Npl3 stabilizes R-loops at telomeres to regulate replicative senescence

Dissertation

zur Erlangung des Grades

„Doktor der Naturwissenschaften“

am Fachbereich Biologie

der Johannes Gutenberg-Universität Mainz

Lara Pérez Martínez

geb. am. 22.08.1992 in Madrid, Spain

Mainz, 2019

Table of Contents

Summary

Telomeres are the end structures of eukaryotic chromosomes, which are subject to the end-replication problem and undergo progressive shortening unless elongated by the reverse-transcriptase telomerase. In the absence of telomerase, short telomeres can stop cell cycle progression in a process called replicative senescence. Telomeres are transcribed into the long non-coding RNA TERRA which is able to hybridize with the telomere, forming a homologous recombination-prone structure called an R-loop. In yeast, telomeric R-loop levels increase during senescence and delay senescence rate, supporting a role of TERRA R-loops in replicative senescence.

In this study, we performed quantitative interactomics to identify proteins binding to telomeres in *S. cerevisiae*. Using a DNA pull-down strategy and protein extracts from telomerase positive and telomerase negative cells, we identified proteins that associate to telomeres. We identified a set of telomere associated proteins that showed enrichment in RNA regulatory functions and helicase functions. This suggests that RNA binding proteins and helicases may be important for telomere integrity and the regulation of senescence in yeast.

Among our candidates, we identified the yeast hnRNP-like protein Npl3 and further characterize its function at short telomeres. We first validated its *in vivo* binding to telomeres and showed that Npl3 displays a strong association to short telomeres. Importantly, deletion of *NPL3* has been reported to cause a fast senescence phenotype. We show that TERRA mediates the Npl3 recruitment to telomeres, as changes in TERRA and TERRA R-loop levels modulate the binding of Npl3 to telomeres. This suggests that the accumulation of TERRA and R-loops at short telomeres may recruit Npl3. Using a combination of genetic and biochemical approaches we also show that *NPL3* can stabilize R-loops when overexpressed, suggesting that local accumulation of Npl3 can stabilize pre-formed R-loops. Further, we demonstrate that Npl3 stabilizes R-loops at telomeres. Altogether, our data supports a model in which TERRA recruits Npl3 to short telomere to stabilize telomeric R-loops and prevent premature senescence in yeast.

Zusammenfassung

Die Telomere sind die Endstrukturen der eukaryotischen Chromosomenenden. Telomere unterliegen dem Endreplikationsproblem und erfahren eine progressive Verkürzung, falls sie nicht durch das reverse Transkriptase Enzym Telomerase verlängert werden. Wenn Telomerase fehlt, dann können kurze Telomere zum Anhalten des Zellzyklus führen. Dieser Prozess heißt replikative Seneszenz. An Telomeren entsteht durch Transkription die lange, nicht-kodierende RNA TERRA. TERRA kann mit dem Telomer hybridisieren, wodurch ein sogenannter „R-Loop“ entsteht. R-Loops sind DNA:RNA-hybride Strukturen, welche die homologe Rekombination begünstigen. In der Bäckerhefe steigen die telomerischen R-Loop Spiegel während der Seneszenz an und verzögern das die Seneszenzrate. Dieser Zusammenhang spricht für eine Rolle von TERRA-R-Loops während der replikativen Seneszenz.

In dieser Studie haben wir quantitative Interaktomik angewandt, um Telomer-bindende Proteine in S. cerevisiae zu identifizieren. Wir nutzen eine DNA Pulldown Strategie und Telomerase-positive sowie Telomerase-freie Proteinextrakte und identifizierten eine Reihe von Telomer-assoziierten Proteinen. Einigen dieser Proteine werden RNA regulierende Funktionen und Helikase-Aktivität zugeschrieben. Dies lässt vermuten, dass RNA-binde Proteine und Helikasen wichtige Faktoren für die Integrität der Telomere sein könnten und bei der Regulierung von Seneszenz in Bäckerhefe mitwirken.

Einer dieser Faktoren ist das hnRNP-like Protein Npl3, dessen Funktion an kurzen Telomeren wir weitergehend charakterisiert haben. Zunächst bestätigten wir die Bindung von Npl3 an Telomere in vivo und zeigten, dass Npl3 verstärkt mit kurzen Telomeren assoziiert. Zudem wurde beschrieben, dass die Deletion des NPL3 Gens einen schnellen Seneszenz Phänotyp verursacht. Wir zeigen, dass TERRA das Npl3 Protein an die Telomere rekrutiert, da Änderungen der TERRA-RNA und TERRA-R-Loop Spiegel den Grad der Bindung von Npl3 an die Telomere moduliert. Dies lässt annehmen, dass die Akkumulation von TERRA und R-Loops an kurzen Telomeren der Rekrutierung von Npl3 zu Grunde liegt. Unter Verwendung von genetischen und biochemischen Methoden zeigen wir des Weiteren, dass die Überexpression von Npl3 zu einer Stabilisierung von R-Loops führt. Wir vermuten deshalb, dass eine lokale Anreicherung von Npl3 bereits vorhandene R-Loops stabilisieren kann. Außerdem demonstrieren wir, dass Npl3 R-Loops an Telomeren stabilisiert. Zusammenfassend unterstützen unsere Daten ein Model in welchem TERRA Npl3 an kurze Telomere rekrutiert, um die telomerischen R-Loops zu stabilisieren und die vorzeitige Seneszenz in der Bäckerhefe zu verhindern.

Abbreviations

a: anti

AGS: Aicardi-Goutieres syndrome

AID: auxin inducible degron

CSR: Ig class switch recombination

ALT: alternative lengthening of telomere

AOA2: Ataxia-ocular apraxia 2

APB: ALT-associated promyelocytic leukemia body

ARIA: telomeric RNA in fission yeast made of C-rich repeats

ARRET: antiparallel telomeric RNA species

ARS: autonomously replicating sequences

ASF/ASF2: SR protein splicing factor ASF/SF2

ATM: Ataxia telangiectasia mutated

ATR: ATM and RAD3-related

ATRX: Transcriptional regulator ATRX

ATRX: Transcriptional regulator ATRX

BIR: break induced replication
RBP: RNA binding protein

BLM: Bloom syndrome protein

ChIP: Chromatin immunoprecipitation

ChRIP: Chromatin-associated RNA immunoprecipitation

CSB: Cockayne syndrome group B

CST: Cdc1, Stn1, Ten1 complex

CTCF: Transcriptional repressor CTCF

DAXX: Death domain-associated protein

DDK: Dbf4-dependent kinase

DDX1: ATP-dependent RNA helicase DDX1

DEAD-box: protein motif containing asp-glu-ala-asp (DEAD) sequence

DNMT1/3b: DNA (cytosine-5)-methyltransferase 1/3b

DNMTs: DNA methyltransferases

ds: double strand

DSB: double strand break

dsDNA: double strand DNA

dU: deoxy-Uracil

ECTR: extra-chromosomal telomeric repeats

eV: empty vector

FACS: Fluorescence-activated cell sorting

FANCM: Fanconi anemia group M protein

GADD45: Growth arrest and DNA damage-inducible protein GADD45A

HATTI: heterochromatin amplification dependent and telomerase independent survivors

HBD: hybrid binding domain

HDR: homology directed repair

hnRNP: heterogeneous nuclear ribonucleoproteins

HP1: heterochromatin protein 1

HR: homologous recombination

hTERT: Telomerase reverse transcriptase

hTR: telomerase RNA component

HU: hydroxyurea

IAA: indol-3-acetic acid

LSD1: Lysine-specific demethylase 1

MiDAS: mitotic DNA synthesis

MRN: Mre11, Rad50, Nbs1

MRX: Mre11, Rad50, Xrs2 complex

MS: mass spectrometry

ncRNA: non-coding RNA

NHEJ: non homologous end joining

oE: overexpression

ORC: Origin recognition complex

PCNA: Proliferating cell nuclear antigen

PIF1: ATP-dependent DNA helicase PIF1

PIP: PCNA_interacting protein motif

POT1: protection of telomeres 1

PP1: PP1 phosphatase

pRB: p16/retinoblastoma

PRC2: Polycomb repressive complex 2

PCNA: Proliferating cell nuclear antigen

RACE: rapid amplification of cDNA ends

RER: ribonucleotide excision repair

RIP: RNA immunoprecipitation

rNMPs: nucleoside monophosphate

RNP: ribonucleoprotein

rNTPs: nucleoside triphosphate

ROS: reactive oxygen species

RPA: replication protein A

RRM: RNA recognition motif

RTEL1: Regulator of telomere elongation helicase 1

SDFs: senescence-associated DNA damage

SETDB1: Histone-lysine N-methyltransferase

SETX: gene encoding Senataxin protein

SLX4: Structure-specific endonuclease subunit

SMARCAL1: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1

ss: single strand

ssDNA: single strand DNA

SUV39H1: Histone-lysine N-methyltransferase

TA-HRR: transcription-associated homologous recombination repair

TAP: tandem affinity purification

T-circles: telomeric circles

TC-NER: transcription-coupled nucleotide excision repair

TERRA: TElomeric Repeat containing RNAs

TET1: ten-eleven translocation 1

THO: Hpr1p, Tho2p, Thp1p, and Mft1p complex

TIN2: TRF1-interacting nuclear protein 2

TPE: telomere position effect

TPE-OLD: telomere position effect over long distances

TPP1: TINT1, PTOP, PIP1

TRF1: telomeric repeat binding factor 1

TRF2: telomeric repeat binding factor 2

T-SCE: telomeric sister chromatic exchanges

Unsynch: unsynchronized culture.

Introduction

The ends of linear chromosomes have attracted the attention of the scientific community and beyond for nearly a century. Early studies in the Muller and McClintock laboratories showed that chromosome ends, thereafter named telomeres, are essential to preserve chromosome integrity (Muller, 1927; McClintock, 1941). Later in the 1970', telomeres were put in the spotlight again, as they are subject to what was then defined as the end replication problem (Watson, 1972; Joachim Lingner, Cooper and Cech, 1995). According to the DNA replication model, chromosome length could not be maintained as cells divide, resulting in chromosome shortening and limited cellular lifespan (Olovnikov, 1973). Chromosome length maintenance remained a mystery until the discovery of telomerase (Greider and Blackburn, 1985, 1987, 1989), an enzyme capable of counteracting telomere loss. This discovery opened up a new field of study that has expanded ever since. For thirty years now, telomeres have proven to be fascinating structures with important implications in human health and disease.

In this thesis, we studied telomere biology using *S. cerevisiae* as a model organism. In the following section, a short summary of telomere biology will be provided. To facilitate discussion of the experimental approach, the most relevant aspects of telomere biology will be highlighted.

Telomere structure and associated proteins

Telomeres are the terminal structures of eukaryotic chromosomes that protect chromosome ends. To ensure this protection, functional telomeres consist of repetitive DNA sequences and associated proteins. In particular, telomeres in *S. cerevisiae* consist of double strand DNA repeats of $C_{1\text{-}3}A/TG_{1\text{-}3}$ that extend for 300 +/- 75bp at the end of the chromosomes (Wellinger and Zakian, 2012). The TG rich sequence displays a single-stranded 3' overhang of 12-15 nts long that expands to 30-100 nts during the late S/G2 phase of the cell cycle as a consequence of telomere replication-related processing (Wellinger, Wolf and Zakian, 1993; Dionne and Wellinger, 1998; Larrivée, LeBel and Wellinger, 2004; Frank, Hyde and Greider, 2006). Telomere proximal regions – called subtelomeres – are centromere-proximal sequences adjacent to the telomeric repeats that promote their integrity and function. In budding yeast, subtelomeres can contain two different types of sequences defined as the X and the Y' elements. The Y' element is located in a subset of telomeres immediately adjacent to the telomeric repeats. It consists of 0-4 copies of tandem sequences, ranging from 5.2 to 6.7 kb in length (Chan and Tye, 1983; Horowitz, Thorburn and Haber, 1984; Wellinger and Zakian, 2012). The centromere-proximal element, referred to as the X' element, is present in all the yeast telomeres and has various lengths longer than 500 bp (Wellinger and Zakian, 2012) (Figure 1).

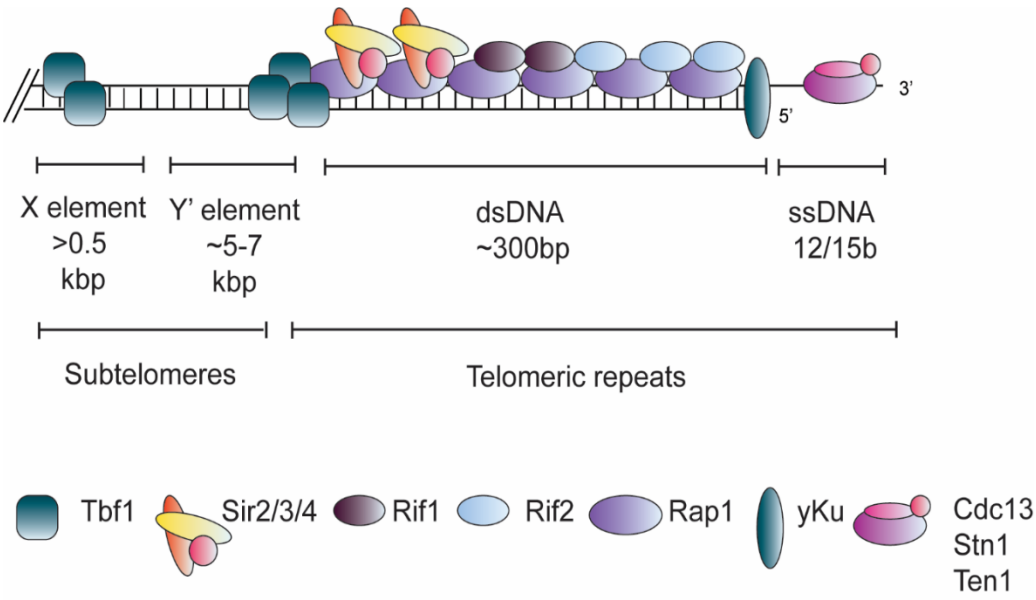


Figure 1 *S. cerevisiae telomeres*.

DNA structure and associated proteins are indicated. DNA elements and length is represented. Proteins are positioned in the telomere representation and symbol legends are located at the bottom of the image. Reviewed by Wellinger and Zakian, 2012. Abbreviations: dsDNA: double strand DNA. ssDNA: single strand DNA.

Mammalian telomeres consist of tandem repeats of TTAGGG sequences with a 3' single strand overhang. As opposed to yeast telomeres, mammalian repeats expand for several kilobases of DNA sequence, whereas the telomeric 3' overhang is less than 500 bp (Palm and de Lange, 2008; de Lange, 2018). Subtelomeric regions in mammalian cells contain degenerate TTAGGG repeats, which may contribute to chromosome end protection (Palm and de Lange, 2008). Interestingly, mammalian telomeres form a structure called t-loop, where the 3' overhang invades the double strand telomeric repeats to provide additional protection to chromosome ends (Griffith *et al.*, 1999; Palm and de Lange,

2008; Doksani *et al.*, 2013). In yeast, the existence of telomeric fold back structures have also been suggested (Poschke *et al.*, 2012).

