein that also has Rap1-independent role in assisting replication and heterochromatin formation, and Rif2 has been suggested to signal the presence of short telomeres (8, 100).

5.3 Sir complex proteins

The Sir complex involves three proteins: Sir2, Sir3 and Sir4. These proteins are recruited to the telomere by interactions with Rap1 (Figure 4C) (101-104). Histone modifications characteristic of the silenced chromatin regions at the TAS elements are known to occur by action of the NAD^+-dependent histone deacetylase Sir2 protein (105). Sir3 is also a histone deacetylase found to be located over the X elements of *S. cerevisiae* chromosomes, where it mediates transcriptional silencing. Sir4 is required for the recruitment and assembly of the complex. The Sir proteins are also suggested to be responsible for the association of the telomeres to the nuclear periphery (69). Outside the telomere, the Sir proteins act at the HM mating type silent locus in yeast where they promote the formation of heterochromatin (101).

Genes located in regions proximal to the telomeres are usually transcriptionally silenced when the telomere heterochromatin is assembled correctly. This phenomenon has been described as the Telome Position Effect (TPE) (106). The silencing of genes due to TPE can extend up to 10-15kb upstream from the telomere, depending on the organism and the chromosomal end involved. TPE occurs due to the nature of the heterochromatin of subtelomeric regions, that is dependent on the Sir-dependent histone modifications and the spreading of the Sir complex into the subtelomeric region (106).

5.4 Yeast Ku complex

The Ku complex is conserved among eukaryotes and in yeast is assembled by two proteins: Ku70 and Ku80. In the nucleus, the Ku proteins are essential in the metabolic pathways for DNA repair of DSBs by NHEJ (107). It is not clear if the complex binds directly to the telomeric DNA or in association with the Sir4 protein (108, 109). Paradoxically, when associated with the telomeres the complex helps preventing NHEJ at the chromosome termini (110). Other roles of the Ku complex involve the protection of the telomeres from nucleolytic degradation, mediating telomere silencing by TPE and the nuclear localization of telomeres (111). Moreover, the Ku complex has been shown to have functions in the recruitment of telomerase and the transport of the *TLC1* transcript to the nucleus (112). Therefore, the Ku complex has roles in telomere length homeostasis and maintenance.

5.5 Telomere ssDNA binding protein: Cdc13

In yeast, Cdc13 is an essential protein that binds to the single-stranded telomeric DNA with high affinity (Figure 4B) (8). Cdc13 was first described as a protein necessary for cell division. The binding of the Cdc13 protein to the telomere provides functions related to telomere replication, maintenance of telomere length, telomere protection and regulation of telomerase activity at the telomeres (81, 89). In absence of its telomeric binding activity, cells quickly arrest in G2/M phase in a Rad9 dependent manner that leads the cell towards apoptosis (113).

In its structure, Cdc13 contains multiple OB-fold domains, a common feature of ssDNA binding proteins (114). The ssDNA binding domain is determined by one of the OB-folds, this conserved region constitutes the DNA binding domain of the protein. Two different OB folds located towards the N-terminal region in the aminoacidic sequence of the protein facilitates the protein homodimerization (115). Additionally, the N-terminal proximal OB-fold is important for the interaction with Pol1, linking its functions to the replisome. The same area of the protein also

contains a domain of interaction with the Est1 protein, needed for telomerase recruitment and the elongation of telomeres. Lastly, the C-terminal region contains an OB-fold needed for the association with the Stn1 protein (116).

While Cdc13 is essential, its main role might be limited to a specific part of the cell cycle. Indeed, telomeric localization of the Cdc13 is mostly visible during the S-phase cycle while during the rest of the cell cycle Cdc13 has decreased localization at the telomeres (117). This selective binding could be partially regulated by a cell cycle regulated nuclear import of Cdc13 (115). Furthermore, this temporal localization of the proteins highlights its importance during the replication of telomeres, a process that involves the DNA replication machinery, telomerase and the processing of newly formed chromosome ends.

5.6 The CST complex

Two additional essential proteins, Stn1 and Ten1, interact with Cdc13 to provide the telomeric protective function (Figure 4C). The Stn1 protein interacts directly with Cdc13 through a C-terminal OB-fold of its own (118, 119). Through its N-terminal domain, Stn1 interacts with the Ten1 protein. The protein-protein interactions between these three proteins form the CST complex that, when bound to the telomeric 3′ overhang, forms the capping structure that maintains the telomeres in yeast (120).

CST complexes seem to be evolutionarily conserved amongst eukaryotes, having been identified in fission yeast, plants and mammals. The human CST complex shares the role of aiding the replication of the telomeres but has not been characterized to have protective roles in the cell. The CST complex is structurally similar to RPA (121). RPA is formed by the interaction of 3 proteins (Rfa1-Rfa2-Rfa3) and it is required to bind ssDNA during replication, repair, and recombination cellular events (8). The main difference between these complexes is that CST binds specifically to telomeric ssDNA.

Stn1 has been proposed to act in cooperation with Cdc13 to provide protection against degradation and telomere length regulation, two of the main roles of the Cdc13 protein (118, 122). Indeed, the lack of Stn1 binding leads the cells to the activation of the Rad9 checkpoint indicating the uncapping of the DNA end structures, a phenotype shared with Ten1 mutants (118). Moreover, the lack of Ten1 binding to the Cdc13-Stn1 complex leads to defects in telomere length regulation where cells accumulate long overhangs (120). Interestingly, overexpression of Stn1 and Ten1 can rescue the phenotype of the Cdc13 temperature sensitive mutants, indicating that the interactions of these two proteins could act independently of Cdc13 to provide telomere protection (123).

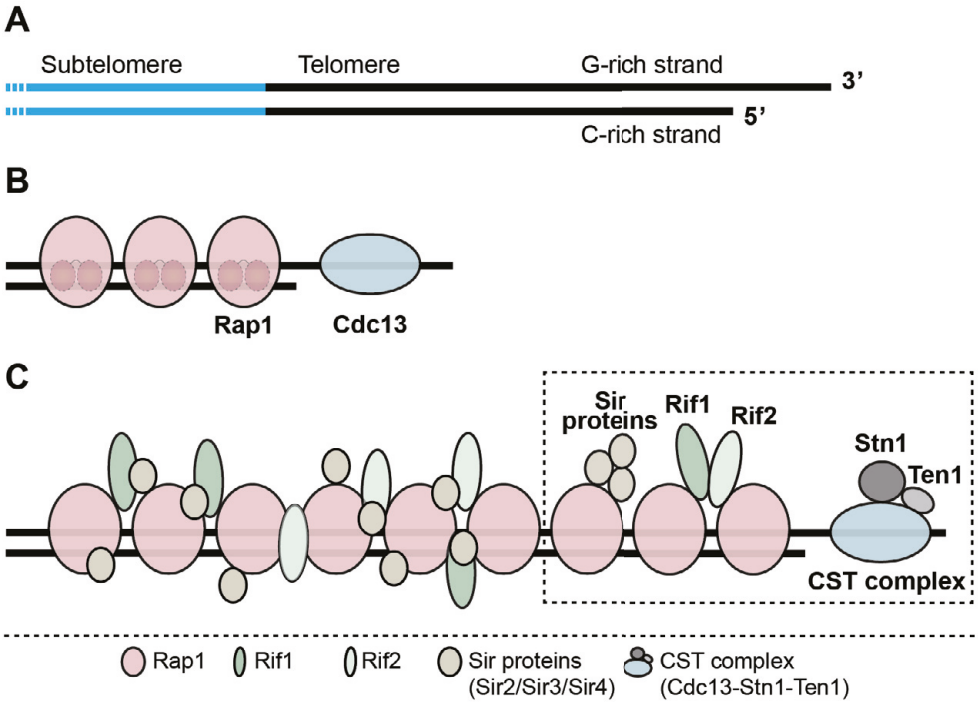

Figure 4. Schematic representation of the binding of the telomeric proteins. A) Telomere DNA structure. Located after the subtelomeric region, the telomeric DNA repeats are located at the end of the DNA strand and culminate in a single-stranded 3′ overhang. B) Binding of the core telomeric proteins. Multiple Rap1 proteins bind the telomeric dsDNA with high affinity through its two Myb-domains and Cdc13 binds the telomeric 3′ overhang. C) Telomere associated proteins interact with the core telomeric proteins. As indicated at the dotted section, multiple proteins can interact with Rap1 including the Rif1, Rif2, Sir2, Sir3 and Sir4 proteins. Cdc13 associates with Stn1 and Ten1 to form the CST complex. The interactions with the telomere associated proteins are needed for the formation of a functional telomeric chromatin. Adapted from Bonnel, et al. (2021) (124).

6 The role of telomeres

Functional telomeres are essential for maintaining the viability of the cells. The effects of complete or partial loss of telomere capping are diverse and reflect the multiple roles of the proteins that interact at the telomeres (Summarized in Figure 5). The absence of Cdc13 generates extensive ssDNA at the telomeres and the subtelomeric regions, which has been linked to the generation of chromosomal instability (125). The study of Cdc13 mutant strains illustrates the functions of its telomere capping: Cdc13-null mutants are not viable and can only survive in the absence of nucleases known to degrade the telomere (Exo1), ligases known to act in the NHEJ repair pathway (Ligase IV complex), and the important recombinases needed for HDR (Rad51 and Rad52) (125). Thus, Cdc13 is essential for its roles in telomere maintenance. Rap1 is also an essential protein, although it is unclear if the cellular requirement is exclusively limited to its roles at the telomeres or for its roles in transcription regulation (8). *S. cerevisiae* Rap1 mutants with impaired telomere binding show an increased telomere length, as telomerase activity is no longer inhibited at the ends. However, the phenotype does not seem to affect senescence as loss of telomeric repeats occurs at an equal rate for these mutants (126). In contrast, in human cells with low telomerase activity, the loss of the dsDNA binding protein TRF2 leads to increased rate of fusions between chromosomes, an increased loss of telomere DNA and the appearance of chromosomal aberrations (127, 128).

The shortening and uncapping of telomeres leads to the activation of signaling paths that act as checkpoints for replication, protecting the integrity of the genome. The DNA damage signal from a single critically short telomere is enough to promote growth arrest (129). Thus, the signal emitted from the telomere is different than those exerted by genomic DSBs, although many of the same proteins are involved in both events. Genomic DSBs are processed by a series of events that start with the association of MRX with the site of damage after phosphorylation by Cdk1 kinase (130), and are explained in detail in Chapter 3.

Checkpoint signaling is important for the maintenance of functional telomeres. At the ends, MRX association to short telomeres leads to the recruitment of Tel1. Tel1 does not elicit a checkpoint signal when associated to the telomeres, instead its action is thought to be involved in telomerase recruitment (131). The MRX-Tel1 interaction at the telomeres is also responsible for the generation of long 3′ overhangs during the S phase in *S.* cerevisiae (80). The binding of the RPA-like CST-complex to the 3′ overhang prevents the association of Mec1 at the telomeres

(132). In Cdc13 deficient cells, extensive resection of the ends by Exo1 generates ssDNA that, when bound by the RPA complex, elicits a DNA damage signal through Mec1 and the arrest of the cell-cycle in a Rad9 dependent manner (129). The 9-1-1 complex is also parallelly loaded into RPA coated ssDNA as a damage signal checkpoint at broken ends (69). Tel1 knockout strains have a short telomere phenotype, and mutants of both Tel1 and Mec1 suffer from telomere attrition that leads to cellular senescence, highlighting the importance of the DNA damage signaling at the telomeres (133, 134). Furthermore Mrc1, a replication stress damage sensor, has also been linked to have roles in the maintenance of the telomeres suggesting that telomeres are also capable of eliciting a replication damage signal (135, 136).

6.1 Protection from degradation

Due to their characteristic DNA structure, telomeres are constantly exposed to the action of different nucleases present in the cell. Moreover, the coordinated action of helicases and exonucleases is necessary for the generation of 3′ overhangs and telomere length homeostasis, further discussed in Chapter 7. Protection from extensive resection of the telomeres is important as the generation of ssDNA leads to an increased recombination activity at telomeres that can lead to the loss of genetic material and genetic instability.

The action of 5′ to 3′ exonucleases has been extensively investigated, as many proteins with this processing characteristics are responsible for the C-strand resection mechanism. Cdc13 is known to protect from extensive degradation of the 5′ end when bound to telomeric DNA (137, 138). The protection is expanded by the association of the protein with Stn1 and Ten1, forming the CST-complex. However, in *S. cerevisiae* the protection against exonucleases provided by Cdc13 seems to be more relevant during the S-phase, as non-dividing cells that lack Cdc13 binding do not activate a DNA damage response or show degraded telomeres (139). Cdc13 temperature sensitive mutants unable to bind the telomeres can transition through the S-phase if the Pif1 helicase and Exo1 activities are inactivated, indicating that the protection of the telomeres against degradation is one of the essential functions of Cdc13 (137, 140). Since *S. cerevisiae* has short 3′ overhangs outside of the S-phase, it is likely that not all overhangs host a binding site for Cdc13.

Indeed, in *S. cerevisiae* it was shown that Rap1, prevents the accumulation of ssDNA caused by resection of the 5′ end in non-dividing and cycling cells (98). The protection exerted is accentuated by the protein-protein interactions of Rap1 with the Rif1 and Rif2 proteins, preventing extensive resection during the G1 and G2 cell cycle phases (98). In *N. castellii*, *in vitro* protection assays based on the binding ability and positioning of Rap1 suggest that the binding of the protein is enough to

protect the 5′ end from degradation (74, 141). Unlike Cdc13, the protection provided by Rap1 might not be limited to a specific time of the cell cycle, instead it could promote the formation of structures that favor telomerase activity or regulate the access of the C-strand resection machinery (74, 141, 142). Rap1 is the most highly conserved telomeric protein and in human cells it has also shown the ability to prevent 5′ end resection of the telomeres (139). Furthermore, the Ku complex has also been shown to prevent degradation from 5′ to 3′ exonucleases during G1-phase in *S. cerevisiae* (98, 139, 143).

The 3′ overhang is also at risk of being degraded by 3′ exonucleases, and for some species the degradation of the telomeric 3′ overhang is regulated to confer a specific end nucleotide to the ssDNA (74, 144, 145). While a large number of 3′ exonucleases are present in eukaryotic cells, little is known about the protection of the G-rich strand against nucleolytic degradation. The telomeric ssDNA binding protein in humans POT1, has been shown to protect the 3′ overhangs against degradation by the WRN helicase, which contains 3′ exonuclease activity and its known to have a role in telomere maintenance. No similar studies have been performed for *S. cerevisiae*, but through telomere-PCR it has been shown that the 3′ overhangs of this species show no preference for a terminal 3′ nucleotide, suggesting that if degradation of the 3′ end occurs it is not regulated (146). In *N. castellii* we have shown that binding of Rap1 and Cdc13 proteins *in vitro* provides protection to the telomeric ssDNA from 3′ exonucleases (74, 142).

6.2 Protection from the DSB repair machinery

The telomeric proteins protect the chromosome ends from the DSB machinery by preventing the signaling of DNA damage at the telomeres, as it is the signaling that recruits the proteins that degrade the end structures and generate long ssDNA that facilitate recombination as a mean to repair the DNA damage. Telomeres are composed of highly repetitive DNA sequences, making them prone to homologous recombination. Resolution of recombination products at the telomeres could lead to catastrophic loss of genomic material and chromosomal instability. The action of telomerase also limits recombination at the chromosomal ends by maintaining a functional DNA structure, and the telomere binding proteins are responsible for preventing unwanted recombination at the ends (147).

The binding of Rap1 is suggested to prevent recombination in yeast (148). *K. lactis* cells with mutant telomeric repeats prevent the proper binding of Rap1 at the telomeres, these cells also obtain longer telomeres by a recombination-based mechanism even while having active telomerase (149). Rif2 can counteract MRX association independently and when interacting with Rap1 in *S.* cerevisiae, and Rap1 can also independently modulate MRX association in a manner dependent on

the way the protein binds the DNA (100, 150). Human Rap1 has also shown to prevent unwanted recombination events at human telomeres, further supporting the idea that Rap1 association to the telomere prevents unwanted homologous recombination (151, 152). Moreover, uncapping of the telomeres by the loss of Cdc13 binding could promote the binding of RPA to the ssDNA, therefore promoting recombination between telomeres (153). Cdc13 can also prevent recombination by aiding the progression of replication forks at the telomeres, thus preventing replications stress (125). *K. lactis* Stn1 mutants have ALT-like telomeres produced by recombination, even in the presence of telomerase, suggesting a specific role in preventing recombination by the CST-complex (154). It has also been suggested that the Ku complex prevents recombination at the telomeres by preventing the extensive resection of the C-rich strand.

6.3 Protection from NHEJ

At the telomeres, NHEJ can lead to chromosomal end-to-end fusions, forming dicentric chromosomes. Dicentric chromosomes form anaphase-bridges that can promote the stop of the cell cycle and eventually cell apoptosis (155). Resolution of fused chromosomes can lead to further genomic rearrangements and instability because these chromosomes break in different positions between the two centromeres in cells that progress through the anaphase.

At DSBs, classical NHEJ occurs mostly during the G1 phase and requires the binding of the Ku complex, MRX and the ligase activity of the Ligase IV complex (107). Paradoxically, the binding of the Ku complex at chromosomal ends seems to prevent NHEJ at the telomeres (156). The binding of the CST complex has also been suggested to prevent fusions at the chromosomes, by preventing damage at the ends but primarily, chromosomal fusions are prevented by Rap1 in mammals, fission yeast and budding yeast. In *S. cerevisiae* Rap1 can independently prevent fusions by binding the telomeric dsDNA (157). Interactions of Rap1 with Rif2 and Sir4 have synergistic roles in providing protection against fusion of short telomeres (99). Since multiple pathways protect the telomere from fusions, NHEJ is not a frequent event at yeast telomeres. Intriguingly, chromosomal fusions at yeast telomeres seem to preferentially break at the telomere fusion site in a mechanism mediated by Rap1, preventing genomic instability (155, 158). Since recombinational repair is not as prominent in human cells (as it is in *S. cerevisiae*), the fusion of telomeres is a common phenotype of uncapped chromosomal ends that is linked to the generation of chromosomal aberrations (127).

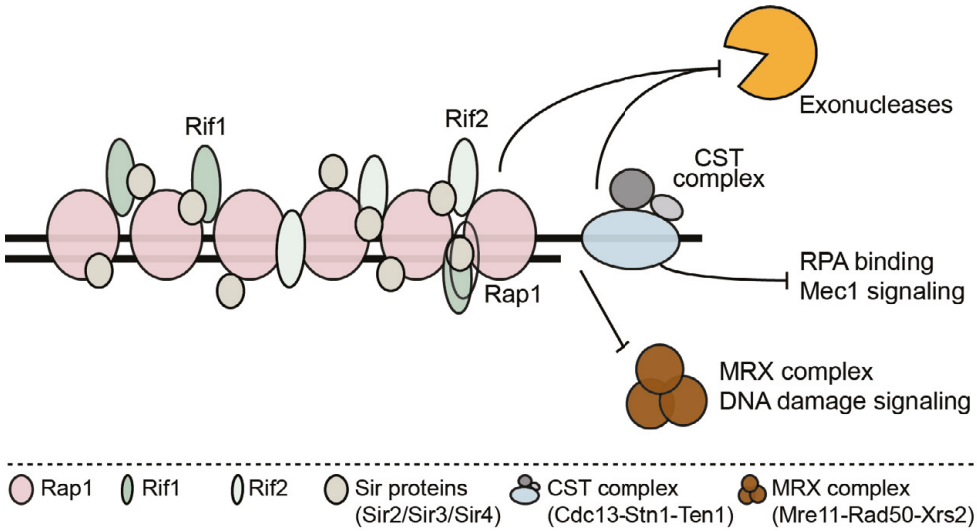

Figure 5. Functional telomeres prevent chromosomal instability. The binding of the telomeric proteins prevent degradation of the DNA by exonucleases. The telomeric proteins also prevent the recognition of the DNA ends as broken ends and, therefore, prevent DNA damage-induced cell cycle arrest. This is achieved by preventing the binding of RPA to the telomeric ssDNA that leads to Mec1-Ddc2 DNA damage signaling or by preventing the binding of MRX-Tel1, which leads to degradation of the ends and DNA damage signaling.