Functional telomeres require the association of specific proteins. In budding yeast in particular, the double strand telomeric repeats associate with Rap1 (Conrad *et al.*, 1990; Lustig, Kurtz and Shore, 1990; Wright and Zakian, 1995). Rap1 is a direct binder of telomeric sequences and recruits indirect binders to promote telomere function. Indeed, Rap1 recruits the Rif1/Rif2 proteins as well as the Sir3/Sir4 complex through its C-terminal domain (Hardy, Balderes and Shore, 1992; Hardy, Sussel and Shore, 1992; Moretti *et al.*, 1994; Wotton and Shore, 1997). The distribution of Rif proteins is nonrandom, with Rif2 localizing more towards the end of the chromosome and Rif1 associating to centromere-proximal telomeric sequences (McGee *et al.*, 2010). The presence of these proteins at telomeres is important to protect chromosome ends, promote heterochromatin formation at subtelomeric regions and regulate telomerase action (Wellinger and Zakian, 2012). The yeast 3' overhang is associated to Cdc13, which additionally recruits Stn1 and Ten1 to form the CST complex (Grandin, Reed and Charbonneau, 1997; Grandin, Damon and Charbonneau, 2000, 2001). This complex is essential to protect chromosome ends, as *cdc13* mutants have unstable telomeres due to excessive resection (Garvik, Carson and Hartwell, 1995). Another telomere factor in yeast is the Ku complex, which associates to telomeres through direct binding of telomeric DNA or through its interaction with Sir4 (Boulton and Jackson, 1996; Porter *et al.*, 1996; Gravel *et al.*, 1998; Roy *et al.*, 2004). Absence of the yeast Ku complex increases telomere resection (Bonetti, Clerici, Anbalagan, *et al.*, 2010a; Vodenicharov, Laterreur and Wellinger, 2010), which demonstrates the importance of this complex in telomere function. Finally, yeast subtelomeric regions enrich Sir2/Sir3 histone deacetylases to promote telomere silencing (Imai *et al.*, 2000), as well as Tbf1, which recruits telomerase (Koering *et al.*, 2000; Arnerić and Lingner, 2007; Preti *et al.*, 2010) (Figure 1).

Mammalian telomeres are coated with a complex of proteins called the shelterin complex (Palm and de Lange, 2008; de Lange, 2018). This complex is composed of telomeric repeat binding factor 1 and 2 (TRF1, TRF2), protection of telomeres 1 (POT1), TRF1-interacting nuclear protein 2 (TIN2), TPP1 and RAP1 (Palm and de Lange, 2008). Importantly, TRF1 and TRF2 bind the double strand telomeric DNA, while POT1 associates to the single strand telomeric repeats (Palm and de Lange, 2008). Altogether, the recruitment of shelterin is essential for telomere function (de Lange, 2018).

End protection

Chromosome ends share similarities with DNA double strand breaks (DSBs). Therefore, they are targeted by DNA repair mechanisms such as Non homologous end joining (NHEJ) or homology directed repair (HDR) when unprotected. Importantly, the aberrant detection of chromosome ends as DSBs activate a cell cycle checkpoint that halts proliferation (Sandell and Zakian, 1993). To prevent this phenomenon, telomeres protect chromosome ends in a process called end protection (de Lange T., 2009; de Lange, 2018).

Activation of DNA repair mechanisms at chromosome ends may have different outcomes. For example, the inability to protect chromosome ends from NHEJ results in chromosome fusions, whereas the activation of HDR at telomeres can result in telomere loss and chromosome exchanges (de Lange T., 2009). These two different phenotypes derive from distinct processing of NHEJ and HDR. Indeed, NHEJ is an error prone DNA repair mechanism that ligates broken ends predominantly in G1 (Chang *et al.*, 2017), while HDR repairs broken DNA using a homologous sequence available after DNA replication (Heyer, Ehmsen and Liu, 2010).

Telomeres have evolved sophisticated mechanisms to promote end protection, which rely on telomere-associated proteins. Budding yeast telomeres prevent aberrant C-strand end resection by hiding the 3' overhang through Cdc13 binding (Garvik, Carson and Hartwell, 1995; Vodenicharov and

Wellinger, 2006). This protective function is not restricted to Cdc13 but rather relies on the complete CST complex (Petreaca *et al.*, 2006; Petreaca, Chiu and Nugent, 2007; Xu *et al.*, 2009; Wellinger and Zakian, 2012). Interestingly, the capping function of Cdc13 seems to be restricted to late S and G2 phases of the cell cycle (Vodenicharov and Wellinger, 2006). In other cell cycle phases, additional proteins participate in telomere capping. This is the case for Rap1, which, together with its protein interactors Rif1 and Rif2, protect telomeres from resection and fusions (Marcand *et al.*, 2008; Bonetti, Clerici, Anbalagan, *et al.*, 2010b; Vodenicharov, Laterreur and Wellinger, 2010). In particular, Rif2 prevents both resection and checkpoint activation by decreasing the association of the Tel1/MRX complex (Mre11, Rad50, Xrs2) to chromosome ends (Hirano, Fukunaga and Sugimoto, 2009; Bonetti, Clerici, Anbalagan, *et al.*, 2010a). Finally, the yKu complex protects chromosome ends from mild resection in G1 (Vodenicharov and Wellinger, 2007; Bonetti, Clerici, Manfrini, *et al.*, 2010; Vodenicharov, Laterreur and Wellinger, 2010). Chromosome end protection may additionally be mediated by telomere structure or telomeric features. Indeed, the formation of G-quadruplexes (G4s) at yeast telomeres prevents chromosome end resection (Smith *et al.*, 2011). Further, it has been proposed that RNA-DNA hybrid structures accumulated at yeast DSBs prevent excessive resection (Ohle *et al.*, 2016). It is therefore tempting to speculate that accumulation of RNA-DNA hybrids at telomeres, perhaps through the formation of G4s on the displaced strand (see below), may participate in end protection, possibly when capping is compromised.

Mammalian telomeres protect chromosome ends through telomere associated proteins and through a lariat structure called t-loop (Griffith *et al.*, 1999; de Lange, 2018). TRF2 cooperates with RAP1 to prevent NHEJ at telomeres (Celli and de Lange, 2005; Bae and Baumann, 2007; Konishi and de Lange, 2008; Sarthy *et al.*, 2009; Benarroch-Popivker *et al.*, 2016) and, together with POT1, RAP1 and Ku70, TRF2 represses HDR at mammalian telomeres (Celli, Denchi and de Lange, 2006; Wu *et al.*, 2006; Rai *et al.*, 2016). Importantly, TRF2 protects chromosome ends from DNA repair pathways, partially through the generation of t-loop structures (Griffith *et al.*, 1999; de Lange, 2018). These structures originate from the invasion of the 3’ single strand overhang into the double strand telomeric DNA and hide the 3’ ends of the chromosomes. In such conformation, chromosome ends are protected from ATM-mediated checkpoint activation and NHEJ, as the ends are not accessible (de Lange T., 2009). To prevent the activation of ATR-mediated checkpoint, which is activated by RPA (replication protein A) binding to single strand DNA, mammalian telomeres rely on POT1 (Denchi and de Lange, 2007). It has been proposed that POT1 outcompetes RPA from the telomeres, therefore preventing aberrant activation of ATR checkpoint (Denchi and de Lange, 2007; de Lange T., 2009). The end protection may require other factors such as heterogeneous nuclear ribonucleoproteins (hnRNPs) and telomeric RNAs, to facilitate an RPA to POT1 switch (Flynn *et al.*, 2011).

End replication

End replication problem

Chromosome ends replicate through semiconservative DNA replication (Wellinger and Zakian, 2012). Due to the nature of DNA replication, where DNA polymerases can only polymerize in the 5’ to 3’ direction, chromosome ends shorten after every replication cycle (Watson, 1972; Joachim Lingner, Cooper and Cech, 1995). This phenomenon is defined as the end replication problem and is counteracted by telomerase, a reverse-transcriptase enzymes that elongates chromosome ends (Watson, 1972; Greider and Blackburn, 1985, 1987, 1989; Joachim Lingner, Cooper and Cech, 1995).

The end replication problem derives from leading strand replication. Indeed, leading strand replication generates a blunt-ended DNA product, which losses DNA sequences in the following rounds of replication (J Lingner, Cooper and Cech, 1995; Soudet, Jolivet and Teixeira, 2014). Therefore, telomeres generated from leading strand replication –named leading-end telomeres- progressively shorten in the

absence of maintenance mechanisms. Lagging-end telomeres on the other hand, maintain telomere length as their replication generates a telomere with a 3' overhang (Figure 2).

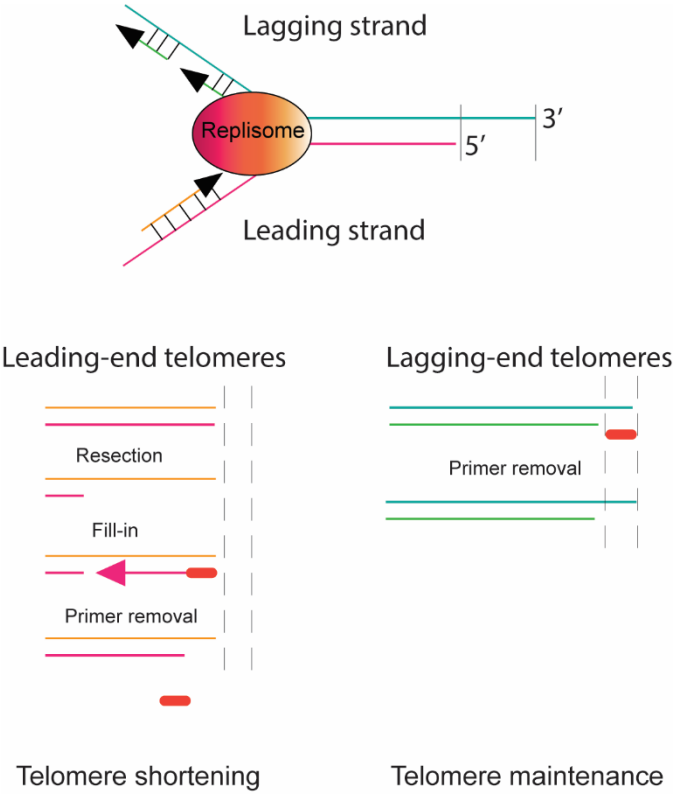


Figure 2 *End replication problem*.

Illustration of telomere length after DNA replication of leading and lagging strands. Blue strand with 3' overhang is the lagging strand and pink strand represents the leading strand. Leading-end telomeres refer to those derived from replication of leading strand. Lagging-end telomeres derive from replication of lagging strand. Replication of lagging strand will reconstruct a telomere with a 3' overhang. Therefore, replication of lagging strand does not shorten telomeres. Replication of the leading strand results in a blunt end and telomere shortening. Replication primers are represented as an orange box. This model is supported by Lingner, Cooper and Cech, 1995 and Soudet, Jolivet and Teixeira, 2014..

To reconstruct the telomeric 3' overhang after replication, C-strand degradation is required (Wellinger *et al.*, 1996; Wu *et al.*, 2010). Indeed, the Texeira lab has recently demonstrated in budding yeast that the blunt-end leading strand replication intermediate is resected in a Tel1-mediated process and generates a product with a 40nt 3'overhang (Soudet, Jolivet and Teixeira, 2014). Several factors including nucleases and helicases (Sae2, Sgs1 and Mre11 in yeast) additionally participate in telomere end-resection in yeast (Larrivée, LeBel and Wellinger, 2004; Bonetti *et al.*, 2009). Importantly, after resection, C-strand fill-in of telomeric intermediates is required to reconstruct a telomere with a 5-10nts overhang (Qi and Zakian, 2000; Soudet, Jolivet and Teixeira, 2014). The generation of this 3' overhang ensures Cdc13 binding to chromosome ends and therefore it promotes end protection and telomerase recruitment.

Similarly, mammalian telomeres require post-replication processing to become fully functional. This processing is required for both telomere replication and generation of t-loops. First, initial cleavage of leading-end telomeres by Apollo provides a substrate for resection (Lam *et al.*, 2010; Wu *et al.*, 2010). Subsequently, products from leading and lagging strand replication are resected by Exo1 (Wu, Takai and de Lange, 2012). Lastly, the CST complex mediates Pol alpha/primase fill-in synthesis, generating a functional telomere with a 3'overhang and a t-loop (Wu, Takai and de Lange, 2012).

Telomere replication problem

Semi-conservative replication through telomeres is subject to many obstacles derived from the telomeric sequences and structures (Maestroni, Matmati and Coulon, 2017a). Therefore, the telomere replication problem refers to the effects that replication fork stalling has on telomere maintenance (Miller, Rog and Cooper, 2006). Fork stalling at telomeres can derive from their compacted heterochromatin structure, the strong association of telomeric binding proteins, the formation of complex nucleic acid structures including G-quadruplexes or RNA-DNA hybrids and, in mammalian telomeres, the formation of the t-loop (Maestroni, Matmati and Coulon, 2017a) (Figure 3).

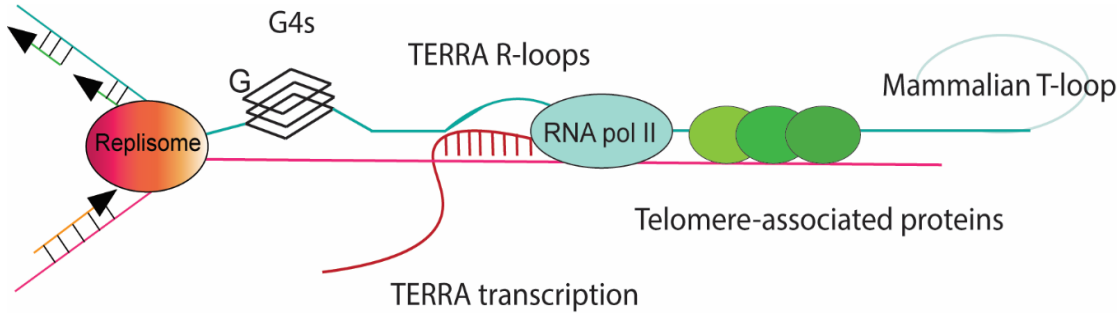


Figure 3 *Telomere replication problem*

Telomere structure is represented. Blue strand represents lagging strand and pink represents the leading strand. Telomere-associated proteins are depicted as green circles. G-rich lagging strand is prone to G4 formation. Leading strand is the DNA template for telomeric transcripts (TERRA). These may hybridize and form three stranded structures called R-loops. Mammalian t-loop is represented at the end of the chromosome as a replication barrier. Reviewed by Maestroni, Matmati and Coulon, 2017a.

To prevent fork stalling, telomeres rely on telomere associated proteins (Miller, Rog and Cooper, 2006; Martínez *et al.*, 2009; Sfeir *et al.*, 2009; Ye *et al.*, 2010) and components of the fork protection complex (FPC) (Leman *et al.*, 2012). Additional factors such as RecQ helicases promote telomere replication by resolving secondary structures in mammalian cells. This is the case for WRN and BLM helicases, which unwind telomeric structures in a POT1-mediated mechanism (Opresko *et al.*, 2005). Other helicases are implicated in telomere replication as well. For example, the Boulton lab has characterized the human RTEL1 (regulator of telomere length 1) helicase as a key regulator of mammalian telomere replication. Indeed, RTEL1 unwinds t-loops and G4s and prevents telomere loss, fragility and telomere catastrophe (Vannier *et al.*, 2012; Sarek *et al.*, 2015; Margalef *et al.*, 2018). Other studies have shown that BLM helicase also participates in telomere replication and prevents fragile-telomeres to ensure proper chromosome segregation (Sfeir *et al.*, 2009; Barefield and Karlseder, 2012). Finally, additional proteins including SMARCAL1 (SWI/SNF-related, matrix associated, actin-dependent, regulator of chromatin subfamily A- like 1) prevent replication stress at mammalian telomeres (Cox, Maréchal and Flynn, 2016; Poole and Cortez, 2016). It is likely that different helicases coordinate telomere replication by resolving different types of topological constraints. For example, helicases like mammalian UPF1 may be specialized in leading strand replication, as they resolve telomeric transcripts associated with the leading strand (Azzalin *et al.*, 2007; Chawla *et al.*, 2011). Additional factors like ATRX, FEN1, FANCM and perhaps PIF1 may regulate telomeric transcripts and thus facilitate leading strand telomere replication (Flynn *et al.*, 2015; Teasley *et al.*, 2015; D. T. Nguyen *et al.*, 2017; Pohl and Zakian, 2019; Silva *et al.*, 2019). On the other hand, human RTEL1 and WRN may facilitate lagging strand synthesis, likely by unwinding G4s (Crabbe *et al.*, 2004; Vannier *et al.*, 2012). Similar to mammalian telomeres,

telomeres in budding yeast require the helicases Rrm3 and Pif1 to prevent fork stalling (Ivessa *et al.*, 2002; Carly L. Geronimo and Zakian, 2016).

Telomere replication timing

Telomere replication is coordinated in a timely manner. For example, in budding yeast, telomeres replicate late in S phase (McCarroll and Fangman, 1988; Raghuraman *et al.*, 2001). In yeast, replication has been propose to originate at an autonomously replicating sequences (ARS) proximal to the telomeric repeats and continues until the end of the chromosomes (Ferguson and Fangman, 1992). Different factors restrict telomere replication to late S in budding yeast. First, the yeast Ku proteins ensure late S replication, as telomeres replicate early in mutants of this complex (Cosgrove, Nieduszynski and Donaldson, 2002). Similarly, Rif1 regulates replication timing, as deletion of *RIF1* anticipates telomere replication (Lian *et al.*, 2011). Indeed, Rif1 interacts with PP1 phosphatase to regulate the DDK-mediated phosphorylation of pre-replication complex (Cooley *et al.*, 2014; Hiraga *et al.*, 2014; Mattarocci *et al.*, 2014). Ultimately, this regulation ensures late origin firing at yeast telomeres.

Several studies suggest that replication timing may be regulated by telomere length. Indeed, Bianchi and Shore demonstrated that engineered short telomeres in yeast switch replication to early S phase and promote telomerase recruitment (Bianchi and Shore, 2007a). Additionally, replication timing and telomere transcription may be closely linked. This is supported by the fact that both short telomeres in telomerase negative cells (*tlc1* cells) and telomeres in *sir2* mutants accumulate telomeric transcripts and replicate early (Stevenson and Gottschling, 1999; Bianchi and Shore, 2007a; Maicher *et al.*, 2012; Graf *et al.*, 2017). As telomeric transcripts may facilitate telomerase recruitment to short telomeres in yeast (Cusanelli, Romero and Chartrand, 2013), these data suggest a correlation between telomere length, telomeric transcription, replication timing and telomerase recruitment.

Replication timing of telomeres differs between species. While budding yeast replicates telomeres in late S phase, human telomeres replicate throughout S phase (Ten Hagen *et al.*, 1990; Wright *et al.*, 1999). The origin of replication in mammalian cells is currently unknown (Gilson and Géli, 2007), although studies have reported that the proximity to telomeric sequences can regulate the firing of adjacent origins (Ofir *et al.*, 1999). Importantly, Verdun and Karlseder showed that replication of human telomeres occurs in two stages, which are required to establish a functional telomere structure. This sequential regulation implies that replication through telomeres activates a DNA damage response in S phase that is necessary to generate 3´overhangs and promote T-loop formation (Verdun and Karlseder, 2006). The second stage of replication processing occurs in late S or G2, showing at least to a certain extent, timing similarities between human and yeast telomere replication.

Mechanism of telomere length maintenance

Telomere length maintenance is essential to promote telomere integrity and protection of chromosome ends. Therefore, eukaryotes and other organisms with linear chromosomes have evolved different strategies to counteract the end replication problem and regulate telomere length maintenance. These include telomerase-mediated telomere maintenance, alternative lengthening of telomeres (ALT) and non-canonical lengthening of telomeres.

Telomerase-mediated telomere length maintenance

Telomerase is a reverse transcriptase enzyme that elongates the ends of linear eukaryotic chromosomes. As a result, telomerase counteracts the end replication problem and telomere loss.

Telomerase was first discovered by Carol Greider and Elisabeth Blackburn in *Tetrahymena thermophila* and contains an RNA subunit that serves as a template to elongate the 3´end of the G-rich strand of telomeres (Greider and Blackburn, 1985, 1987, 1989). Once elongated, the conventional DNA replication machinery replicates the complementary strand.

Telomerase activity in budding yeast compensates for telomere shortening and promotes cell division. However, long-lived organisms tightly regulate telomerase activity, as it may allow proliferation of transformed tumor cells (Maciejowski and De Lange, 2017). Indeed, telomere shortening acts as a tumor suppressor mechanism in higher eukaryotes. Therefore, understanding telomerase function and regulation is critical for the study of cancer cells. Importantly, yeast models offer great advantages for the study of telomerase, as its activity may be controlled in genetically engineered cells.

In budding yeast, telomerase is composed of three protein subunits (Est1, Est2, Est3) and an RNA component (TLC1) (Lundblad and Szostak, 1989; Singer and Gottschling, 1994; Lendvay *et al.*, 1996). Deletion of any telomerase component results in progressive telomere shortening, although the catalytic activity of telomerase lies in the Est2 subunit (Lingner *et al.*, 1997). The other protein subunits mediate the recruitment of telomerase to telomeres. Indeed, Est1 recruits telomerase to telomeres by interacting with Cdc13 (Evans and Lundblad, 1999; Qi and Zakian, 2000; Wu and Zakian, 2011). Finally, the RNA component TLC1 provides the template for the reverse transcriptase activity of Est2 to allow the addition of repeats to the 3’ end of chromosomes (Singer and Gottschling, 1994). In mammalian cells, reverse transcriptase enzyme (TERT) and its RNA moiety (TR) coordinate telomere elongation in a similar manner (Feng *et al.*, 1995; Jiang *et al.*, 2018).

Telomerase activity is regulated by telomere length in a cell-cycle dependent manner. In yeast, telomerase elongates telomeres at the end of S phase (Diede and Gottschling, 1999; Marcand *et al.*, 2000; Gallardo *et al.*, 2011), likely due to the accumulation of Cdc13 at telomeric G-tails (Wellinger, Wolf and Zakian, 1993; Evans and Lundblad, 1999; Qi and Zakian, 2000; Wu and Zakian, 2011). Similarly, human telomerase elongates telomeres in S phase (Tomlinson *et al.*, 2005). Telomerase activity preferentially elongates short telomeres to prevent telomere dysfunction (Marcand, Brevet and Gilson, 1999; Teixeira *et al.*, 2004; Bianchi and Shore, 2007b; Jacobs, 2013). To achieve this, telomere associated proteins like yeast Rif1 and Rif2 (Teng *et al.*, 2000) and human TRF1 (van Steensel and de Lange, 1997; Smogorzewska *et al.*, 2000) act as negative regulators of telomerase. Reductions in telomere associated proteins therefore permit the preferential elongation of short telomeres, as they would associate fewer telomerase negative regulators, a phenomenon referred to as the protein counting model. Interestingly, short telomeres may accumulate additional factors to coordinate telomerase activity. For example, yeast short telomeres accumulate the MRX complex (McGee *et al.*, 2010) and Tel1 (Sabourin, Tuzon and Zakian, 2007), which may recruit and activate telomerase (Goudsouzian, Tuzon and Zakian, 2006). Other factors like yeast Pif1 helicase promote telomerase-mediated elongation of short telomeres in late S by removal of telomerase from long telomeres (Schulz

and Zakian, 1994). Additionally, the specific recruitment of telomerase to short telomeres relies, among other factors, on telomeric transcripts in yeast (Cusanelli, Romero and Chartrand, 2013; Moravec *et al.*, 2016) and hnRNP proteins in human cells (Zhang *et al.*, 2006).

The data presented thus far supports a model in which telomere length regulates telomerase function. In particular, the ´protein counting model´ proposes that telomere length determines the amount of negative regulators associated to telomeres, and this coordinates the accessibility of telomerase (Marcand, Gilson and Shore, 1997). In 2016, Carol Greider proposed an alternative model known as the 'replication fork model' (Greider, 2016). She proposed that telomerase may travel with the replication forks through its interaction with RPA at the lagging strand. As a consequence, replication through nucleosomes or telomere associated proteins may dissociate telomerase from travelling forks and prevent telomere elongation. Telomerase dissociation would therefore be stronger at long telomeres, while shortened telomeres would allow telomerase to travel till the end of the chromosome (Greider, 2016). This model would explain the close link between telomere replication and telomerase-mediated telomere elongation. Unfortunately, this model does not account for how telomeres can be elongated in G1 in certain genetic contexts (Gallardo *et al.*, 2011). In the future, research will contribute to our understanding of telomerase regulation, in particular in the context of telomere structure, replication stress and fork reversal.

ALT

In the absence of active telomerase, some cells maintain their proliferative potential through Alternative Lengthening of Telomeres (ALT). Canonical ALT mechanisms are active in 10-15% of human cancers and rely on homologous recombination (HR) (Bryan *et al.*, 1997).

Several features characterize human ALT cells (Figure 4). First, as HR-mediated telomere elongation requires a telomeric DNA template on a different chromosome or sister chromatid, ALT cells often show telomeric sister chromatic exchanges (T-SCEs) (Bailey, Brenneman and Goodwin, 2004) and a very heterogeneous telomere length (Murnane *et al.*, 1994; Londoño-Vallejo *et al.*, 2004). Secondly, ALT cells accumulate several extra-chromosomal telomeric repeats (ECTRs) including telomeric circles (t-circles) (Cesare and Reddel, 2010; Apte and Cooper, 2017). Extra-chromosomal telomeric DNA structures localize to a specialized compartment termed ALT-associated promyelocytic leukemia body (APBs). In addition to telomeric DNA, APBs contain telomere associated factors, DNA repair proteins like RAD51D, helicases including BLM and WRN, and other factors (Apte and Cooper, 2017). Likely, APBs are specialized compartments that facilitate ALT (Wu, Lee and Chen, 2000; Nabetani, Yokoyama and Ishikawa, 2004; Chung, Leonhardt and Rippe, 2011; Osterwald *et al.*, 2015; Min, Wright and Shay, 2019; Zhang *et al.*, 2019).

Recent studies suggest that ALT telomeres experience a balanced instability, as the latter may promote chromatid exchanges and telomere elongation (Apte and Cooper, 2017; Sobinoff and Pickett, 2017). Possibly, replication stress and telomere DNA damage trigger break-induced replication (BIR) and mitotic DNA synthesis (MiDAS) in ALT cells (John R. Lydeard *et al.*, 2007; O'Sullivan *et al.*, 2014; Robert L Dilley *et al.*, 2016; Roumelioti *et al.*, 2016b; Min, Wright and Shay, 2017; Sobinoff and Pickett, 2017; Özer and Hickson, 2018). The activation of these mechanisms would therefore promote HR-mediated telomere elongation and maintenance. Indeed, induction of telomeric DNA damage using a Fok-I nuclease-TRF1 fusion protein promotes telomere movement and clustering, thus facilitating HR, telomere synthesis and ALT activities in human cells (Cho *et al.*, 2014).

One interesting characteristic of human cells with ALT is that they accumulate non-canonical telomeric repeats (Conomos *et al.*, 2012; Lee *et al.*, 2013). This is perhaps a consequence of low fidelity DNA polymerases acting at ALT telomeres (Roux, Kim and Burke, 2013). Another feature of ALT human cells is that they often harbor mutations or deletions in ATRX and DAXX chromatin remodelers (Heaphy *et al.*, 2011; Flynn *et al.*, 2015). These mutations may promote ALT activities through different mechanisms, including the accumulation of telomeric R-loops (Ramamoorthy and Smith, 2015; Chu *et al.*, 2017; D. T. Nguyen *et al.*, 2017). Indeed, telomeric R-loops may promote replication stress at telomeres. Further, balanced R-loop levels may be required to facilitate ALT without compromising telomere integrity (Arora *et al.*, 2014). To prevent excessive replication stress, toxic recombination intermediates and telomere catastrophe, ALT telomeres rely on a myriad of factors including human helicases RTEL1, SMARCAL1, FANCM or SLX4 (Apte and Cooper, 2017; Sobinoff and Pickett, 2017). These factors coordinate a balanced instability that facilitates ALT without compromising cell viability.

In budding yeast, propagation of telomerase negative cells progressively decreases viability and eventually results in the generation of viable cells called yeast 'survivors'. Similar to human ALT, budding yeast survivors promote telomere length maintenance through HR mechanisms (Lundblad and Blackburn, 1993; Claussin and Chang, 2015). Two types of survivors have been described depending on the amplified sequences and the proteins required for their maintenance. Interestingly, both types of survivors sustain telomere elongation through homologous recombination proteins and a Pol32-mediated mechanism, supporting the implication of BIR in yeast telomere maintenance (Lundblad and Blackburn, 1993; Le *et al.*, 1999; John R Lydeard *et al.*, 2007). Type I survivors amplify Y' elements and additionally require Rad51, Rad54 and Rad57 (Le *et al.*, 1999). On the other hand, Type II survivors require MRX, Rad59 and Sgs1 to amplify long heterogeneous telomeric repeats (Teng and Zakian, 1999; Chen, Ijpma and Greider, 2001; Huang *et al.*, 2001; Johnson *et al.*, 2001). Additional factors may also contribute to the formation of yeast survivors (Claussin and Chang, 2015). Altogether, the HDR-mediated telomere maintenance and the accumulation of extra chromosomal telomeric DNA (Larrivée and Wellinger, 2006), makes budding yeast survivors a good model to study ALT.

Certainly, HDR also promotes yeast telomere maintenance after telomerase inactivation. In budding yeast, one critically short telomere in telomerase negative cells is sufficient to activate a checkpoint-mediated cell cycle arrest in a process called replicative senescence (Abdallah *et al.*, 2009). To prevent accelerated senescence onset, telomerase negative cells repair spontaneously shortened telomeres

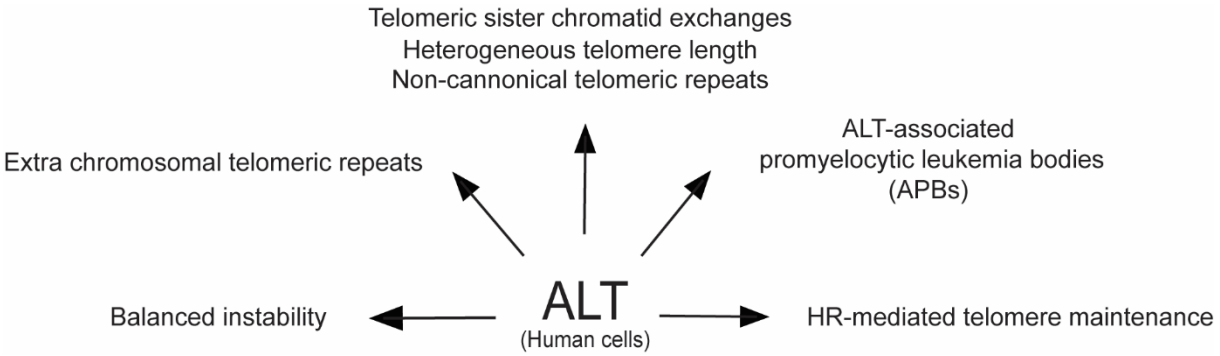


Figure 4 *Features of human ALT cells*

ALT requires balanced instability and HR-mediated telomere maintenance. ALT cells characteristics in human cells are represented. Reviewed by Sobinoff and Pickett, 2017. Abbreviations: ALT: alternative lengthening of telomeres.

via HDR (Le *et al.*, 1999; John R. Lydeard *et al.*, 2007). This mechanism requires the action of several factors including telomeric R-loops, RecQ helicases like Sgs1 or hRNP-like proteins (Lee *et al.*, 2007; Lee-Soety *et al.*, 2012; Balk *et al.*, 2013; Yu, Kao and Lin, 2014; Graf *et al.*, 2017; García-Rubio *et al.*, 2018). Similar to human ALT, telomerase negative yeast cells likely require balanced replication stress levels that promote HDR-mediated telomere elongation yet allow cell proliferation (Simon, Churikov and Géli, 2016)

Non-canonical ALT

Different organisms have evolved distinct mechanisms to protect their chromosome ends and preserve genome integrity through alternative lengthening mechanisms.