7 Replication of telomeres

The bulk of telomeric and subtelomeric DNA replication occurs by semi-conservative DNA replication by the cell's DNA replication machinery. The replication of the telomeres is one of the last events that occur during the S-phase of the cell cycle of yeast cells. This is due to a late firing of the replication origins located proximal to the telomere sequences (159). In *S. cerevisiae* Sir3 depleted cells there is no change in the timing of replication for origins located closely to the telomere, thus the late start of these replication origins seems not to be an effect of the tightly bound telomeric chromatin (160). Interestingly, replication origins near short telomeres fire earlier in the S-phase than those with a wild type length (161). This implies that there is an association between the binding of the telomeric proteins and telomere associated proteins and the regulation of the start of replication at the chromosomal ends.

However, the G-rich sequence and the binding of the multiple telomeric proteins makes replication of telomeric regions a difficult task that can lead to the generation of DNA damage due to replication stress (124, 148, 162). Replication stress, defined as the slowing or stalling of the replication fork progression, can generate replication fork breakage, leading to DSBs and possibly unstable chromosomes (163, 164). Progression of replication forks through the telomere repetitive sequences is also challenged by the formation of DNA tertiary structures such as G4-DNA, the condensed chromatin structure and the binding of TERRA around telomeres (162, 165). Because stalled replication forks at the telomere cannot be rescued by an oncoming replication fork, DNA recombination is a common answer to replication stress which can cause genomic instability.

The CST complex has different roles in the replication of telomeres, its involvement with the DNA replication machinery mediates its association to the DNA polymerase α complex, needed for the initiation and priming of Okazaki fragments during lagging strand synthesis (81). The CST complex in yeast, and CST-like complexes in plants and mammals, has been identified to have functions in rescuing stalled replication forks at the telomere and the sequences near the telomere (81). Recently, it was shown that Cdc13 essential function is linked to its binding at the telomere during the S-phase, where it prevents the formation of unstable chromosomes that originate from the accumulation of ssDNA due to extensive resection at the chromosomal ends (125). CST is therefore needed during replication, although it is unclear whether the mechanism of action concerns the

ability of the complex to help overcome replication stress or its requirement for C-strand synthesis of the lagging strand.

7.1 The end replication problem

The end-replication problem surges from the limitation of the DNA replication machinery to replicate the full extent of the lagging strand (166, 167). This happens because the mechanism of nucleotide addition of DNA-polymerases can only add nucleotides in the 5′ to 3′ direction and require a short RNA primer to start the elongation process. For this reason, during lagging strand replication, when the last primer is removed there is no available 3′ OH for DNA polymerase to fill-in the gap until the end of the template strand (96). The result of this phenomenon, in the absence of a mechanism that fills in the gap, is the loss of genetic material that leads to the shortening of the telomere with every subsequent cellular division (Figure 6).

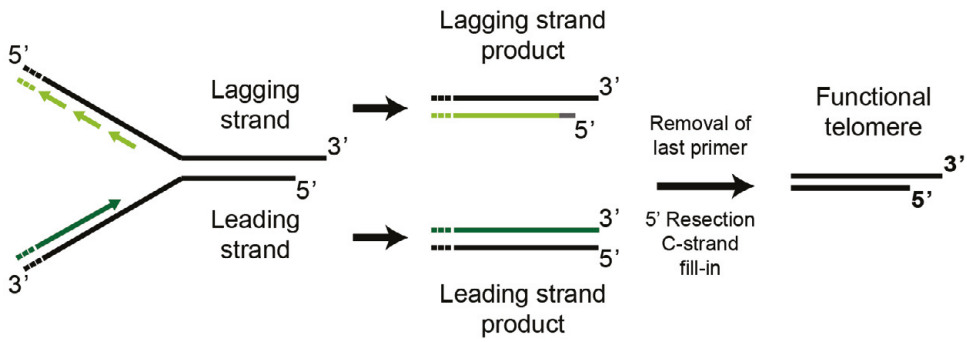

Figure 6. Telomere replication schematic. During replication of the telomeres by the canonical DNA replication machinery, the lagging strand cannot be fully replicated, leaving a gap between the end of the DNA strand and the position of the last primer used for replication (grey, middle pannel). The removal of the last primers facilitates the formation of a 3′ overhang. The leading strand is fully replicated, leaving a blunt ended telomere (middle pannel), however functional telomeres require the presence of a 3′ overhang. Therefore, the leading strand is processed by 5′ end resection and C-strand fill-in to generate a functional end structure.

7.2 Telomere processing after replication

The telomeric 3′ overhang is important as substrate for telomerase and the binding site for the protection proteins. Semi-conservative replication of the telomeres is theorized to produce a blunt-ended telomere at the leading strand. Since all telomeres contain a ss 3′ overhang, there are mechanisms that process the telomeres to generate such structures (Figure 6). In order to generate 3′ overhangs, telomeres have been shown to be subject to many different processing events that involve 5′ end resection, extension by telomerase and C-strand fill in (168-170).

In the cell, the telomeric 3′ overhangs are highly regulated, having a species-specific length and a dynamic behavior during the cell cycle in many organisms. *S. cerevisiae* has been characterized to have short telomeric 3′ overhangs for most of the cell cycle and acquire longer overhangs from processing of the C-rich strand during the S-phase (80, 168). While active telomerase can generate long 3′ overhangs, it has been shown that mouse cells and *S. cerevisiae* can also process 3′ overhangs in the absence of the enzyme in a process regulated by Cdk1 activity (171-173). Human cells with active telomerase generate telomerase-dependent 3′ overhangs during the S-phase of the cell cycle. In *S. cerevisiae*, C-strand fill-in is coupled to the replication complex and elongation of telomeres by telomerase in the late S-phase (174). In human cells the processes are uncoupled, and telomeres elongated by telomerase early in the S-phase are not subjected to C-strand fill-in mechanism until late in the S-phase (few hours later) (169, 175). The generation of 70 nt long 3′ overhangs in *N. castellii*, in a manner dependent on telomerase, might also suggest that the C-strand fill-in mechanism is uncoupled from telomerase elongation in this species (76).

Not much is known about the molecular mechanisms governing telomeric 5′ end resection, however they seem to be tightly regulated in ciliates and human cells, as their telomeres show a clear preference for one or two 5′ end nucleotides (144, 145). The 5′ end nucleotide is also regulated in the budding yeast *N. castellii*, but the ends might be differently regulated from those found in mammalian cells (74). In mammalian telomeres the 5′ exonucleases Apollo, WRN and Exo1 are known to act on the resection of the telomeric 5′ strand, while the actions of other exonucleases are still unknown (170, 176-178). In *S. cerevisiae* the 5′ resection mechanism seems to involve several proteins involved in double-strand break processing. The MRX complex mediates the processing of the 5′ end by the Sae2 nuclease, this mechanism is able to generate short 3′ overhangs (43, 168). The action of Sae2 is limited to the S-phase of the cell cycle by phosphorylation of the protein by Cdk1. Since MRX association to the telomere is prevented by the Rif2 protein, it has been suggested that, in short telomeres, the lack of Rif2 association may promote the formation of a short 3′ overhang by MRX-Sae2 to generate the substrate for Cdc13 binding and telomere elongation (179). Extensive resection of the telomeres can be promoted by an alternative 5′ end resection mechanism, where the action of helicase Sgs1 at the telomere allows for extensive digestion of the C-rich strand by the Exo1 or Dna2 exonucleases (43, 80, 168).

Telomere processing modifies the region of the telomeric DNA where the dsDNA becomes ssDNA is referred to as the ds-ss junction. The ds-ss junction is critical for the protection of the telomeres as this region dictates the positioning of the ds and ss core protection proteins necessary for the capping of the end structures. The maintenance of a specific ds-ss junction structure becomes evident in organisms that keep a preferred terminal nucleotide at the 5′ end of the DNA, such as ciliates, humans and the budding yeast *N. castellii* (74, 145, 180-182).

7.3 Telomerase

Telomerase is the physiological response to the end replication problem and has been conserved in most eukaryotic organisms. First described in the ciliate Tetrahymena, telomerase is a specialized enzymatic complex that has telomere-specific reverse transcriptase activity to fulfill the function of elongating the ends of the chromosomes, mediated by the sequence of a short RNA molecule associated to its structure (183). The catalytic reverse transcriptase function of telomerase seems to have phylogenetically originated early in the evolution of eukaryotic organisms (184). Although the maintenance of the telomeres requires the addition of telomeric repeats by telomerase, the enzyme is not always active in the cells. In some unicellular organisms like budding yeast, telomerase is constitutively active during each cell cycle, fulfilling its activity during the late S phase. However, in humans telomerase is only active in some particular cells, such as stem cells and germline cells, while somatic cells suffer from progressive telomere shortening (185).