Some fly telomeres are composed of three repeat elements that are maintained through retro-transposition (Capkova Frydrychova, Biessmann and Mason, 2009; Apte and Cooper, 2017). More specifically, telomeres in *Drosophila* contain three retrotransposon elements called Het-A, TART and Tahre (termed HTT) (Pardue and Debaryshe, 2008). To counteract the end replication problem, these telomeres transcribe HTT elements that encode GAG-like proteins and reverse transcriptases. Subsequently, GAG proteins and associated mRNAs target chromosome ends and synthesize one DNA strand in a reverse transcriptase mechanism. Similar to telomerase-mediated telomere elongation, retrotransposon elements are copied into chromosome ends, with variable amounts of polyA tail at every transposition (Pardue and Debaryshe, 2008). Second strand synthesis allows for the addition of new repeats at the chromosome ends (Capkova Frydrychova, Biessmann and Mason, 2009).

Fission yeast display three different mechanisms to preserve genome integrity in the absence of telomerase. On the one hand, *S. pombe* strains can circularize their chromosomes and thereby circumvent the biological drawbacks of linear chromosomes (Apte and Cooper, 2017). Additionally, *S. pombe* can preserve telomere integrity through a Rad52-dependent HR mechanism (Nakamura, Cooper and Cech, 1998; Subramanian, Moser and Nakamura, 2008; Rog *et al.*, 2009). Finally, telomerase negative fission yeast can form heterochromatin amplification dependent and telomerase independent survivors (HATTI) (Jain *et al.*, 2010). HATTI survivors replace telomere repeats by heterochromatin repeats such as rDNA repeats (Jain *et al.*, 2010). This exciting phenomenon highlights the importance of heterochromatin marks in the protection of linear chromosome ends.

Interestingly, a few infectious bacteria and viruses harbor linear chromosomes. This is the case for Lyme disease-causing *Borrelia* or Herpesvirus. *Borrelia* spirochetes have linear genomes with ends closed in telomere hairpins that are maintained through telomere resolvase ResT (Chaconas, 2005). On the other hand, Herpesvirus uses GC-rich terminal repeats to integrate viral genome into host telomeres, likely through HDR mechanisms (Morissette and Flamand, 2010; Kaufer, Jarosinski and Osterrieder, 2011).

In conclusion, many different systems have evolved to ensure telomere length maintenance without telomerase activity. Insights into their mechanistic features may provide useful knowledge and perhaps future options for therapies in telomere disorders.

Senescence

The term cellular senescence refers to a permanent cell cycle arrest that limits cell proliferation (Hayflick, 1965). In complex organisms, senescence limits the proliferative potential of cells to counteract cancer progression (Campisi and D'Adda Di Fagagna, 2007). However, accumulation of senescent cells may negatively impact tissue regeneration and contribute to aging (Baker *et al.*, 2011, 2016; Hernandez-Segura, Nehme and Demaria, 2018). For example, senescence may limit the proliferative capacity of progenitor cells and therefore decrease the regenerative potential of different tissues. Therefore, senescence onset must be balanced to prevent premature aging and at the same time prevent cancer development (Figure 5A).

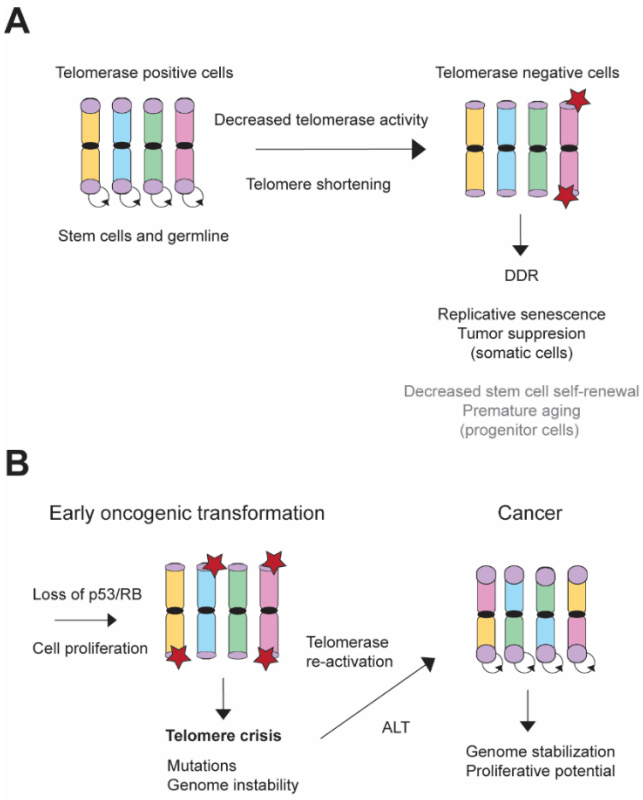


Figure 5 *Senescence is a tumor suppressor mechanism*

A) Decreased telomerase activity shortens telomeres, initiates senescence and prevents cells proliferation. This turns senescence into a tumor suppressor mechanism. Senescent cells harbor a few unprotected telomeres, represented with red stars. Unprotected telomeres activate checkpoint-mediated cell cycle arrest. This phenomenon limits proliferative potential of cells and prevents cancer progression. Accumulation of dysfunctional telomeres in progenitor cells (grey) may limit the regenerative potential of tissues and contribute to premature aging B) Cell proliferation of senescent cells resulted from loose of p53/RB activity results in telomere crisis. Under this condition, cells harbor many unprotected telomeres, represented with red stars. Reactivation of a telomere length maintenance mechanism results in cancer development. Circled arrows represent telomere maintenance mechanisms. Reviewed by Maciejowski and De Lange, 2017 and Hernandez-Segura, Nehme and Demaria, 2018. Abbreviations: DDR: DNA damage response. ALT: alternative lengthening of telomeres. p53/RB: p53/retinoblastoma cell cycle regulators; signaling from these proteins prevents cell proliferation.

In multicellular organisms, senescence onset derives from a series of stimuli that include DNA damage, oncogene expression, chromatin alterations, continued cytokines signaling and telomere dysfunction (Campisi and D'Adda Di Fagagna, 2007). Senescent cells are characterized by a permanent growth arrest, apoptosis resistance and altered gene expression (Campisi and D'Adda Di Fagagna, 2007). Indeed, such altered gene expression facilitates the permanent growth arrest in senescent cells. For example, mammalian senescent cells activate p53 and p16/retinoblastoma (pRB) tumor suppressor pathways and subsequently express cell cycle inhibitors including p21. The coordination of these pathways prevents the E2F-mediated transcription of proliferative genes (Campisi and D'Adda Di Fagagna, 2007).

Additionally, senescent cells can be identified by positive staining of senescence-associated b-galactosidase (SA-b-gal) (Dimri *et al.*, 1995) and expression of specific proteins including p16, DEC1, p15 and DCR2 in mammals (Collado and Serrano, 2006; Campisi and D'Adda Di Fagagna, 2007). Further, senescent cells can be identified by the senescence-associated heterochromatin foci (SAHF) (Narita *et al.*, 2003) and senescence-associated DNA damage foci (SDFs) (Fagagna *et al.*, 2003; Takai, Smogorzewska and de Lange, 2003; Herbig *et al.*, 2004). In both human cells and yeast, senescent cells increase in size (MORTIMER and JOHNSTON, 1959; Hayflick and Moorhead, 1961). Recently, Neurohr *et al*., have shown that senescent cells increase in size and dilute their DNA content. This phenomenon results in impaired gene expression and cell cycle progression, likely contributing the limited proliferative capacity of senescent cells (Neurohr *et al.*, 2019). Telomere shortening and derived telomere dysfunction activate a DNA-damage response that triggers replicative senescence (Fagagna *et al.*, 2003; Takai, Smogorzewska and de Lange, 2003; Herbig *et al.*, 2004). In cancer cells, germ cells and stem cells telomerase can maintain telomere length and prevent senescence onset (Kim *et al.*, 1994). However, most human cells do not harbor sufficient telomerase activity to prevent telomere shortening (Bodnar *et al.*, 1998). As a result, short telomeres in most human cells no longer protect chromosome ends and activate replicative senescence (Hayflick, 1965; Lundblad and Szostak, 1989; Campisi and D'Adda Di Fagagna, 2007) (Figure 5A). Indeed, five dysfunctional telomeres in human cells are sufficient to drive cells into senescence (Kaul *et al.*, 2012). This phenomenon prevents proliferation of transformed cells and turns replicative senescence into a potent tumor suppressor mechanism.

Some cells can overcome senescence-mediated arrest by inactivating inhibitory pathways such as p53 or pRB (Beauséjour *et al.*, 2003; Campisi and D'Adda Di Fagagna, 2007; Maciejowski and De Lange, 2017) (Figure 5B). Proliferation of cells with short dysfunctional telomeres increases genome instability in a stage called telomere crisis (Artandi *et al.*, 2000; Artandi and DePinho, 2009). Finally, restoring the proliferative capacity of newly transformed cells after telomere crisis results in cancer progression (Maciejowski and De Lange, 2017). The majority of transformed cancer cells re-activate telomerase to promote telomere maintenance, although a minority of cancer cells rely on the alternative lengthening of telomeres (ALT) system (Figure 5B) (Kim *et al.*, 1994; Bryan *et al.*, 1997; Pickett and Reddel, 2015; Maciejowski and De Lange, 2017). Importantly, re-activation of telomerase *per se* does not result in malignant transformation (Morales *et al.*, 1999) but additional mutations and genome instability are required.

Budding yeast is a very interesting model to study telomeres and replicative senescence. In particular, deletion of any component of telomerase results in senescence onset after several population doublings (Lendvay *et al.*, 1996) and one critically short telomere is sufficient to drive cells into senescence (Abdallah *et al.*, 2009). In addition, similar to human ALT cancer cells, yeast survivors that rely on HDR to maintain their telomeres have also been characterized (Lundblad and Blackburn, 1993; Claussin and Chang, 2015).

Studies in yeast have characterized different steps of telomere shortening and senescence and attempted to characterize the proteins involved in the regulation of senescence rates. For example, the Teixeira lab has demonstrated that after telomerase loss, telomeres are targeted by Rad52 and Mms1 in a pre-senescence state (Abdallah *et al.*, 2009) to repair prematurely arising short telomeres. Subsequently the inevitable accumulation of very short telomeres activates the Mec1 pathway in a senescence state (Abdallah *et al.*, 2009). Additionally, the composition of the yeast telomere associated proteins changes during senescence (McGee *et al.*, 2010; Platt *et al.*, 2013) and activates a specific gene expression profile that regulates senescence rate (Nautiyal, DeRisi and Blackburn, 2002; Platt *et al.*, 2013). Further, several proteins and repair factors associate preferentially to short telomeres (Fallet *et al.*, 2014). This and other features including telomeric R-loops (Balk *et al.*, 2013; Graf *et al.*, 2017) dictate the rate of senescence.

Recent studies in yeast suggest that short telomere-mediated cell cycle arrest derives from both gradual telomere shortening and stochastic telomere damage in the absence of telomerase (Xu *et al.*, 2015). Interestingly, follow up studies suggest that adaptation to DNA damage derived from stochastic short telomeres may contribute to survival of telomerase negative cells at the cost of high genome instability (Coutelier *et al.*, 2018; Coutelier and Xu, 2019). This is particularly interesting in the context of cancer, as adapted cells with short telomeres increase their mutation rate. It is therefore possible that adaptation in response to short telomere-derived instability allows pre-cancer cells to acquire mutations that may trigger malignant transformation. Ultimately, yeast short telomeres derived from stochastic damage or gradual shortening facilitate recombination-mediated telomere elongation, likely through increased replication stress (Pickett and Reddel, 2015; Simon, Churikov and Géli, 2016). As a result, HDR regulates senescence rate and the generation of yeast survivors (Lundblad and Blackburn, 1993).

Telomere chromatin

Maintenance of telomere integrity relies, among other factors, on the chromatin structure of both, telomeric repeats and subtelomeric regions. Therefore, epigenetic modifications at telomeres or subtelomeres may impact telomere function differently and contribute to genome integrity.

In budding yeast, only subtelomeric regions but not telomeric repeats harbor nucleosomes (Wright, Gottschling and Zakian, 1992). Yeast subtelomeres accumulate transcriptional silencing marks such as deacetylated H4K16 (Zhu and Gustafsson, 2009), which is mediated (in part) by the SIR complex (Imai *et al.*, 2000). Different modifications regulate telomere integrity and dynamics. For example, while the SIR complex mediates histone deacetylation to promote telomere silencing (Imai *et al.*, 2000; Maicher *et al.*, 2012), Bre1 and Rad6 mediate ubiquitination of H2B (K123) to promote end-resection and sustain telomere replication and elongation (Wu *et al.*, 2017, 2018). Interestingly, H2B ubiquitination precedes and allows H3K4 methylation (Sun and Allis, 2002), which may regulate telomere silencing and lifespan in yeast (Rhie *et al.*, 2013). These results raise the intriguing possibility that positioning of initial histone modifications orchestrate subsequent marks that dictate the epigenetic state of the telomere. Interestingly, mutations in mammalian BRE1 increase replication stress and genome instability, likely by impacting R-loop regulation and contributing to cancer development (Chernikova *et al.*, 2012). It is therefore possible that mutations in BRE1 impact telomere silencing, telomere transcription and perhaps telomeric R-loop accumulation to promote cancer progression in mammalian cells.

In yeast, loss of Sir2-mediated transcriptional silencing increases telomeric transcript (TERRA) levels, shortens telomeres and anticipates senescence onset in certain genetic contexts (Maicher *et al.*, 2012). However, NuA4 acetyltransferase also associates to telomeres, presumable to restrict excessive SIR complex association to telomeres (Zhou *et al.*, 2011). These studies suggest that balanced histone

modifications are required for telomere function and transcription regulation. A link between TERRA and yeast histone methyltransferase Dot1 has also been proposed to regulate telomere length and senescence rate (Wanat *et al.*, 2018), although the exact role of Dot1 at telomeres is not fully understood. Altogether, epigenetic modifications in yeast coordinate telomere integrity by mediating different processes that include telomere transcription and silencing.

Mammalian telomeres contain nucleosomes and are subject to histone modifications that promote telomere integrity. Indeed, both histone methylation mediated by SUV39H1/H2 and direct telomeric DNA methylation by DNA methyltransferases (DNMTs) can regulate telomere length (García-Cao *et al.*, 2004; Gonzalo *et al.*, 2006; Benetti *et al.*, 2007; Schoeftner and Blasco, 2010). Additionally, epigenetic modifications may determine distinct telomere maintenance mechanisms in mammalian cells. For example, it has been proposed that heterochromatin formation mediated by SUV39H1/H2 and other factors may prevent ALT, likely by promoting a degree of telomere condensation that would interfere with HDR (Schoeftner and Blasco, 2010). Studies from the Decottignies lab showed that human ALT telomeres decrease the heterochromatin mark H3K9me3, which supports that compaction of telomeres may be important for ALT (Episkopou *et al.*, 2014). However, more recent studies have challenged this model and propose that human telomeres may be euchromatic while ALT telomeres may accumulate heterochromatin marks (Cubiles *et al.*, 2018; Gauchier *et al.*, 2019). In particular, the Dejardin lab has proposed that SETDB1-mediated heterochromatin formation promotes telomere recombination and ALT (Gauchier *et al.*, 2019). A very complex regulatory network can be anticipated, as telomeric transcripts may additionally interact with chromatin remodelers and promote heterochromatin marks at ALT telomeres in mammalian cells (Arnoult, Van Beneden and Decottignies, 2012; Montero *et al.*, 2018; Bettin, Oss Pegorar and Cusanelli, 2019). Further research will elucidate the epigenetic nature of telomeres depending on their length, maintenance mechanism and transcription status in both yeast and mammalian cells. Additionally, it would be interesting to elucidate the epigenetic changes at telomeres that may determine telomere chromatin dynamics during development and disease (Tardat and Déjardin, 2018).