7.3.1 Telomerase structure

The telomerase enzyme is a ribonucleoprotein structurally composed of two components: A catalytic subunit that acts as a reverse transcriptase (TERT – Telomerase reverse transcriptase), and a non-coding telomeric RNA (TERC – Telomerase RNA component) which function is to be the template for the synthesis of the telomeric G rich strand at the 3′ end (186). In humans, only the TERT and TERC components are needed to guarantee telomerase activity *in vitro*, but it is estimated that at least 32 different proteins can be associated to telomerase *in vivo* to keep its functionality (187).

The TERT domains vary substantially in size between species, but the majority of the protein domains have been phylogenetically conserved between organisms. In general, TERT has 3 functional domains in its structure: The N-terminal domain, which contains a docking region that binds telomeric DNA; the RNA binding domain and the reverse transcriptase domain this last one being highly conserved. The RNA component varies considerably in size, sequence, and secondary structure between species. This subunit is known as TLC1 in budding yeast. There are also several accessory factors of telomerase that assist in the assembly, maturation, recruitment, and activation of telomerase (186-188).

Yeast telomerase assembly and function requires five different genes, some of which are named *EST* from “ever short telomeres”, because their gene function deficiency leads to a gradual shortening of the telomeres (189, 190). The telomerase components include the TLC1 RNA transcript and the proteins Est1, Est2, Est3 and Cdc13. From these, Cdc13 is the only essential component for the viability of the cell, as its functions are not limited to the telomere length maintenance (8). Est1

interacts directly with Cdc13, and it is therefore necessary for the recruitment of telomerase to the telomeres where Cdc13 binds (191). Est1 is also thought to recruit Est3 to telomere and aid with the activation of telomerase (8). Est2 is the catalytic subunit of telomerase and it possesses the reverse transcriptase activity (192). Est2 can interact directly with the TLC1 transcript and telomerase activity can be observed *in vitro* with only these two components by telomerase primer extension assays (193). Est3 is a component unique for budding yeast as part of the telomerase holoenzyme and has roles in activation of telomerase and telomere maintenance (8).

7.3.2 Regulation of telomerase activity

Telomerase binds and elongates the telomeric 3′ overhang. The catalytic cycle of telomerase consists of several sequential stages that lead to the addition of the telomeric repeats to the overhang substrate. The association of telomerase to the telomeric DNA occurs by a rather short (4-7 bp) complementation of bases which promotes the docking of the protein (194). As other polymerases, telomerase can translocate the RNA-DNA duplex in its active site after the addition of a single nucleotide during elongation. Telomerase also has the capability of translocating the DNA template after a telomeric repeat has been added, to allow the continuous elongation of the DNA primer (186, 195).

Telomerase activity is highly regulated in yeast (Figure 7). More specifically, telomerase activity is restricted to the late S phase of the cell cycle, where it preferentially elongates short telomeres (196, 197). The preference of telomerase to target short telomeres might be influenced by the amount of Rap1 binding sites, as suggested by the telomere length counting mechanism. This effect could be a consequence of the interaction of Rap1 with the Rif1 and Rif2 proteins, which are known to inhibit telomere elongation by telomerase. While the interaction of these proteins is present in short telomeres, there is a lower abundance of Rif2 association at short telomeres which could be marking these ends for elongation (131).

Cdc13 binding to the telomeric 3′ overhang and its interactions with the Est proteins is necessary for the recruitment of telomerase to the telomere. The dynamics of Cdc13 import to the nucleus and retention at the telomere, post-transcriptional modifications and the formation of the CST complex has a function in regulating the association of telomerase to the G-rich telomeric DNA (115). Assembly and disassembly of telomerase also occurs in a cell cycle dependent manner to regulate telomerase activity to occur specifically at the time of replication (198). Matured TLC1 transcripts are transported into the nucleus by associating with Est2 and the Ku complex (199). Retention of nuclear TLC1 involves the binding of Ku and its association with Sir4 during the G1 phase (112, 199). Through a different region, the TLC1 transcripts recruit Est1, which is more abundant during the late S-phase and is needed for the association with Est3 (200). The assembled telomerase holoenzyme then needs to be recruited to the telomere by its interaction with Cdc13.

However, many factors such as SUMOylation of the Cdc13 protein and interactions with Rif2 promote the formation of the CST complex at the 3′ overhang limiting telomerase activity (117). Therefore, even after telomerase is assembled in the nucleus during the late S-phase its activity is highly regulated by the telomere structure. The length of the telomeric DNA is important for maintaining the functionality of the end structures and its further discussed in chapter 9.

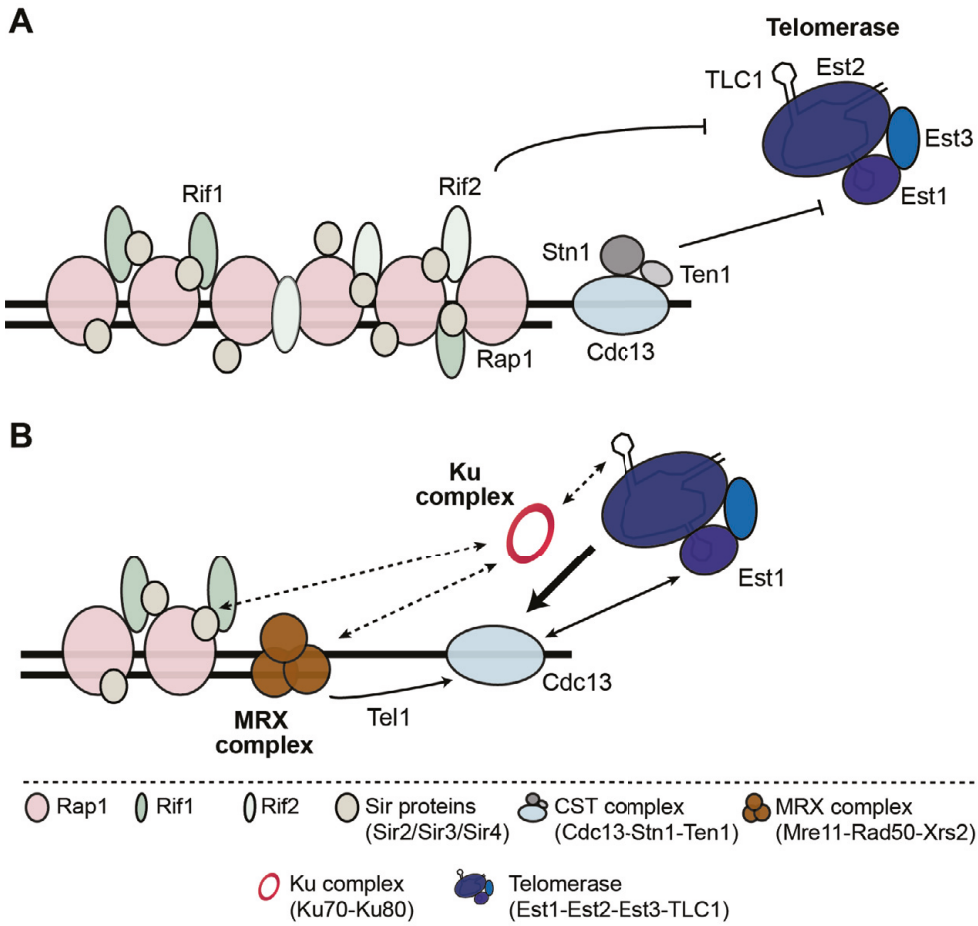

Figure 7. Elongation of telomeres by telomerase. A) Long telomeres prevent telomerase activity. The action of telomerase is prevented by the binding of Rap1, Rif1 and Rif2 proteins. Moreover, the association of Cdc13 with Stn1 and Ten1 prevents the interactions between Cdc13 and Est1. B) Short telomeres are preferentially elongated by telomerase. Shortening of the telomere limits the number of Rap1 proteins bound at this region. Short telomeres can be bound by MRX-Tel1, promoting ssDNA generation and the dissociation of the CST complex. Cdc13 can then promote the recruitment of telomerase to its substrate through its interactions with Est1. The Ku complex also facilitates the recruitment of telomerase to the telomere, however it is unclear if the heterodimer is recruited to the telomere by interactions with TLC1, Sir4, the MRX complex or directly with the telomeric DNA. Elongated 3′ overhangs are filled in with the complementary strand by the DNA replication machinery.

8 Alternative mechanisms for the lengthening of telomeres

The use of telomerase to solve the end-replication problem is the most common pathway followed in eukaryotes. However, some organisms have lost telomerase activity and replaced its function with already existing genetic mechanism such as retrotransposition, recombination and/or rolling circle replication (201). For example, the fruit fly *Droshophila melanogaster* utilizes a mechanism based on retrotransposons to replenish the ends of their chromosomes (202). Some yeast cells activate different telomere maintenance mechanisms that generally rely on recombination between subtelomeric elements or between telomeric sequences (67, 203), referred to as Alternative Lengthening of Telomeres (ALT). Human cancer cells reverse and prevent further replicative senescence mainly by upregulating telomerase expression or by activating recombination-based ALT mechanisms (3, 204, 205).

8.1 Telomere recombination

Recombination is normally repressed at the telomeres by the binding of the telomeric proteins. This is important because the telomere structure resembles a DSB and thus becomes target of the DDR machinery. The G-rich and tandemly repeated sequence facilitates recombination between telomeres and interstitial telomeric sequences (ITS) present distally from the telomeres (206). Homologous recombination at the chromosomal ends is also promoted by the clustering of the telomeres at the nuclear periphery (207). Moreover, TERRA binding at shortened telomeres can also promote the signaling of DNA damage and repair of the telomere by HDR (20, 208). Unregulated telomere recombination can be deleterious for the cell as it can lead to chromosomal instability by generating gross chromosomal rearrangements including translocations by HDR and end-to-end fusions by NHEJ. However, recombination is needed to prevent senescence triggered by short telomeres generated from replication stress and in the absence of telomerase, critically shortened telomeres are rescued by recombination to prevent further erosion (209).