Epigenetic modifications and heterochromatin formation at telomeres regulate gene expression of nearby genes in a process called telomere position effect (TPE) (Gottchling zakian 1990). In yeast, several telomere associated factors regulate TPE (Wellinger and Zakian, 2012) to control the expression of metabolic and stress response genes (Ai *et al.*, 2002; Robyr *et al.*, 2002). Of note, telomere length modulates TPE, as longer telomeres increase TPE (Kyrion *et al.*, 1993). Not surprisingly, telomerase negative yeast cells with short telomeres upregulate the expression of metabolic and stress response genes (Nautiyal, DeRisi and Blackburn, 2002; Platt *et al.*, 2013). These changes in gene expression may be perhaps due to TPE, as those genes locate close to telomeres. Likely, a myriad of factors including DNA repair factor like Smc5/6 participate in TPE through different regulatory pathways (Moradi-Fard *et al.*, 2016).

Similar to yeast, mammalian telomeres regulate TPE (Baur *et al.*, 2001; Blasco, 2007). Further, telomere length correlates with TPE (Baur *et al.*, 2001; Koering *et al.*, 2002). TPE-mediated silencing extends over long distances in a process called TPE-OLD, which regulates the expression of different genes including telomerase component h*TERT* (Robin *et al.*, 2014; Kim and Shay, 2018). This discovery suggests that shortening of telomeres promotes their re-elongation by de-repressing telomerase in mammalian cells. Moreover, it suggest that TPE regulates different metabolic pathways depending on the telomere length. In conclusion, TPE-mediated gene expression regulation may have interesting consequences for aging, life span and cancer through the expression of specific genes and pathways.

Telomere transcription

In 2007, transcription of telomeres was first described in mammalian cells (Azzalin *et al.*, 2007). This discovery challenged the view of telomeric regions being transcriptionally silenced and opened up new possibilities for telomere function. Since then, much progress has been made on understanding the role of telomeric transcripts in telomere stability, integrity and human disease (Bettin, Oss Pegorar and Cusanelli, 2019).

Telomere transcription and regulation of telomeric transcript levels

TElomeric Repeat containing RNAs (TERRA) are transcribed from the C-rich strand of telomeres in eukaryotes (Azzalin *et al.*, 2007; Luke *et al.*, 2008; Schoeftner and Blasco, 2008; Bettin, Oss Pegorar and Cusanelli, 2019). In addition, telomeres in fission yeast transcribe both C-rich and G-rich repeats into TERRA and ARIA transcripts, and subtelomeric regions are transcribed into ARRET and alpha-ARRET transcripts (Bah *et al.*, 2011; Greenwood and Cooper, 2011; Azzalin and Lingner, 2015). Importantly, the transcriptional regulation of fission yeast telomeric transcripts depends on telomere-associated proteins and heterochromatin marks (Greenwood and Cooper, 2011).

Even though several telomeric transcripts have been described in fission yeast, TERRA seems the most conserved telomeric transcript in the eukaryotic kingdom (Azzalin and Lingner, 2015). Human TERRA transcription starts in the subtelomeric regions and proceeds into telomeric repeats, as demonstrated by rapid amplification of cDNA ends (RACE experiments) (Nergadze *et al.*, 2009). Similarly, yeast TERRA transcription starts within subtelomeric regions (Pfeiffer and Lingner, 2012).

TERRA transcription is mainly carried out by RNA polymerase II and results in heterogeneous lengths of TERRA molecules (Azzalin *et al.*, 2007; Luke *et al.*, 2008; Schoeftner and Blasco, 2008). In mammalian and yeast cells, many studies propose that telomeric transcription originates from a diversity of telomeres (Luke *et al.*, 2008; Nergadze *et al.*, 2009; Arnoult, Van Beneden and Decottignies, 2012; Deng *et al.*, 2012; Balk *et al.*, 2013; Porro *et al.*, 2014; Mazzolini *et al.*, 2017; Feretzaki, Renck Nunes and Lingner, 2019). However, conflicting results have been obtained in different laboratories, where one single telomere was identified as the main source of TERRA transcripts in both human and murine cells (López de Silanes *et al.*, 2014; Montero *et al.*, 2016).

In humans and budding yeast, TERRA carries a 7-methyl-guanosine cap at its 5’ end and is poly-adenylated on its 3’end (Azzalin *et al.*, 2007; Luke *et al.*, 2008; Schoeftner and Blasco, 2008; Porro *et al.*, 2010). These modifications likely promote TERRA stabilization, localization and function.

TERRA expression is regulated by the epigenetic state of telomeres. For example, methylation of subtelomeric CpG islands by DNMT1 and DNMT3b repress active telomere transcription in human cells (Nergadze *et al.*, 2009). In addition, the cooperative action of CTCF and cohesin subunit Rad21 may regulate the association of RNA polymerase 2 to subtelomeres, thus positively regulating TERRA transcription (Deng *et al.*, 2012). Finally, TERRA expression is regulated by SUV39H1, HP1 or ATRX-mediated epigenetic marks (Arnoult, Van Beneden and Decottignies, 2012; Flynn *et al.*, 2015). As TERRA may interact with some of these factors (Porro *et al.*, 2010; Arnoult, Van Beneden and Decottignies, 2012; Deng *et al.*, 2012), it is possible that TERRA regulates its own transcription in a feedback loop. In budding yeast, the subtelomeric X and Y’ elements determine distinctive TERRA transcription at different chromosome ends (Iglesias *et al.*, 2011). This regulation is mediated by the yeast telomeric binding protein Rap1, which recruits Sir proteins to repress TERRA at X-only telomeres and Rif proteins to repress TERRA and Y’ telomeres. Altogether, these studies suggest that different epigenetic marks modulate TERRA expression. In particular, methylation of H3K9 and H4K20 may positively regulate TERRA, while histone acetylation may promote TERRA transcription in different organisms (Bettin, Oss Pegorar and Cusanelli, 2019). This regulation may differ between species and

perhaps depending on the telomere context. Indeed, human ALT cells may facilitate TERRA expression by maintaining a less compacted telomere status (Ng *et al.*, 2009; Schoeftner and Blasco, 2010; Episkopou *et al.*, 2014), although this hypothesis remains controversial (Cubiles *et al.*, 2018; Gauchier *et al.*, 2019).

Budding yeast regulate TERRA levels through transcriptional regulation as well as RNA degradation. In fact, TERRA transcripts are degraded by the exonuclease Rat1 (Luke *et al.*, 2008) which prevents the accumulation of TERRA in cells with long telomeres. Upon telomere shortening, TERRA transcripts accumulate due to decreased Rat1 localization to short telomeres (Graf *et al.*, 2017). This likely promotes telomere elongation through telomerase recruitment or HDR (Cusanelli, Romero and Chartrand, 2013; Graf *et al.*, 2017). Similarly, short telomeres in fission yeast display increased TERRA levels, which possibly facilitates the recruitment of telomerase (Moravec *et al.*, 2016).

Budding yeast survivors display increased TERRA levels (Misino *et al.*, 2018), which reminds of TERRA regulation in ALT human cells (Ng *et al.*, 2009; Episkopou *et al.*, 2014). In human cells, several hnRNP proteins regulate stability of TERRA (De Silanes, D'Alcontres and Blasco, 2010) and members of the non-sense mediated decay pathway (NMD) coordinate the displacement of TERRA from telomeres (Azzalin *et al.*, 2007; Chawla *et al.*, 2011). Altogether, a complex network of TERRA-interacting factors regulate TERRA expression and stability, with important implications in telomere pathologies (Scheibe *et al.*, 2013).

TERRA levels are regulated in the cell cycle both in human cells and yeast (Porro *et al.*, 2010; Graf *et al.*, 2017). Human cells accumulate TERRA at the G1/S transition point and progressively decrease its levels during S and G2 (Porro *et al.*, 2010). In budding yeast, TERRA levels also increase in early S and decrease in late S (Graf *et al.*, 2017). This regulation likely facilitates telomere replication, as TERRA accumulation may stall replication forks at telomeres (Graf *et al.*, 2017; Maestroni, Matmati and Coulon, 2017a). Interestingly, human ALT cells with mutations in ATRX have a compromised cell cycle regulation of TERRA (Flynn *et al.*, 2015). It is therefore possible that in these conditions, TERRA may be a source of replication stress and genome instability in ALT cells.

Function of TERRA

Several lines of evidence demonstrate that TERRA is functionally relevant at telomeres. Indeed, TERRA downregulation increases the formation of telomere dysfunction-induced foci (TIFs) (Deng *et al.*, 2009, 2012; López de Silanes *et al.*, 2014). On the other hand, unscheduled TERRA accumulation may negatively impact telomere integrity (De Silanes, D'Alcontres and Blasco, 2010). Therefore, balanced TERRA levels must be regulated to promote telomere function. Among others, TERRA has been proposed to regulate telomere replication, telomere length, DNA damage response at telomeres and telomere epigenetic state (Azzalin and Lingner, 2015; Bettin, Oss Pegorar and Cusanelli, 2019) (Figure 6).

TERRA may facilitate telomere replication through different mechanisms in human cells. One possibility is that TERRA regulates heterochromatin marks and localization of ORC proteins to telomeres (Deng *et al.*, 2009; Takahama *et al.*, 2013). Another possibility is that telomere transcription *per* se influences telomere replication, as RNA Polymerase II-mediated transcription may regulate DNA replication initiation sites (Gros *et al.*, 2015). In budding yeast, cells with short telomeres or mutants that replicate telomeres early have increased levels of TERRA (Stevenson and Gottschling, 1999; Bianchi and Shore, 2007a; Maicher *et al.*, 2012; Graf *et al.*, 2017). However, it is not yet known if TERRA transcription directly regulates telomere replication or replication timing.

TERRA may also regulate telomere length maintenance, as TERRA levels inversely correlate with telomere length. Inducing strong TERRA transcription shortens telomeres in yeast due to excessive

Exo1-mediated resection (Pfeiffer and Lingner, 2012). Therefore, it has been proposed that TERRA sequesters yKu from telomeres, allowing Exo1-mediated resection of telomeres (Pfeiffer and Lingner, 2012). In mammalian cells, TERRA may regulate telomere length by inhibiting telomerase activity, as *in vitro* studies suggest that TERRA associates with hTR (Redon, Reichenbach and Lingner, 2010). These artificial systems suggest that TERRA may promote telomere shortening although this does not account for technical difficulties. Follow up *in vivo* data, on the other hand, rather supported a role of TERRA in promoting telomere elongation of short telomeres. Yeast TERRA transcripts accumulate in cells with short telomeres and associate back to their telomere of origin (Cusanelli, Romero and Chartrand, 2013; Moravec *et al.*, 2016; Graf *et al.*, 2017). These observations, together with the fact that TERRA can interact with TLC1 in yeast, argues that TERRA nucleates telomerase *in cis* specifically to shortened telomeres, where it accumulates (Cusanelli, Romero and Chartrand, 2013; Moravec *et al.*, 2016). Additionally, TERRA may promote telomere elongation in telomerase negative cells through different mechanisms, including R-loop-mediated telomere recombination (Balk *et al.*, 2013; Graf *et al.*, 2017; Hu *et al.*, 2019).

TERRA may also prevent activation of DNA damage response at telomeres, thereby promoting telomere stability. One possibility is that TERRA regulates *in cis* the stability of the telomere from which it is transcribed. Another option is that TERRA transcribed from one telomere acts *in trans* to regulate stability of all telomeres. Most studies support that TERRA acts *in cis* to promote telomere integrity (Episkopou *et al.*, 2014; Porro, Feuerhahn and Lingner, 2014; Feretzaki, Renck Nunes and Lingner, 2019). However, studies from Maria Blasco's group suggest that TERRA can act *in trans*, as removal of TERRA locus from chromosome 20q in human ALT cells (Montero *et al.*, 2016) and chromosome 18q in murine cells (López de Silanes *et al.*, 2014) increases telomere dysfunction.

The mechanisms through which TERRA promotes telomere stability are not fully understood, particularly in human cells. One possibility is that TERRA facilitates POT1 localization to telomeres and RPA displacement in human cells (Flynn *et al.*, 2011; Flynn, Chang and Zou, 2012). Another possibility is that TERRA promotes telomere integrity upon stress conditions. In support of this, TERRA levels in human cells increase upon heat stress and treatment with chemotherapeutic drug etoposide (Tutton *et al.*, 2016; Koskas *et al.*, 2017). Likely, increased TERRA levels prevent telomere dysfunction under these conditions.

Notably, TERRA transcription may regulate the epigenetic status of telomeres, which may affect the regulation of telomere length and telomere stability. Indeed, data from the Decottignies lab suggest that TERRA transcripts originated from long telomeres facilitate the accumulation of H3K9me3 and HP1 to repress their own transcription (Arnoult, Van Beneden and Decottignies, 2012). In addition, TERRA may associate to heterochromatin marks like H3K9me3 and heterochromatin factors including HP1, SUV39H1 or ORC complex (Bettin, Oss Pegorar and Cusanelli, 2019). Possibly, the interaction of TERRA with these factors nucleates heterochromatin marks at telomeres (Deng *et al.*, 2009; Takahama *et al.*, 2013; Bettin, Oss Pegorar and Cusanelli, 2019). In human ALT cells, TERRA mediates heterochromatin formation in a process mediated by the Polycomb Repressive Complex 2 (PRC2) (Montero *et al.*, 2018). It is therefore possible that by maintaining a heterochromatic state, TERRA facilitates ALT activities (Montero *et al.*, 2018; Gauchier *et al.*, 2019). Future research will elucidate the mechanisms that drive TERRA-mediated telomere length maintenance or telomere integrity.

Conversely, aberrant accumulation of TERRA may promote telomere dysfunction (Azzalin *et al.*, 2007; De Silanes, D'Alcontres and Blasco, 2010; Montero *et al.*, 2016). Further, dysfunctional human telomeres increase TERRA levels (Porro *et al.*, 2014), which results in the recruitment of factors like LSD1/MRN or SUV39H1 (Porro *et al.*, 2014). These factors may further contribute to telomere dysfunction.

Altogether, these studies suggest that TERRA acts as a scaffold to recruit different factors to telomeres. Recruitment of these factors may therefore promote telomere function (Figure 6). However, balanced TERRA levels are required to promote telomere stability, as unscheduled accumulation of TERRA may impair telomere integrity.

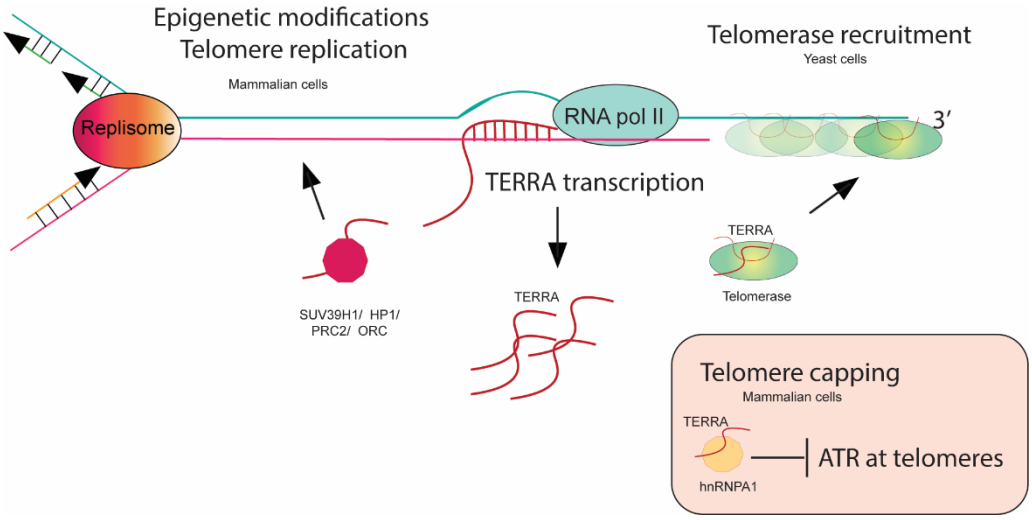


Figure 6 *TERRA interacts with different factors to promote telomere integrity*

TERRA transcripts (represented as red lines) recruit different factors that contribute to epigenetic modifications at telomeres. Recruitment of certain factors may facilitate telomere replication and epigenetic modifications. TERRA transcripts may recruit telomerase to short telomeres in yeast cells. TERRA transcripts may promote telomere capping by interacting with hnRNPA1 in human cells. Reviewed by Bettin, Oss Pegorar and Cusanelli, 2019.Abbreviations: TERRA: telomeric repeat containing RNAs. ATR: ataxia telangiectasia and Rad3 related. Checkpoint kinase implicated in DNA damage signaling and cell cycle arrest.