8.2 Telomerase-independent telomere maintenance in yeast

Early after losing telomerase activity *S. cerevisiae* and *K. lactis* cells experience a severe growth crisis, from which only few survivor cells manage to avoid the lethal consequences of telomere shortening (66, 147, 190). *S. cerevisiae* survivors have been classified as Type I or Type II cells based on the ALT pathway employed for telomere maintenance in the absence of telomerase (66, 67). ALT pathways commonly utilize BIR to elongate the telomeres and therefore, depend on strand invasion mediated by Rad52. Rad52 has also been shown to prevent extensive resection at the telomere, possibly in association with the MRX complex (209, 210). However, few rare mutants have been found to survive with a Rad52-independent mechanism in yeast (211, 212). The ALT mechanisms of *S. cerevisiae* also depend on the DNA polymerase δ subunit, Pol32, necessary for BIR DNA synthesis (65, 66).

While BIR is employed as a telomere rescue and maintenance mechanism (Figure 8), it remains unclear what triggers the initiation of telomere recombination and if it occurs between a homologous DNA template from the sister chromatid, another chromosome or extrachromosomal circles containing telomere repeats (ECTR). The origin and function of ECTR (also known as t-circles) remains unknow, but they have been proposed to be a hallmark of ALT (213). ECTR is commonly found as partially double-stranded molecules containing G-rich, C-rich or subtelomeric sequences (214). They have been described in *S. cerevisiae* (207, 215), *K. lactis* and human ALT cancer cells. In *K. lactis*, telomerase-deficient mutants use a “roll and spread” mechanism where the sequence found on a t-circle is used as template for the elongation of a shortened telomere, subsequent elongation by BIR is then favored to elongate other telomeres (216, 217).

A growth crisis, however, is not a hallmark of ALT in yeast. A lack of a prominent senescence phenotype has been observed in *N. castellii* and *Candida albicans* telomerase-deficient mutants and, recently, it was shown that *S. cerevisiae* strains containing a Y′ element in every chromosomal end can also avoid the characteristic growth crisis without the appearance of senescence phenotypes, due to an increase in the efficiency of the Type I ALT pathway (218-220).

8.2.1 Telomere maintenance by *RAD51*-dependent BIR

The majority of BIR events in *S. cerevisiae* happen through a *RAD51*-dependent mechanism which involves the resection of the damaged end, DNA-Rad51 filament

formation and homology-based invasion with at least 200 bp of DNA template (64). Type I survivors extend the telomeres by frequently multiplying the Y′ subtelomeric elements utilizing a *RAD51*-dependent BIR mechanism, while keeping only short telomeric regions at the ends (48, 67, 221, 222). Besides requiring Rad52 and Rad51, the pathway also requires the Rad54, Rad55 and Rad57 proteins to facilitate the formation of the Rad51 filament and the homology search. It is unclear whether extensive resection of telomere into the subtelomeric region is necessary to expose the Y′ element sequence for homology, if the unprotected telomeric sequences from a critically short telomere mediate recombination between the ITS regions present between Y′ elements or if the copying mechanism initiates from a collapsed replication fork located in the subtelomere region (64). Given the higher efficiency of Rad51-dependent BIR, it has been recently proposed that Type I recombination is the first step in the formation of ALT survivors, providing an efficient rescue of short telomeres early after the loss of telomerase activity for both Type I and Type II survivors (223).

8.2.2 Telomere maintenance by *RAD51*-independent BIR

Type II survivors maintain long telomeres by utilizing *RAD51*-independent BIR. For this mechanism, strand annealing is promoted by the Rad52-Rad59 complex through short sequence homology between the telomeric repeats. Rad51-independent BIR additionally requires the MRX complex, the recombination factor Rdh54 and the helicase Sgs1 (67, 221, 224). Type II survivors appear with less frequency than Type I survivors, however, Type II cells grow faster and outgrow Type I cells in liquid media cultures. The telomeres of Type II cells have heterogeneous lengths, possibly because BIR template switching can promote different rearrangements during the DNA synthesis of the telomeres (64).

8.2.3 Chromosome circularization and abnormal telomeric sequences

As mentioned above, Rad52 is essential for the functioning of the ALT mechanisms in *S. cerevisiae*. Without telomerase activity and Rad52 the cells rapidly lose their viability as their telomeres shorten. The deletion of Exo1, involved in extensive resection of broken DNA ends, allows for few cells to proliferate with linear chromosomes that commonly lose the telomeric and subtelomeric sequences and instead replace them with large palindromes, originated from inverted repeats, at the ends of the chromosomes (225). These palindrome-survivors (PAL-survivors) depend on the gene function of *RIF1*. Moreover, PAL-survivors are prone to genomic instability, experiencing changes in their chromosomal arrangements in later generations.

In telomerase-deficient cells of the fission yeast *Schizosaccharomyces pombe*, cells experience a pronounced growth crisis that lead to three types of surviving cells

(226, 227). Survivors with linear chromosomes utilize recombination to maintain long and heterogeneous telomeres similar to *S. cerevisiae* Type II survivors. Survivors with circular chromosomes lacking telomere sequences are believed to be form by end-to-end fusions occurring at each of its 3 chromosomes. Circular chromosome survivors display morphological differences and significant growth defects in comparison to the WT-like morphology and growth of the survivors with linear chromosomes. *S. cerevisiae* genetically modified cells containing only a single linear chromosome also promote circularization in the absence of telomerase and when Cdc13 is hindered (228). Lastly, heterochromatin amplification-mediated and telomerase independent (HAATI) survivors maintain linear chromosomes by amplifying ribosomal DNA repeats or subtelomeric elements. Interestingly, the mechanism requires RNAi machinery components and is also dependent on Rhp51 (Rad51 ortholog) and Rad50 (227, 229).

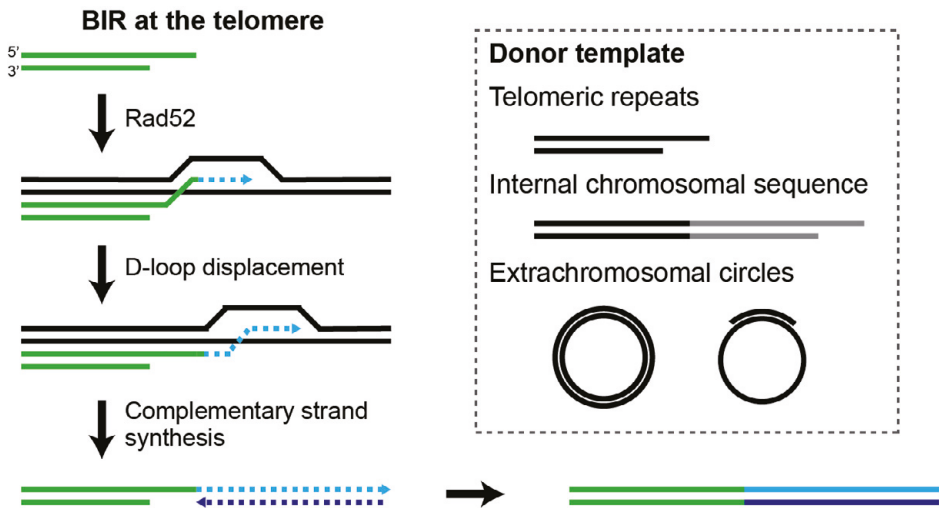

Figure 8. Schematic of telomere recombination by break-induced recombination. Telomere recombination by break-induced recombination (BIR) requires Rad52 to promote the invasion of the 3′ overhang at the region of homology within the donor template. The donor template can vary but it includes the telomere sequences of different chromosomal ends, internal sequences in the subtelomeric region of the chromosome (telomeric or non-telomeric) and extrachromosomal circles containing telomere repeats (ECTR). Invasion of the ssDNA creates a D-loop at the donor template. DNA synthesis at the invading strand proceeds along the donor template, displacing the D-loop, until the end of the sequence (if the template is linear) or until the strand dissociates from the donor template. Complementary strand synthesis generates duplex DNA.

9 Telomere dynamics

Telomeres are highly dynamic structures that constantly shift in levels of organization to maintain its functionality and provide the optimal protection to the chromosome (Figure 9). This concept becomes more intriguing as telomeres are different at each chromosomal end in the genome. Preservation of the primary DNA structure is a conserved feature among all eukaryotic organisms, the secondary structure formed by DNA-protein interactions at the telomeres guarantees the functional aspects of the end structures. Lastly, different protein-protein interactions provide an additional layer of protection and directs the interactions between the nuclear environment and the telomeres.

Telomere length regulation is carried out by the telomere binding proteins, the holoenzyme telomerase and the DNA replication machinery (230). Due to the end replication problem and telomere processing events, telomeres shorten progressively after every cell cycle. Moreover, recombination events that occur at the telomeres can also contribute to telomere attrition. Cells that cannot maintain uniform telomere length homeostasis eventually reach replicative senescence and cannot replicate further, leading the cell to apoptosis (66, 190). Replicative senescence is thought to be triggered by the accumulation of DNA damage signals elicited by few or several short telomeres. The attrition of the telomeres has been estimated to be around three to five nt per S-phase in *S. cerevisiae* (124). Without telomerase to replenish the loss of telomeric DNA, cells accumulate enough short telomeres to undergo replicative senescence after 60-80 generations of growth (66).

There is a shifting balance between the elongated and shortened states of telomeres in every chromosomal end of the cell. In yeast, only a few telomeres are elongated by telomerase during a single cell cycle (146). The addition of sequences varies depending on the length of the telomeric tract as telomerase can dissociate from the telomere after the addition of a single telomeric repeat in non-critically short telomeres. The dissociation of telomerase from the substrate has been suggested to be modulated by the Pif1 helicase, a protein also responsible from preventing the action of telomerase on DSBs (231). While all telomeres can be elongated by telomerase, short telomeres elicit a DNA damage signal that can only be mended by the action of telomerase. Indeed, it has been shown that telomeres do not shorten beyond a length of 100bp in wild type *S. cerevisiae* cells (146). Telomere recombination can further rescue short telomeres in telomerase-deficient strains or in ends that suffer from replication stress.

The dynamics of the length of the telomeric 3′ overhang play an important role in the maintenance of the telomeric structure (232). The action of telomerase is limited by the access to its substrate by the binding of the CST complex. At the same time, the association between Cdc13 and Est1 is necessary for elongation of telomeres by telomerase in the cell (119). Short overhangs do not allow for the binding of the Cdc13 and therefore interfere with its functioning. While long telomeres do not seem to affect the fitness of budding yeast, long telomeric 3′ overhangs generate instability in Cdc13 unbound telomeres (126).

Telomeric DNA binding proteins are essential for the capping of the telomeres. The dsDNA tract of the telomeres is bound by multiple Rap1 proteins, while the ssDNA is bound by Cdc13 with high affinity. The DNA-protein interactions of these two proteins provide protection to the end structures, particularly at the ds-ss junction. The dynamics between the length of the telomere are heavily related to the binding of the telomeric proteins. Telomeres with short 3′ overhangs sometimes cannot host a Cdc13 binding site sequence, and therefore are bound only by Rap1. On the other hand, short telomeres contain less Rap1 binding sites, are more prone to digestion and, therefore, generate longer 3′ overhangs that can be bound by Cdc13 and function as a loading platform for telomerase.

The formation of telomeric chromatin relies on the protein-protein interactions of the core telomeric proteins and their correct assembly at the telomeres (7). The interaction of the telomeric accessory proteins enhance the protection provided by the core proteins and are necessary for the formation of high-order structures. There is experimental evidence that supports that yeast telomeres have the ability to bend over the subtelomeric region in a process mediated by Rap1 and the Sir2, Sir3 and Sir4 proteins, forming a fold-back structure (106). The fold-back structure of the telomere is hypothesized to aid on the silencing of genes located in the vicinity of the telomeres by TPE. Telomere fold-back might also be required for regulating telomerase activity, the clustering of telomeres in the nuclear periphery and the late firing of replication origins near the telomere, although these two last processes might just be mediated by the Sir complex and Ku proteins independently of the fold back structure. Human telomeres have long (100-200bp) 3′ overhangs that invade the telomeric dsDNA to form a high-order structure known as the T-loop, first visualized by electron microscopy (233). Although T-loop formation is promoted by interactions with the telomeric proteins specifically at the ds-ss junction, they are established by DNA-DNA interactions (234). The plasticity of yeast telomeres to adopt different structural configurations does not seem to affect cellular viability and it has been proposed that fold-back structures might only occur under specific conditions. However, T-loops have been suggested to enhance the protection provided by the telomere by physically hiding away the exposed DNA end, thus preventing the activation of DNA damage signals (235).

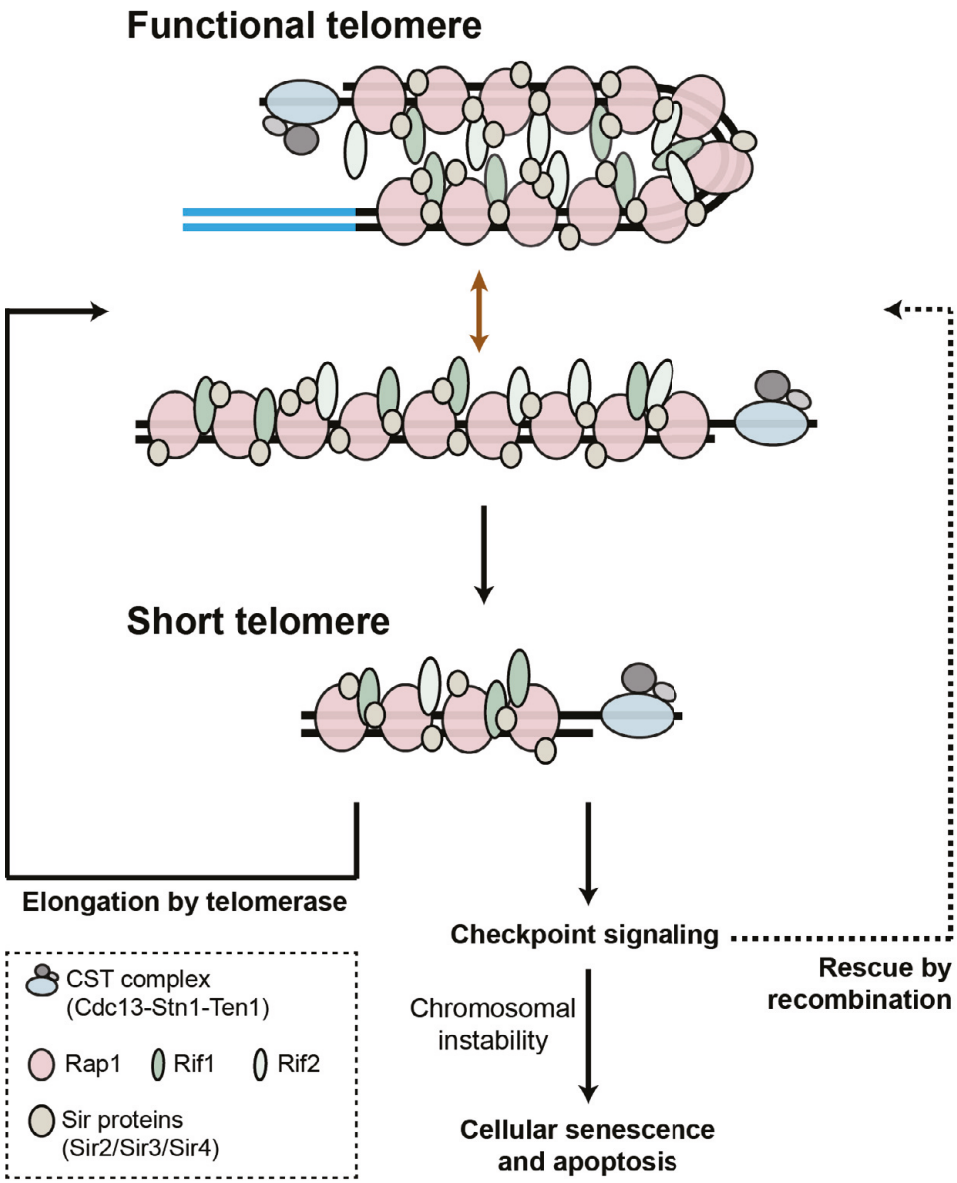

Figure 9. Telomere dynamics in yeast. A functional telomere is bound by the telomeric proteins and sometimes adopts a fold-back structure to provide protection to the genomic DNA. As telomeres progressively shorten, the protection of the telomere diminishes, and a DNA damage signal is elicited at the end. Short telomeres are normally elongated by telomerase and quickly bound by the telomeric proteins to reestablish a functional structure. Critically short telomeres with extensive ssDNA can be rescued by telomere recombination. However, telomere recombination and end-to-end fusions can generate chromosomal instability that could lead the cell to senescence and apoptosis if the damage is irreparable.

10 Telomeres in a human perspective

In somatic human cells, the presence and action of the telomerase enzyme is limited, therefore telomeres shorten throughout human lifespan. The degree of telomere shortening correlates to an increased risk of development of age-related diseases (17). Telomere maintenance is determined by genetic and non-genetic factors. Defects of the mechanisms needed for maintenance of telomeres have physiological and pathological effects on the cells. In general, the syndromes associated to telomere shortening are characterized by the manifestation of pulmonary fibrosis, aplastic anemia and failure of the bone marrow (6, 16). It has been suggested that telomere dysfunction is a direct consequence of telomere shortening, since attrition prevents the formation of a functional protective complex at the telomere. Uncapped chromosomes lead to the activation of mechanisms mediated by p53 to arrest the cell cycle, leading the cell to senescence and apoptosis. Therefore, there is a progressive structural and functional weathering of high turnover tissues such as stem cells, hematopoietic cells and heart tissue cells (236).

The main syndromes associated with telomere defects are characterized by mutations in fundamental genes involved in the maintenance of these structures (17). Individuals who suffer from hereditary telomeric diseases are more likely to develop symptoms at early ages, as they inherit short telomeres from one or both parents (17). Mutations in TERT and TERC are some of the monogenic defects more commonly identified for the different telomere syndromes (237). While these diseases are rare and the symptoms diverse, they generally manifest following the shortening of the telomeres (238).

When telomeres shorten to critical levels a cellular response marks the exit of the cell cycle, leading to cellular senescence. This natural process indicates that the cell has reached its maximum proliferation capacity. Some cells accumulate mutations, abnormalities or chromosomal aberrations that allow them to overwrite the cellular checkpoints. If these cells also develop mechanisms to restore telomere length they become immortal, and are considered the precursors of oncogenic cells (17). Indeed, the expression of telomerase is essential for the proliferation of 85-90% of all human cancers (239, 240). 10-15% of cancers restore the telomere by recombination-based ALT mechanisms (204). Moreover, the shortening of telomeres is not the only pathogenic factor. The appearance of some types of cancer have been linked to a genetic predisposition to have longer telomeres (241). A cell with long telomeres will be able to sustain an increased number of replication cycles, thus a higher risk

of accumulating oncogenic mutations. The understanding of the mechanisms behind telomere length homeostasis, telomere maintenance and the role of the different telomeric proteins is, therefore, essential for the understanding of cellular aging and the associated pathologies. Furthermore, knowledge about telomerase and the different ALT mechanisms is necessary for the development of treatments against cancer.