TERRA localization

Telomere transcripts localize to the nucleoplasm and associate to chromosome ends (Azzalin *et al.*, 2007; Luke *et al.*, 2008; Porro *et al.*, 2010; Balk *et al.*, 2013). In yeast and human cells, telomere associated TERRA forms a three-stranded nucleic acid structure called an R-loop, where the association of TERRA to the C-rich telomeric strand displaces the G-rich telomeric strand (Balk *et al.*, 2013; Arora *et al.*, 2014). TERRA R-loops may negatively affect telomere replication, as they pose barriers to the replication machinery (Aguilera and García-Muse, 2012; Graf *et al.*, 2017). Therefore, different mechanisms regulate TERRA R-loop balance to preserve telomere integrity. On the other hand, non-chromatin associated TERRA may serve as a scaffold to recruit different nuclear factors that promote telomere integrity (De Silanes, D'Alcontres and Blasco, 2010; Porro *et al.*, 2010; Scheibe *et al.*, 2013).

Localization of TERRA RNA may change depending on physiological conditions. For example, in yeast, TERRA relocalizes to the nuclear periphery and cytoplasm during diauxic shift (Perez-Romero *et al.*, 2018). In these conditions, oxidative stress increases as a consequence of oxidative respiration. Therefore, TERRA relocalization may be linked to telomere oxidative damage, at least in budding yeast (Perez-Romero *et al.*, 2018). Additionally, the Lieberman lab has characterized a fraction of cell free TERRA that localizes to human exosomes (Wang *et al.*, 2015; Wang and Lieberman, 2016). Presumably, after telomere dysfunction, accumulated TERRA molecules get released into body fluids in the form of exosomes. This facilitates cytokine production and macrophage-mediated elimination of cells with dysfunctional telomeres (Wang *et al.*, 2015; Wang and Lieberman, 2016). As a result, this mechanism

would likely prevent tissue degeneration. However, it is also possible that excessive cell-free TERRA-containing exosomes affect telomere integrity of surrounding cells. Future investigation will shed light into the function of cell-free TERRA and its implication in human disease.

RNA-DNA hybrids

RNA-DNA hybrids are molecules composed of ribo- and deoxyribo- nucleotides. Two types of RNA-DNA hybrids have been described, depending on their origin, nature and structure. First, RNA-DNA hybrids may result from rNTPs incorporation into a DNA backbone during DNA replication (Joyce, 1997; McElhinny *et al.*, 2010; Nick McElhinny *et al.*, 2010). Therefore, the resulting molecule is composed of a DNA backbone with intercalated rNMPs, which increase the risk of DNA hydrolysis especially in alkaline conditions (McElhinny *et al.*, 2010). Second, RNA molecules can base-pair with DNA strands. In particular, when an RNA molecule associates with one strand of DNA and displaces its pairing DNA strand, the resulting three-strand nucleic acid structure is called an R-loop (Aguilera and García-Muse, 2012; Skourti-stathaki and Proudfoot, 2014; Costantino and Koshland, 2015; José M. Santos-Pereira and Aguilera, 2015). R-loops arise from transcription processing, and maybe also from *in trans* annealing of RNAs to a DNA duplex in a RecA/Rad51-dependent manner (Kasahara *et al.*, 2000; Zaitsev and Kowalczykowski, 2000; Wahba *et al.*, 2011). R-loops are localized to different regions in the genome including telomeres and non-coding RNAs (ncRNAs) and participate in several biological processes (José M. Santos-Pereira and Aguilera, 2015). Unscheduled R-loop formation on the other hand, poses a threat to genome stability, as R-loops may cause transcription-replication conflicts and replication stress (Aguilera and García-Muse, 2012). As a consequence, cells have evolved several mechanisms to regulate R-loop levels and therefore preserve genome integrity.

Several studies indicate that R-loops accumulate at telomeres (Balk *et al.*, 2013; Arora and Azzalin, 2015). In fact, several studies suggest that telomeric R-loops are particularly important in ALT and telomere replication stress (Arora *et al.*, 2014; Maestroni, Matmati and Coulon, 2017b). Understanding the function and regulation of both R-loops and RNA-DNA hybrids genome-wide may provide insights into the mechanisms that regulate telomeric R-loops. In the future, this may help understanding the impact of telomeric R-loops in telomere integrity.

In the following section, different roles of R-loops will be discussed, particularly in the context of genome instability and telomeres.

R-loop formation

The exact mechanism by which R-loops are formed remains unclear, although many studies propose that R-loops form *in cis* during the transcription process. One possibility, called the extended hybrid model, is that the RNA transcribed by the RNA polymerase remains annealed to its DNA template due to the high stability of the interaction between RNA and DNA molecules (Roberts and Crothers, 1992). After this initial association, the RNA polymerase may extend the R-loop as it transcribes the DNA template. Another possibility, known as the thread back model, is that nascent transcripts denature shortly from their DNA template but associate back at a later stage, before the two complementary DNA strands anneal together (Roy, Yu and Lieber, 2008). In support of this, the Lieber lab demonstrated that R-loop formation during transcription is sensitive to RNase treatment, which suggests that transcripts exist shortly in a ‘non associated form’ (Roy, Yu and Lieber, 2008).

Formation of an R-loop relies, at least partially, on the transcribed DNA template sequence. Several studies have shown that R-loops are more prone to form at loci where the non-template DNA strand is G rich (Aguilera and García-Muse, 2012). The asymmetric distribution of Gs and Cs on the template DNA is called GC skew and it is one of the positive regulators of R-loop formation. Indeed, G-clustering initiates R-loop formation and subsequent G-rich sequences on the template strand facilitates R-loop

elongation (Roy and Lieber, 2009). Further, the distance from G-rich sequences to promoter regions may determine the efficiency of R-loop formation, as G-rich sequences located further from promoters decrease R-loop formation (Roy *et al.*, 2010). As chromosome ends contain GC rich sequences, telomeres are prone to R-loop formation.

In addition to the GC skew, two other factors contribute to R-loop formation. On the one hand, negative DNA supercoiling behind the transcription machinery may promote R-loop formation, as it may transiently open the transcribed DNA and facilitate RNA association (Roy *et al.*, 2010). On the other hand, single strand nicks on the non-template DNA may increase R-loop formation. This is likely due to the nicked non-template DNA being transiently displaced, thus facilitating the RNA binding to the template DNA strand (Roy *et al.*, 2010). Similarly, formation of G4 quadruplexes on the non-templated strand may promote R-loop formation by trapping out the non-template DNA strand away from re-annealing (Duquette *et al.*, 2004).

The previous studies suggest that R-loops form *in cis* during transcription. However, several observations in bacterial systems showed that, at least *in vitro*, R-loops can form *in trans* in a RecA-mediated reaction (Kasahara *et al.*, 2000; Zaitsev and Kowalczykowski, 2000). Similarly, the Koshland lab reported a similar mechanism using budding yeast, where R-loop formation *in trans* is mediated by Rad51 (Wahba, Gore and Koshland, 2013). Additional evidence for the *in vivo* function of R-loop formation *in trans* is still missing and remains to be elucidated.

As mentioned above, telomeric R-loop formation occur co-transcriptionally, (Episkopou *et al.*, 2014; Porro, Feuerhahn and Lingner, 2014; Feretzaki, Renck Nunes and Lingner, 2019), although the possibility of *in trans* R-loop formation at telomeres remains open (López de Silanes *et al.*, 2014; Montero *et al.*, 2016).

R-loop regulatory functions

RNA-DNA hybrids and R-loops are obligatory intermediates of DNA lagging strand replication and transcription. In addition, RNA-DNA hybrids participate in several biological processes that regulate, among other, gene expression, DNA replication and DNA repair.

R-loop regulation of DNA replication and gene expression

RNA-DNA hybrids allow DNA replication by providing 3'OH substrates to DNA polymerases that cannot synthesize DNA *de novo* (Watson, 1972). Therefore, short stretches of RNA-DNA hybrids called Okazaki fragments facilitate lagging strand DNA replication. Additionally, R-loops coordinate replication initiation of bacterial, viral and mitochondrial DNA. In bacteriophage T4, DNA replication start requires R-loops at origins of replication to provide a 3'OH substrate (Kreuzer and Brister, 2010). Similarly, replication of ColE1-type plasmids in *E. coli* requires R-loops to generate a 3'OH that primes DNA replication (Itoh and Tomizawa, 1980). Studies in mitochondria suggest that processed R-loops initiate replication by providing an RNA primer for DNA polymerases (Baldacci, Chérif-Zahar and Bernardi, 1984; Xu and Clayton, 1996). These studies suggest that R-loop accumulation and processing allows replication of circular DNA in several systems. Therefore, it is tempting to speculate that R-loops might also sustain replication of other circular DNAs, such as T-circles in ALT cells (Pickett and Reddel, 2015; Doksani, 2019).

R-loops coordinate gene expression through different mechanisms including epigenetic regulation (Skourti-Stathaki, Proudfoot and Gromak, 2011; Castellano-Pozo *et al.*, 2013; Skourti-Stathaki and Proudfoot, 2013). Indeed, studies by the Chedin lab demonstrated that active mammalian promoters have a strong GC skew and form R-loops (Ginno *et al.*, 2012). These R-loops prevent DNMT3B1-mediated methylation at promoters, therefore maintaining the corresponding loci transcriptionally active (Ginno *et al.*, 2012). Further, R-loops recruit GADD45 and TET1 to promote local DNA

demethylation at the *TCF21* promoter (Arab *et al.*, 2019). Similarly, R-loops coordinate gene expression through histone modifications that triggers chromatin condensation. This is the case for H3S10-phosphorylation (H3S10-P), an R-loop associated mark that condensates chromatin in different organisms (Castellano-Pozo *et al.*, 2013). At telomeres, little is known about R-loop mediated epigenetic modifications. However, it would be interesting to determine the possible link between R-loop accumulation and telomeric chromatin. In addition to the R-loop mediated histone modifications, R-loops can recruit RNA interference factors to promote heterochromatin formation in fission yeast (Nakama *et al.*, 2012; Skourti-Stathaki, Kamieniarz-Gdula and Proudfoot, 2014). In particular, heterochromatin formation and R-loops accumulated at gene terminators may facilitate transcription termination, possibly by pausing RNA polymerase II (Mischo *et al.*, 2011; Skourti-Stathaki, Proudfoot and Gromak, 2011; Ginno *et al.*, 2013; Skourti-Stathaki, Kamieniarz-Gdula and Proudfoot, 2014). In support of this model, Senataxin-mediated R-loop degradation promotes subsequent Xrn2 recruitment (5'-3' exoribonuclease 2) and transcription termination in mammalian cells (Skourti-Stathaki, Proudfoot and Gromak, 2011). Similarly in yeast, R-loops may pause RNA polymerase II to allow Rat1 exonuclease-mediated transcription termination (Mischo *et al.*, 2011).

Examples of R-loop-mediated gene expression include the floral repressor gene *FLC* in *Arabidopsis* (Sun *et al.*, 2013), Ig class switch recombination in mammalian cells (Aguilera and García-Muse, 2012) and cellular differentiation (Chen *et al.*, 2015). In plants, R-loop stabilization represses the expression of anti-sense long non-coding RNA COOLAIR upon heat conditions to allow flowering (Sun *et al.*, 2013). In B-cells, the displaced G-rich ssDNA strand generated after R-loop formation is targeted by the activation-induced cytidine deaminase (AID). This likely generates a DSB that facilitates CSR (Aguilera and García-Muse, 2012). Finally, R-loops mediate the recruitment of chromatin remodelers and influence pluripotency factors (Chen *et al.*, 2015). These examples show that R-loops allow a very dynamic modulation of gene expression. The implication of telomeric R-loops in gene expression is unclear, although it is possible that they mediate the expression of genes located close to telomeres, depending on the biological contexts.

RNA-DNA regulation of DNA repair

Recent studies suggest that R-loops play a role in DNA repair. Initial observations reported that transcription starts in the proximity of a DSBs and that R-loops accumulate at these sites to facilitate recruitment of repair factors (Britton *et al.*, 2014; Michelini *et al.*, 2017). In 2016, Ohle et al. proposed that RNA-DNA hybrids act as DSB repair intermediates in fission yeast (Ohle *et al.*, 2016) . The authors suggested that, after the generation of DSBs, transcription initiates at DNA breaks. This facilitates chromatin remodeling and initiates DNA repair processes, such as Exo1-mediated resection. RNA-DNA hybrids may subsequently stall RNA polymerase II to prevent excessive transcription and aberrant resection around the DSB. To complete DNA repair, RNA-DNA hybrids must be degraded by RNase H enzymes. Although the results of this study are controversial (Zhao *et al.*, 2018), they raised the possibility of RNA-DNA hybrids and perhaps R-loops participating in DNA repair.

Similarly, in human cells, the accumulation of RNA-DNA hybrids at DSBs facilitates the recruitment of repair factors and mediates DNA repair (D'Alessandro *et al.*, 2018; Teng *et al.*, 2018). This RNA-DNA hybrid accumulation at DSBs may derive from active *de novo* transcription after DSB resection and Drosha-mediated processing (D'Alessandro *et al.*, 2018; Lu *et al.*, 2018).

The importance of the RNA-DNA hybrids in the repair process is clear, as impairment of RNA-DNA hybrid formation at DSBs severely impairs the recruitment of HR factors in mammalian cells (D'Alessandro *et al.*, 2018; Teng *et al.*, 2018). However, the exact mechanism through which RNA-DNA hybrids promote DNA repair is unclear and may depend on the nature of the DNA damage. For example, while RNA-DNA hybrids accumulating at ROS-induced DNA damage sites activate HR repair

through transcription-coupled nucleotide excision repair (TC-NER) factor CSB (Cockayne syndrome group B) (Teng *et al.*, 2018), nucleases-induced DSB repair may require RNA-DNA hybrids in a BRCA1/2-mediated mechanism (D'Alessandro *et al.*, 2018). Additionally, RNA-DNA hybrids may promote different types of repair, including NHEJ and HR in human cells (Lu, 2018). Indeed, Yasuhara et al. postulated that RNA-DNA hybrids drive a specific type of repair called transcription-associated homologous recombination repair (TA-HRR) (Yasuhara *et al.*, 2018). Further, the authors suggest that RNA-DNA hybrids regulate repair pathway choice at DSBs, when generated at transcriptionally active sites (Yasuhara *et al.*, 2018). Nevertheless, RNA-DNA hybrids must be tightly balanced to ensure successful DNA repair, as illegitimate RNA-DNA hybrid accumulation at DSB interferes with HR and results in translocations and cell death (Li *et al.*, 2016; Cohen *et al.*, 2018). Several proteins including RNase H2 (D'Alessandro *et al.*, 2018), DDX1 (Li *et al.*, 2016), Senataxin (Cohen *et al.*, 2018) or XPG (Yasuhara *et al.*, 2018) regulate RNA-DNA hybrids at DSBs to ensure DNA repair in human cells. RNA may also template DNA repair after a DSB (Keskin *et al.*, 2014; Mazina *et al.*, 2017). It is therefore possible that RNA-DNA hybrids mediate, at least partially, RNA-templated repair. These data highlight the importance of balanced RNA-DNA hybrid levels in DNA repair.