11 The model organism: *Naumovozyma castellii*

Different species of non-conventional yeast recently become popular research organisms as they possess characteristics that are beneficial for the study of different research topics or have traits beneficial for specific biotechnological applications. The budding yeast *N. castellii* (syn. *Naumovia castellii, Saccharomyces castellii*) is a member of the Saccharomycetaceae family. *N. castellii* is used as a model organism for telomere studies due to its prominent telomerase activity and human-like telomere structure (193). Its speciation from *S. cerevisiae* began after a whole genome duplication event. Unlike *S. cerevisiae*, *N. castellii* lost a great number of duplicated genes and different chromosomal rearrangements led to the reduction of its haploid number of chromosomes to 10 (16 chromosomes in *S. cerevisiae)* (242, 243). Despite their evolutionary distance, many of the tools and molecular methods utilized and developed for *S. cerevisiae* can be used or modified for its use in *N. castellii* (244, 245). The whole genome sequencing of this species has also facilitated the application of different genetic approaches and molecular techniques. For example, the application of different microscopy approaches allowed us to image cells grown under different conditions and characterize them (Figure 10).

Yeast species have evolved very diverse telomeric sequences. In *N. castellii* the octameric regular telomeric repeat 5′-TCTGGGTG-3′ shares structural resemblance to the mammalian telomeric sequence. The telomeric DNA structure has been advantageous for the studies and characterization of the binding of the protection proteins Rap1 and Cdc13 (71, 90, 246, 247). As in human cells, the 5′ terminal nucleotide is regulated in *N. castellii* suggesting that a specific ds-ss junction structure may be necessary for the establishment of the functional protective telomere chromatin cap structure (74). Interestingly, in an alternative binding mode *N. castellii* Rap1 can bind the telomere across the ds-ss junction (90). In this binding mode Rap1 recognizes the same dsDNA sequence as in the canonical binding mode, except part of the dsDNA is replaced with the ssDNA of the 3′ overhang. This characteristic has allowed us to demonstrate novel roles of Rap1 in the protection of the end structures against degradation and in the maintenance of telomeres (74, 141, 142). Furthermore, telomerase-deficient *N. castellii* cells efficiently maintain their telomeres through a novel ALT mechanism which rapidly activates as the

telomeres of the yeast shorten, allowing for sustained long-term growth of the cells (220).

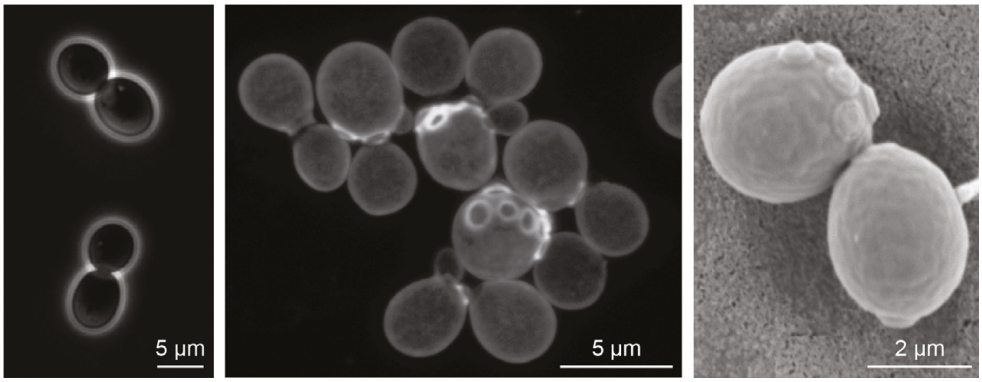

Figure 10. Microscopy of the budding yeast *Naumovozyma castellii*. A) Phase contrast microscopy image of G2/M arrested cells. The cells were arrested in the G2/M phase by threatment with the spindle poison Nocodazole. Because the cell cycle progression is stopped late, the daughter cell has a similar size as the mother cell and the unbudded cells show a characteristic dumbell shape. B) Confocal laser scanning microscopy (CLSM) image stacked by maximum intensity after alignment and deconvolution. The cells were stained with calcoflour white before imaging in CLSM using a DAPI filter. Budding scars are visible on some of the mother cells. C) Scanning electron microscopy (SEM) image. The cells were place on the surface of 3% agarose.

Summary of papers

Paper I

Either Rap1 or Cdc13 can protect telomeric single-stranded 3′ overhangs from degradation *in vitro*. We aimed to study how the binding of the telomeric proteins at the ds-ss junction provide protection to the 3′ overhang against exonucleolytic degradation. The regular telomeric repeats of *N. castellii* allowed us to create oligonucleotides that mimic the telomere, with specific lengths of ds and ssDNA. The telomere mimic also contained specific locations for the binding of Cdc13 and Rap1, based on their minimal binding site (MBS). With our 3′ DNA-end protection assay we provided experimental evidence that shows that the binding of Cdc13 to the 3′ overhang provides protection against 3′ -> 5′ exonucleases in yeast. Interestingly, we found that Rap1 is also able to provide protection to the 3′ overhang, a role that has not been described previously for the protein. When Rap1 binds adjacent to the ds-ss junction a short 3′ overhang is protected, most likely by steric hindrance of the protein. In an alternative binding mode, where Rap1 partially bind across the ds-ss junction, a longer 3′ overhang is protected from degradation. This protection extends further from the Rap1 MBS, suggesting that this binding mode either promotes a different configuration of the protein that provides increased steric hindrance or that, in this binding configuration, the protein is able to interact with the distal part of the ssDNA. While we only evaluated the protection provided by Cdc13 when bound adjacent to the ds-ss junction, it is likely that the protein will be able to protect the 3′ overhang when bound closer to the terminal 3′ nucleotide of a longer overhang. However, not all 3′ overhangs can host a Cdc13 binding site and it is unclear if the protein binds the telomeric DNA to the same extent outside of the S-phase. Therefore, the protection provided by Rap1 is extremely relevant in situations where Cdc13 cannot bind the telomere. When binding to its canonical MBS, the protection of a short 3′ overhang could favor the processing of the ssDNA to generate a longer overhang, as in the 5′ to 3′ resection process shown in *S. cerevisiae.* The human Rap1 protein favors the binding to ds-ss junctions. In yeast the protection provided to the 3′ overhang by the alternative binding mode of Rap1 could provide a substrate to the telomerase holoenzyme to elongate the telomere. Furthermore, our methodology provides mechanistical insights on the protection provided by binding of the core telomeric proteins and opens for future protection studies regarding the different telomere associated proteins.

Paper II

The telomeric 5′ end nucleotide is regulated in the budding yeast *Naumovozyma castellii*. To better understand how the core telomeric proteins assemble at the ds-ss junction, we aimed to determine the 5′ end terminal nucleotide of the telomeres in the budding yeast *N. castellii*. To this end, we developed a PCR based methodology termed the permutation-specific telomere PCR (PST-PCR) that allowed us to determine the terminal 5′ nucleotide of the majority of the chromosomal ends. While the terminal permutation has been determined in ciliates and human cells, this is the first time it has been described in yeast. We found that the telomeres commonly end with one of two specific 5′ end permutations, both corresponding to a terminal adenine nucleotide. These results suggest that there is a mechanism that regulates the formation and maintenance of a specific ds-ss junction during logarithmic growth. The absence of some terminal permutations indicated that some ds-ss junctions do not allow for the establishment of a functional telomeric chromatin. We found no change in the distribution of the terminal nucleotides in telomerase-deficient strains, indicating that the maintenance of the permutation is independent of telomerase activity. Using *in vitro* DNA end protection assays as in Paper I, we determined that the binding of Rap1 and Cdc13 around the most abundant ds–ss junction ensures the protection of both 5′ ends and 3′ overhangs from exonucleolytic degradation. This was important because Cdc13 and Rap1 can compete for binding at certain ds-ss junctions, possibly hindering the protection provided by each of the proteins (90). We determined that for the most abundant ds-ss junctions of *N. castellii*, the core telomeric proteins can bind in an optimal arrangement where the proteins avoid competition. Interestingly, we observed that one type of ds-ss junction allows for Rap1 to bind across the ds-ss junction in its alternative binding mode. For this binding mode, we observed an extended protection of the 3′ overhang and we also show that the binding of Rap1 does not interfere with the binding of Cdc13, suggesting that Rap1 could be protecting a short overhang that will, during telomere replication, be bound by Cdc13 and possibly extended by telomerase. Furthermore, with our methodology we were able to determine the length of the telomeres in *N. castellii*, a fundamental aspect of the telomere structure. Based on these results and the results from our *in vitro* protection assays (141, 142), we presented a model of the telomere DNA telomere structure and how the telomeric proteins bind to the preferred ds-ss junction and provide protection against exonucleases. In our model we highlighted how Cdc13 and Rap1 can work together to maintain the structure of the telomeric DNA at the ds-ss junction, however, more data is necessary to understand if the binding of the telomeric proteins is responsible for establishment of the 5′ end terminal nucleotide.

Paper III

Characterization of the *RAD52* gene in the budding yeast *Naumovozyma castellii*. DNA damage compromise the integrity of the genome; thus, DNA repair mechanisms are essential to prevent genome instability. Recombination is also essential for rescuing the maintenance of telomeres in telomerase-deficient strains. In this study, we aimed to characterize *RAD52,* the main recombination gene in yeast, in our model organism. To this end, we identified the *RAD52* gene and compared its protein sequence to those of other organisms, including several members of the Saccharomycetaceae family. We found the sequence of *N. castellii* Rad52 to be highly conserved, specifically the primary amino acid sequence of *N. castellii* Rad52 shares 70% similarity (61% identity) with *S. cerevisiae* Rad52. *S. cerevisiae* Rad52 has been extensively studied, and we found that essential residues at the three functional domains described for *S. cerevisiae* Rad52 remain highly conserved. To characterize the gene function, we developed *rad52Δ* mutant strains by targeted gene replacement transformation. Although we did not observe any differences in colony morphology, we found that *rad52Δ* mutants have slightly bigger cell size and slower growth rate, particularly when grown at higher temperatures. We then tested whether growth was affected by the presence of DNA damaging agents. Specifically, we tested Ultraviolet (UV) radiation as a physical agent capable of inducing DSBs by creating pyrimidine dimers in the DNA that later generate replication stress; Hydroxyurea as a compound that can indirectly cause DSBs by generating replication stress and the radiomimetic compound Bleomycin that directly causes DSBs. We found that *N. castellii rad52Δ* mutants are mildly sensitive to UV irradiation but highly sensitive to Hydroxyurea and the radiomimetic compound Bleomycin. The lower tolerance against DNA damaging agents showed that the *RAD52* gene function in DNA repair is conserved. The construction and characterization of recombination mutant strains in different organisms will be useful for research in topics pertaining recombination, DNA damage repair as well as telomere maintenance.

Paper IV

Alternative Lengthening of Telomeres is dependent on *RAD52* and *RAD51* gene function in the budding yeast *Naumovozyma castellii*. The genetic requirements of the ALT mechanism in *N. castellii* was studied. Specifically, we aimed to determine if the establishment of the ALT mechanism requires homologous recombination. We first developed a new diploid strain line of our model organism containing a deletion of the *HO* and *URA3* genes, the latter which we deleted by targeted gene replacement transformation followed by removal of the marker gene. In this strain background, we knocked out the *EST2* and *TLC1* genes coding for the catalytic core of the telomerase holoenzyme. *S. cerevisiae* cells lacking any of these two components develop a severe growth crisis, which only few cells manage to survive by activating an ALT pathway. *N. castellii* is capable of rapidly activating an ALT mechanism that prevents the appearance of the onset of senescence phenotypes. Because *est2Δ* mutants of *N. castellii* had never been characterized, we sporulated an *EST2* heterozygote diploid mutant and studied the growth capabilities of the *est2Δ* haploids, as well as their telomere structure. We observed that *est2Δ* mutants do not exhibit a severe growth crisis and rapidly activate an ALT mechanism that effectively maintains short length telomeres. We made similar observations from *tlc1*$^{-}$ haploid strains derived from the same strain line. To study the genetic requirements for different recombination genes in the establishment of the ALT mechanism, we developed a senescence growth analysis that allowed us to study multiple clonal populations in a quantitative manner. In this assay, we sporulate double-mutant heterozygote strains, germinate the tetrad spores after microdissection and passage colonies of each haplotype obtained subsequently, culminating at the third passage of the cells. We deleted a single copy of the *RAD52* in the telomerase deficient (*EST2* and *TLC1* mutants) heterozygote diploid strains. With the senescence growth assay, we quantitatively demonstrated that telomere-deficient strains do not exhibit an early growth crisis and most importantly we found that the establishment of the ALT mechanism requires *RAD52*, as mutant haploids lacking telomerase activity and *RAD52* are unable to sustain growth. We performed the same steps to knockout and study mutants of the *RAD51, RAD59* and *RAD50* genes respectively, and found that the *RAD51* gene function is necessary for the establishment of the ALT mechanism in *N. castellii*. We found a strong senescence phenotype in the double mutant strains lacking telomerase activity and either *RAD52* or *RAD51*, characterized for an inability to grow beyond 60 generations in liquid or solid media. Although the ALT-deficient cells senesce rapidly, analysis of the telomere structure of theses cell did not reveal accelerated telomere shortening. Our results demonstrate that the *N. castellii* ALT mechanism maintains the telomeres by homologous recombination mediated by Rad52 and Rad51.

Concluding remarks and future perspectives

The work presented in Paper I and II is centered around the structural basis of telomere protection in the budding yeast *N. castellii*. Through knowledge of the fundamental aspects of the telomeric DNA structure, the length of the telomeric tract and the arrangement of the ds-ss junction, we were able to generate a model based on our experimental evidence that explains how the binding of the core telomeric proteins provides protection to the ends of the DNA. Our observations can be further expanded by studying the role of the telomere associated proteins, which have been also described to play roles in telomere protection.

Our results provide insights for better understanding of different physiological processes such as the replication of telomeres, the elongation of telomeres by telomerase and the processing of the DNA ends by different exonucleases. In future work, it would be interesting to gain a better understanding of the dynamics of the telomere structure in *N. castellii* by studying the changes that take place during the transition of the cells through the cell cycle. While we have already established that *N. castellii* accumulates 70nt long telomeric 3′ overhangs during the S-phase of the cell cycle, in a telomerase-dependent process (76), there is much to uncover about the timing of action of the C-strand fill in mechanism by the replication machinery. With the implementation of the methodologies detailed in this work, we can further evaluate the structure of the ds-ss junction before such processing events. Synchronizing cells at the S and G2/M phases and performing the PST-PCR method, can allow us to study the structure of the telomeres before and after the complete replication of the telomeres and gain insights of the processes governing the establishment of the terminal permutation. Similarly, this approach could be further performed in mutant strains to evaluate the dependency of different proteins for the regulation of the DNA structure of the ds-ss junction.

We have been working extensively in developing tools and strains to promote the use of the non-conventional yeast *N. castellii* as a model organism for telomere biology research. The development and characterization of the mutant strains presented in Paper III will provide useful tools also in topics pertaining recombination, DNA damage and DNA repair. From the research presented in Paper IV we have obtained multiple deletion haploid mutants of different members of the *RAD52* epistasis group of genes that may also be further utilized to study

recombinational events that could lead to the discovery of different molecular pathways or further the understanding of existing ones. Therefore, the library of strains created and the development of a technique to efficiently replace genes of interest in *N. castellii* presented in our studies promotes the use of non-conventional yeast in molecular biology.

The work presented in paper IV showed that *N. castellii* telomerase-deficient strains bypass cellular senescence by activating a Rad52 and Rad51 dependent ALT mechanism that quickly reestablishes telomere maintenance. This ALT mechanism is similar to the Type I survivor ALT pathway of *S. cerevisiae*, however, the most important distinction is the high efficiency of the mechanism and the copying of a short subtelomeric element (TelKO). It is remarkable how the *N. castellii* ALT mechanism maintains short telomeres containing a single TelKO element, as it implies that there is an underlying mechanism that regulates the addition of TelKO or that the chromosomal ends are processed to contain mainly a single subtelomeric repeat. *S. cerevisiae* Y′ elements can be present in tandem copies just before the telomere, but perhaps the TelKO element cannot be maintained as tandems because its genetic structure easily promotes recombination between chromosomes. It would be interesting to further investigate the structural DNA requirements underlying the addition of the TelKO element by obtaining sequencing data of the subtelomeric region of the chromosomes, searching for recombination byproducts such as extrachromosomal circles and by introducing small changes in different regions of the TelKO sequence and a tag to track if a spreading of the element occurs.

Further research applying the techniques used to study the structure of the telomeres in our telomerase-deficient strains are needed to understand the mechanisms underlying the efficiency of ALT. For example, the presence of long 3′ overhangs in *N. castellii* and the evidence for Rap1 binding across the ds-ss junction suggest the possibility that high-order structures, such as the T-loops seen in mammalian cells, could develop transiently in our model organism. Hypothetically, this could be favoring recombination events that would be implicated in the rescue of short telomeres in the absence of telomerase and are worth investigating further.

There are many genes which can be tested to challenge the hypothesis that *RAD51*-dependent BIR mechanism is responsible for the copying of TelKO element, for example *POL32, RAD55* and *RAD57*. Furthermore, during my studies we have started investigating the structural changes of the 3′ overhangs in ALT strains, employing the duplex-specific nuclease reaction to measure the length of the overhangs in different conditions, together with the application of the PST-PCR. Single-stranded DNA is necessary for strand-invasion during recombination and furthering this research would provide insights in the mechanisms underlying the formation and dynamics of the 3′ overhangs in the absence of telomerase, as well as their role in the ALT mechanism.

To study how the telomerase-dependent and telomerase-independent mechanism interact and compete for telomere maintenance, reactivating telomerase in our telomerase-deficient strain could prove to be a valuable experiment. Evaluation of the morphology and telomere structure after the reintroduction of telomerase could answer interesting questions about the functioning of these two highly efficient pathways and coupling these studies with next generation long-read sequences techniques would provide useful insights regarding the structure of the subtelomeric regions before and after the reactivation of telomerase in ALT cells.

Until now, I have proposed immediate continuations of my work that I believe would further progress our understanding of telomere maintenance. One particularly challenging aspect of telomere research is that telomeres are intrinsically heterogenous at the cell level, which generates a great deal of variance when studying cell populations. For example, we have not observed a senescence phenotype in our cultures of telomerase-deficient cells but that does not discard the possibility that individual cells are experiencing replicative senescence. Thus, we do not know how our generalized observations affect the single cells. A way to study single-telomere dynamics would be the application of the PST-PCR method with primers targeted towards individual telomere ends, which could reveal novel insights regarding the dynamics of the 5' end structure and the length of individual telomere ends during the cell cycle. Moreover, while the mechanistic aspects of ALT mechanisms remain elusive, the propensity of *N. castellii* cells to switch to ALT mechanism could be further investigated with single-cell analysis techniques that currently are implemented in *S. cerevisiae*, to study the efficiency of the ALT mechanism at the cellular level and to reveal the lifespan of individual ALT cells.

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