The implication of RNA-DNA hybrids in DNA repair is particularly interesting in the context of telomeres and ALT, as ALT cells require DNA repair mechanisms such as HR to regulate telomere length. Therefore, telomeric R-loops may directly promote or maybe facilitate telomere recombination in the absence of telomerase. To do so, telomeric R-loops may perhaps facilitate the recruitment of specific factors that promote HDR and coordinate telomere maintenance.

R-loop associated genome instability

Aberrant R-loop accumulation can be a source of genome instability in multiple ways. On the one hand, the exposed ssDNA strand may be a target for specific enzymes that introduce DNA modifications or generate single-strand DNA breaks (Aguilera and García-Muse, 2012; Hamperl and Cimprich, 2014; Skourti-stathaki and Proudfoot, 2014). On the other hand, R-loop accumulation may cause DSBs as a consequence of transcription-replication conflicts (Aguilera and García-Muse, 2012; Hamperl and Cimprich, 2014; Skourti-stathaki and Proudfoot, 2014; Crossley, Bocek and Cimprich, 2019). Understanding R-loop regulation is therefore critical, as dysregulated R-loops may be an important source of genome instability in cancer cells and other pathologies. In particular at telomeres, R-loops may be a source of replication stress and telomere dysfunction.

The displaced ssDNA of an R-loop may be targeted by DNA modifying enzymes such as AID or Top1. While modifications of R-loop's ssDNA may be beneficial for processes such as CSR (Muramatsu *et al.*, 2000; Belen Gomez-Gonzalez and Andres Aguilera, 2007; Conticello, 2008; Petersen-Mahrt, Harris and Neuberger, 2015), they may also generate DNA nicks that increase genome instability. For example, AID-mediated deamination of cytosines may accumulate deoxy-Uracil (dU) on the ssDNA in mammalian cells. dU may be subsequently removed by the base excision repair (BER) machinery and generate abasic sites and single strand breaks (Hamperl and Cimprich, 2014) (Figure 7A). Additionally, R-loops may generate single strand breaks in a Top1-mediated cleavage in yeast cells (Takahashi *et al.*, 2011) or through RPA-mediated processing (Hamperl and Cimprich, 2014). Replication through nicked DNA as well as spontaneous mutations may generate DSBs and compromise genome integrity (Figure 7B).

Alternatively, R-loops may generate DSBs as a consequence of transcription-replication collisions (Aguilera and García-Muse, 2012; Hamperl and Cimprich, 2014; Skourti-stathaki and Proudfoot, 2014). First, the R-loop may be a physical obstacle that interferes with replication (Gan *et al.*, 2011; Gómez-González *et al.*, 2011). Second, collisions between the replisome and the transcription machinery may

stall the replication fork which, if not repaired, lead to a DSB (Tuduri *et al.*, 2009; Hamperl and Cimprich, 2014). Altogether, these studies suggest that R-loops may trigger DSB formation (Figure 7).

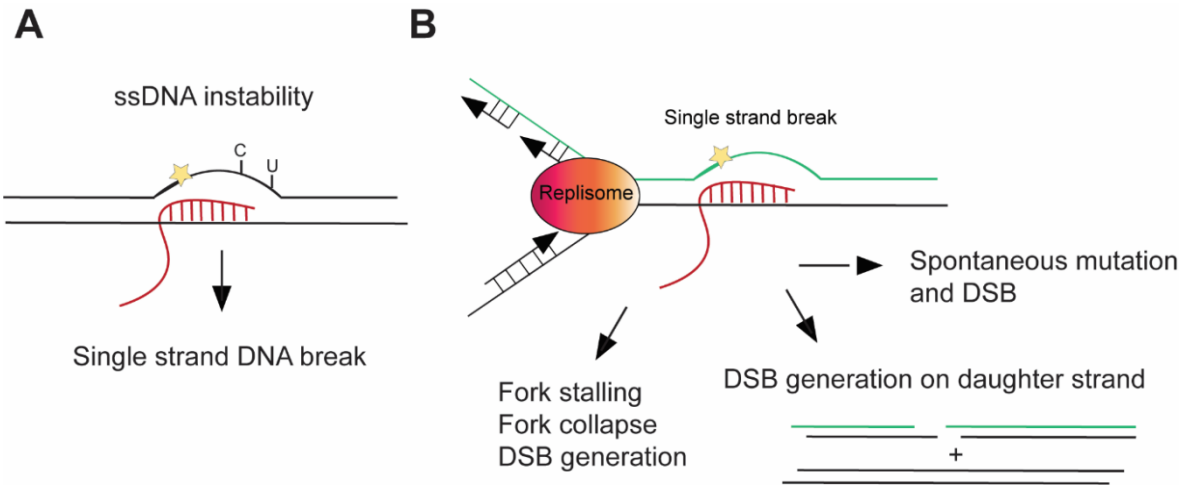


Figure 7 *R-loops trigger genome instability*

A) R-loops are three stranded structures, where one DNA strand anneals with RNA, leaving a single strand DNA strand displaced. Single strand DNA may be modified by specific enzymes (see text), which lead to single strand DNA breaks. Single strand breaks are represented with yellow star. B) R-loop-replisome encounters may generate double strand breaks. Approaching replisomes may replicate loci with accumulated R-loops. Replication through single strand nicked DNA results in a double strand break. Stalling of replisome may collapse replication forks and result in double strand breaks. Spontaneous mutations on single strand nicked DNA may result in double strand breaks. Reviewed by Hamperl and Cimprich, 2014. Abbreviations: ssDNA: single strand DNA. DSB: double strand break.

Transcription-replication collisions may generate DSBs. However, it seems that the orientation of the collision influences the DNA repair response. While co-directional collisions are less severe and decrease the amount of detected R-loops, head-on collisions are more severe and increase the amount of detected R-loops at the site of collision in mammalian cells (Hamperl *et al.*, 2017). This is explained by the fact that a moving replisome may displace the transcription machinery and the R-loop in a co-directional collision, while it may not do so if it encounters transcription machinery or R-loops in a head-on direction (Hamperl *et al.*, 2017). Likely, co-directional collisions are able to displace R-loops because the replisome-associated helicases encounter the intercalated RNA on the leading strand. This phenomenon may allow the unwinding of the RNA component and displacement of the R-loop as replication progresses. Occasionally, co-directional collisions can activate an ATM-mediated checkpoint, suggesting that those collisions generate DSBs (Hamperl *et al.*, 2017). Thus, accumulation of R-loops at different genomic loci may result in different DNA damage responses. At telomeres in particular, transcription and replication conflicts occur in a co-directional orientation, as TERRA transcripts associate with the leading strand template. This would likely promote telomere integrity when telomeric R-loops accumulate, as they may be displaced by a moving replisome. However, data from the Aguilera lab suggests that R-loop binding proteins over stabilize R-loops and protect them from approaching replisomes even in a co-directional encounter (García-Rubio *et al.*, 2018). As a consequence, telomeric R-loops may be a source of genome instability depending on their associated proteins. It is therefore possible that a tight regulation of R-loop regulatory proteins at telomeres may

determine the context when they increase replication stress and genome instability, perhaps depending on telomere length and maintenance mechanism.

The outcome of R-loop-mediated DSBs may depend on the loci where R-loops accumulate (Costantino and Koshland, 2018). Indeed, when R-loops accumulate at unique sequences, they generate DSBs that are processed with extensive resection (Costantino and Koshland, 2018). As a consequence, accumulation of large stretches of ssDNA increase genome instability. Interestingly, if R-loops accumulate at repetitive regions, they increase genome instability in a different way. In fact, R-loop-derived DSBs originated at repetitive regions can prime unidirectional replication and result in genome rearrangements and duplications (Costantino and Koshland, 2018). This mechanism may be relevant for ALT telomeres, as R-loop-mediated replication conflicts may therefore facilitate break-induced replication and telomere elongation.

Finally, it has been proposed that R-loops *per se* may not be the source of genome instability, but rather the chromatin modifications that derive from R-loop accumulation. Indeed, R-loops increase the H3S10-P modification and generate chromatin condensation regions (Castellano-Pozo *et al.*, 2013). Further, the Aguilera lab confirmed that preventing H3S10-phosphorylation using specific histone mutants abolished R-loop-mediated genome instability (García-Pichardo *et al.*, 2017). These studies suggest that R-loop mediated chromatin condensation is a source of genome instability, presumably by increasing transcription-replication conflicts. Conversely, R-loop levels may also be affected by histone levels, as histone 1 depletion in *Drosophila* increases the amount of R-loops particularly at heterochromatic regions (Bayona-Feliu *et al.*, 2017). Similarly, certain histone modifications may be required to prevent unscheduled R-loop accumulation (Wahba *et al.*, 2011; Salas-Armenteros *et al.*, 2017). Future research may help understand the interplay between histone levels, histone modifications and R-loop-mediated genome instability. Interestingly, yeast cells with short telomeres show low histone levels (Platt *et al.*, 2013), raising the possibility that low histone levels facilitate the accumulation of telomeric R-loops at short telomeres.

R-loop levels regulation

R-loop levels must be tightly regulated to prevent genome instability. For this reason, cells have evolved several mechanisms that regulate R-loops genome-wide. On the one hand, several proteins coordinate transcription to prevent unscheduled R-loop formation. On the other hand, specialized enzymes that unwind or degrade the RNA within the R-loop regulate R-loop levels.

Scheduled transcription and coordination of R-loop formation

Coordination of transcription and proper packaging of nascent RNAs into functional ribonucleoproteins (RNPs) prevent unscheduled R-loop formation (Aguilera and García-Muse, 2012) (Figure 8). For this reason, mutations in transcription factors and RNA binding proteins (RBP) increase R-loop levels in yeast (Aguilera and Huertas, 2003; Santos-Pereira *et al.*, 2013; Gavaldá *et al.*, 2016). RBP include a myriad of factors including heterogeneous nuclear ribonucleoproteins (hnRNPs) or splicing factors. One hypothesis is that functional RNPs trap the nascent RNA and physically separate it from its DNA template. This separation is possible by targeting the RNPs to the nuclear pores (García-Benítez, Gaillard and Aguilera, 2017). Indeed, increased physical distance to nuclear pores increases R-loop levels in yeast, likely because transcripts accumulate in the nucleoplasm and re-anneal with their DNA templates (García-Benítez, Gaillard and Aguilera, 2017). Similarly, proteins implicated in splicing prevent unscheduled R-loop formation. For example, the vertebrate splicing factor ASF/SF2 regulates R-loop levels, as ASF/SF2 depletion facilitates R-loop accumulation (Li and Manley, 2005). Other RNA regulatory proteins additionally contribute to the regulation of R-loop levels at different stages of the transcription process (José M Santos-Pereira and Aguilera, 2015).

One important feature of transcription is that it generates negative DNA supercoiling behind the RNA polymerase. This potentially opens up the DNA template and facilitates nascent RNA association to DNA strands (José M. Santos-Pereira and Aguilera, 2015). Regulation of this negative supercoiling by topoisomerases like Top1 can therefore prevent unscheduled R-loop formation and associated transcription-replication conflicts (Tuduri *et al.*, 2009; El Hage *et al.*, 2010).

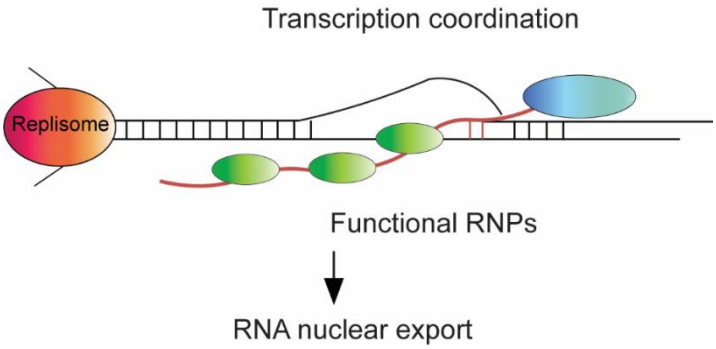


Figure 8 *Transcription coordination prevents unscheduled R-loop formation*

Unscheduled R-loop formation is regulated by transcription coordination, packing of RNA into functional ribonucleoproteins and targeting of RNA to nuclear pores. Blue circle represents RNA polymerases. Green circles represent RNA binding proteins and export factors. Reviewed by Aguilera and Garcia-Muse, 2012. Abbreviations: RNP: ribonucleoprotein.

Importantly, human hnRNPs and yeast hnRNP-like proteins regulate R-loops genome-wide and at telomeres (De Silanes, D'Alcontres and Blasco, 2010; Flynn *et al.*, 2011; Aguilera and García-Muse, 2012; Pfeiffer *et al.*, 2013; Yu, Kao and Lin, 2014; Montero *et al.*, 2016; García-Rubio *et al.*, 2018). In human cells, functional hnRNPs prevent aberrant association of TERRA to telomeres and promote telomere integrity (De Silanes, D'Alcontres and Blasco, 2010). Additionally, human hnRNPs interact with TERRA to promote end protection (Flynn *et al.*, 2011). Yeast hnRNP-like proteins regulate telomeric R-loops to promote telomere stability and prevent anticipated senescence onset (Lee-Soety *et al.*, 2012; Pfeiffer *et al.*, 2013; Yu, Kao and Lin, 2014; García-Rubio *et al.*, 2018). Altogether, these studies show the importance of hnRNPs and hnRNP-like proteins in the regulation of balanced telomeric R-loops.

R-loop resolution

R-loop levels can be balanced by resolution of their three-stranded structure. This may be achieved, for example, by unwinding or degradation of the R-loop RNA component. In particular, specialized helicases unwind the RNA component of R-loops.

One of the best characterized helicases resolving R-loops is the human 5' to 3' helicase Senataxin, encoded by the gene SETX (Skourti-Stathaki, Proudfoot and Gromak, 2011). Physiologically, Senataxin promotes transcription termination (Skourti-Stathaki, Proudfoot and Gromak, 2011) and efficient DSB repair (Cohen *et al.*, 2018) by unwinding R-loops. Mutations in SETX result in Ataxia-ocular apraxia 2 (AOA2) (Moreira *et al.*, 2004), which demonstrates the importance of balanced R-loop levels in human cells. Similarly, yeast helicase Sen1 regulates R-loops and therefore prevents transcription replication conflicts (Mischo *et al.*, 2011).

Additional helicases like Pif1 or yeast Rrm3 may participate in R-loop unwinding to promote DNA replication, particularly at telomeres and centromeres (Pohl and Zakian, 2019). Interestingly, replisome associated helicases may facilitate replication progression through R-loop-containing regions, if they encounter the R-loop RNA on the leading strand (Hamperl and Cimprich, 2014; Hamperl *et al.*, 2017).

This mechanism may be important at telomeres, as replisome encounters TERRA molecules associated to the leading strand.

Recently, Dead-box helicases have been identified as R-loop regulatory proteins. For example, in yeast, Dbp2 regulates R-loop levels likely by cooperating with Sen1 (Tedeschi *et al.*, 2018). In mammalian cells, DDX21 resolves R-loops to promote genome stability (Song *et al.*, 2017). Interestingly, dead-box helicases like DDX39 play a role in telomere protection (Yoo and Chung, 2011), which opens ups new possibilities for the regulation of telomeric R-loops.

R-loop degradation

R-loop levels can be regulated by hydrolyzing their RNA moiety. This important function is carried out by RNase H enzymes (Stein and Hausen, 1969; Cerritelli and Crouch, 2009). Two types of RNase H enzymes have been described in eukaryotes (Figure 9). RNase H type I (RNase H1) is a monomeric protein, with highly conserved N- and C- terminal regions among eukaryotes (Cerritelli and Crouch, 2009). The N-terminal region contains the hybrid binding domain (HBD), which is highly specific for RNA-DNA hybrids (Nowotny *et al.*, 2008). The central region of RNase H1, named connection domain, is less conserved among eukaryotes. Presumably, this domain provides a flexible linker between the N- and C- terminal regions so that they can act on their substrates (Cerritelli and Crouch, 2009). The C-terminal regions of RNase H1 contain the RNase H domain, which cleaves RNA associated to DNA. The RNase H domain of RNase H1 requires at least four consecutive ribonucleotides to degrade the RNA in the hybrid (Cerritelli and Crouch, 2009). This turns RNase H1 into a specialized enzyme that hydrolyses 'long R-loops'. In a recent study, Nguyen et al., showed that RPA promotes RNase H1 recruitment to R-loops and increases its cleavage activity (H. D. Nguyen *et al.*, 2017). These data suggest that RNase H accessory factors cooperate for proper R-loop regulation genome-wide.

RNase H1 activity is important to preserve genome integrity, as it prevents toxic accumulation of R-loops and mediates certain biological processes (Cerritelli and Crouch, 2009; José M. Santos-Pereira and Aguilera, 2015). Indeed, RNase H1-mediated processing facilitates DNA replication in bacteria and mitochondrial DNA (Itoh and Tomizawa, 1980; Xu and Clayton, 1996; Cerritelli *et al.*, 2003). As mentioned above, RNase H1 is also important for efficient DSB repair, as prolonged accumulation of R-loops at DNA breaks impairs DNA repair mechanisms (Ohle *et al.*, 2016; Cohen *et al.*, 2018; D'Alessandro *et al.*, 2018). It would be interesting to study if RNase H1 activity generates an RNA template that primes DNA repair, similar to its role in DNA replication. Further, it would be very interesting to study if this putative mechanisms regulates telomere recombination in ALT cells.

RNase H type II (RNase H2) is a trimeric complex (Jeong *et al.*, 2004; Rice *et al.*, 2007; Chon *et al.*, 2008). In yeast, RNase H2 comprises Rnh201, 202 and 203 (Jeong *et al.*, 2004) whereas in human cells the RNase H2 subunits are called RNASEH2A, H2B and H2C (Rice *et al.*, 2007; Chon *et al.*, 2008). The catalytic activity of RNase H2 lies within the Rnh201subunit in yeast (Nguyen *et al.*, 2011) and the human subunit RNASEH2A (Rice *et al.*, 2007). The other two subunits are likely structural components required for either RNase H activity or localization (Cerritelli and Crouch, 2009). Active RNase H2 degrades stretches of RNA-DNA hybrids as well as single ribonucleotides incorporated into DNA (Eder, Walder and Walder, 1993; Murante, Henricksen and Bambara, 1998; Cerritelli and Crouch, 2009).

RNase H2 activity may be an important mediator of replication and ribonucleotide excision repair (RER), as it may regulate short RNA-DNA hybrids and miss-incorporated ribonucleotides into newly synthesized DNA (Rydberg and Game, 2002; McElhinny *et al.*, 2010; Sparks *et al.*, 2012). In particular, RNase H2 participates in Okazaki primer removal to promote successful replication (Murante, Henricksen and Bambara, 1998). This mechanism might facilitate discrimination between parental and daughter DNA strands, as newly synthesized strands may include rNMPs and recruit RER factors. At least in mammalian cells and yeast, it is likely that these two activities of RNase H2 are facilitated by

the interaction between RNASEH2B/Rnh202 and PCNA through their PIP domain (Chon *et al.*, 2008; Nguyen *et al.*, 2011).

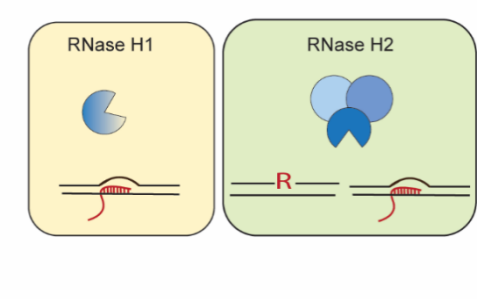


Figure 9 *RNase H enzymes regulate RNA-DNA hybrids levels*

RNase H1 enzymes (left) are monomeric and degrade the RNA moiety in long stretches of R-loops. RNase H2 enzymes (right) are trimeric complexes and degrade both ribonucleotides incorporated into a DNA backbone, as well as R-loops. Reviewed by Cerritelli and Crouch, 2009.

The majority of the RNase H activity in yeast cells is carried out by RNase H2 (Zimmer and Koshland, 2016). This phenomenon may be explained by several factors. First, cell cycle regulation of RNase H activity may distinguish the two types of enzymes (Lockhart *et al.*, 2019). Second, RNase H1 activity may be restricted to specific loci, while RNase H2 may have a more general activity (Skourti-stathaki and Proudfoot, 2014; Zimmer and Koshland, 2016). Third, it is possible that different types of R-loops exist in cells and are regulated differently by the two RNase H enzymes. Deletion of *RNH1* or *RNH201* increases genome instability in yeast, although the damage extent and impact seems to differ between the two genes (Wahba *et al.*, 2011; Conover *et al.*, 2015; O'Connell, Jinks-Robertson and Petes, 2015).

RNase H enzymes regulate R-loop levels genome wide and at telomeres (Balk *et al.*, 2013; Arora *et al.*, 2014; Graf *et al.*, 2017). In particular, RNase H1 is important in human ALT cells to keep balanced levels of telomeric R-loops that sustain HR (Arora *et al.*, 2014). Additionally, yeast RNase H2 is recruited to long telomeres to prevent aberrant R-loop accumulation (Graf *et al.*, 2017). Interestingly, this association decreases at short telomeres, which allows R-loop accumulation and HR (Graf *et al.*, 2017). Therefore, RNase H enzymes contribute to telomere length regulation and cell viability.

Lastly, it is important to note that RNase H enzymes provide a useful tool to study R-loop regulation, both *in vitro* and *in vivo* (Rydberg and Game, 2002; Sparks *et al.*, 2012).

Other R-loop regulatory mechanisms

Different pathways regulate R-loops genome wide. For example, histone deacetylation coordinates chromatin compaction through its interaction with human THO complex and prevent unscheduled R-loop formation during transcription (Salas-Armenteros *et al.*, 2017). Therefore, it is possible that at yeast long telomeres, Sir proteins-mediated deacetylation regulates balance telomeric R-loops.

Additionally, sirtuin proteins regulate dead-box helicase activity to prevent harmful R-loop accumulation in mammalian cells (Song *et al.*, 2017). Unexpectedly, cohesin SA1/2 also regulates R-loop levels (Wang *et al.*, 2019). This phenomenon may increase genome instability and facilitate cancer progression in cells with mutations in SA1/2 such as Ewing sarcoma cells (Wang *et al.*, 2019).

Altogether, these studies suggest that a complex network of regulatory factors cooperate to maintain balanced R-loop levels genome wide and at telomeres.

Genome instability and human disease

R-loop and RNA-DNA hybrids misregulation increase genome instability and impact human health. For this reason, mutations in R-loop regulatory genes may result in different human diseases. For example, mutations in *SETX* cause different neurodegenerative diseases (Chen *et al.*, 2004; Moreira *et al.*, 2004).

Similarly, mutations in RNase H2 cause Aicardi-Goutieres syndrome (AGS), a neurological disease characterized by increased accumulation of ribonucleotides in DNA (Crow *et al.*, 2006; Crow and Rehwinkel, 2009). Interestingly, R-loops affect the expansion of repetitive sequences, which may therefore have implications in nucleotide expansion diseases such as Friedreich ataxia (Groh *et al.*, 2014). Other mutations that perturb the physiological R-loop balance may cause different disorders including Angelman syndrome and Prader-Willi syndrome (Groh and Gromak, 2014).

In addition to neurological disorders, R-loops may contribute to cancer progression, as they trigger genome instability when dysregulated. Indeed, mutations in R-loop regulatory genes are associated to cancer (Chernikova *et al.*, 2012; Bhatia *et al.*, 2014; Groh and Gromak, 2014). Telomeric R-loops may particularly contribute to cancer progression, as their balanced levels ensure cell proliferation of transformed cells (Arora *et al.*, 2014; Silva *et al.*, 2019). Further insights into the mechanisms that drive R-loop associated diseases may facilitate potential therapeutic approaches.

Telomeric R-loop regulation and function

Telomeres are prone to R-loop formation due to their high GC content. Indeed, the existence of telomeric R-loops has been demonstrated in yeast, humans and *Trypanosoma brucei* (Balk *et al.*, 2013; Arora *et al.*, 2014; Nanavaty *et al.*, 2017). Further, studies so far suggest that telomeric R-loops are physiologically relevant.

The origin of telomeric R-loops remains unclear, as telomeric R-loops have been proposed to form both co-transcriptionally and *in trans* (López de Silanes *et al.*, 2014; Arora and Azzalin, 2015; Montero *et al.*, 2016). Further research is required to dissect the mechanisms that regulate telomeric R-loop formation, which may be regulated differently depending on the biological context.

Telomeric R-loop levels inversely correlate with telomere length in yeast (Balk *et al.*, 2013; Graf *et al.*, 2017). At long telomeres, telomeric R-loop levels are kept low, likely to prevent transcription-replication conflicts and telomere shortening (Pfeiffer *et al.*, 2013; Gavaldá *et al.*, 2016; Graf *et al.*, 2017; García-Rubio *et al.*, 2018). To ensure low telomeric R-loop levels at long telomeres, several mechanisms coordinate their formation and degradation. First, long telomeres degrade telomeric R-loops by recruiting RNase H2 in a Rif2-mediated mechanism (Graf *et al.*, 2017). Second, yeast hnRNPs cooperate to prevent unscheduled R-loop formation (Pfeiffer *et al.*, 2013; Yu, Kao and Lin, 2014). As the regulation of telomeric R-loop levels resembles that of genome-wide R-loops, it is tempting to speculate that other R-loop-regulatory proteins, such as helicases like Pif1, may also regulate telomeric R-loops at long telomeres.

When yeast telomeres shorten in the absence of telomerase, telomeric R-loops accumulate at short telomeres due to decreased localization of RNase H2 (Balk *et al.*, 2013; Graf *et al.*, 2017). Particularly, the shortest telomere in a mixed population accumulates telomeric R-loops (Graf *et al.*, 2017). This accumulation allows HDR-mediated telomere maintenance in the absence of telomerase (Balk *et al.*, 2013; Graf *et al.*, 2017). The exact mechanism by which telomeric R-loops promote HDR is not yet known. However, it has been proposed that it depends on replication stress (Balk *et al.*, 2013; Simon, Churikov and Géli, 2016; Graf *et al.*, 2017), as the latter can trigger HDR (Aguilera and Gómez-González, 2008; Hamperl and Cimprich, 2014; Simon, Churikov and Géli, 2016). Possibly, telomeric R-loops generate transcription-replication conflicts that trigger break-induced replication. As a result, R-loop-mediated telomere maintenance may regulate senescence rate and yeast survivor formation (Yu, Kao and Lin, 2014; Graf *et al.*, 2017; Misino *et al.*, 2018; Hu *et al.*, 2019).

Importantly, balanced R-loop levels are required for proper telomere maintenance, especially in ALT cells (Apte and Cooper, 2017; Sobinoff and Pickett, 2017). Therefore, telomeric R-loops accumulated at short telomeres may require processing to prevent excessive replication stress and impaired

recombination. Indeed, over stabilized telomeric R-loops is detrimental for HDR-mediated telomere maintenance in yeast, as they impair type II survivor formation and accelerate senescence rate (García-Rubio *et al.*, 2018). Further, deletion of *SGS1* in telomerase negative cells accelerates senescence onset in yeast (Lee *et al.*, 2007), suggesting that accumulated R-loops may interfere with recombination by generating toxic intermediates. For this reason, functional R-loops accumulated at yeast short telomeres recruit regulatory factors such as the Mph1 helicase (Lafuente-Barquero *et al.*, 2017) and possibly RNase H1. In particular, Mph1 resolves telomeric R-loops, prevents replication stress and regulates senescence rate (Luke-Glaser and Luke, 2012; Lafuente-Barquero *et al.*, 2017). In conclusion, different factors participate in balancing R-loop levels at telomeres to coordinate HDR in the absence of telomerase without resulting in excessive replication stress (Figure 10).

Mammalian telomeres also accumulate R-loops at telomeres (Arora *et al.*, 2014; Pan *et al.*, 2017; Silva *et al.*, 2019). Telomeric R-loops are particularly important in ALT cells, where they sustain recombination of telomeres and cell survival (Arora *et al.*, 2014; Sobinoff and Pickett, 2017). In mammalian cells, telomeric R-loops have been proposed to increase replication stress, generate a DSB at telomeres and initiate telomere recombination through break-induced replication or MiDAS (Robert L. Dilley *et al.*, 2016; Roumelioti *et al.*, 2016a; Min, Wright and Shay, 2017). However, similar to what was observed in yeast, telomeric R-loops must be tightly regulated, as excessive accumulation of telomeric R-loops impairs telomere replication (Arora *et al.*, 2014). For this reason, cells rely on sophisticated mechanisms that counteract R-loop-derived replication stress and telomere loss (Arora *et al.*, 2014; D. T. Nguyen *et al.*, 2017; Pan *et al.*, 2017; Lee *et al.*, 2018; Silva *et al.*, 2019). In addition to the well described RNase H1 (Arora *et al.*, 2014), the ATRX chromatin remodeling factor (D. T. Nguyen *et al.*, 2017) and the helicase FANCM (Pan *et al.*, 2017; Silva *et al.*, 2019) participate in the removal of telomeric R-loops. Further, members of the shelterin complex may directly participate in the regulation of telomeric R-loops and telomere stability (Lee *et al.*, 2018). Altogether, a myriad of proteins participate in telomeric R-loop regulation in ALT cells to sustain telomere recombination and prevent severe replication stress.

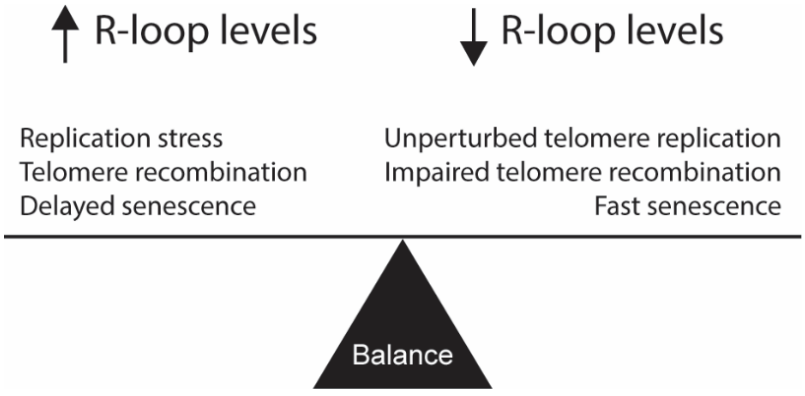


Figure 10 *Balanced telomeric R-loop levels are required for ALT*

Increased R-loop levels at telomeres increases replication stress, telomere recombination and delays senescence onset (left). Decreased R-loop levels at telomeres facilitates telomere replication but impair telomere recombination and accelerate senescence onset. Balanced R-loop levels are required at telomeres to sustain ALT activities and cell proliferation.

Perspectives in telomere regulation and associated proteins

Many studies have demonstrated the importance of telomere associated proteins in telomere function. In the recent years, several new factors were linked to telomere biology. In particular, hnRNPs and other R-loop regulatory factors are drawing much attention, as they may be important regulators of ALT activities and telomere integrity.

hnRNPs, telomeres and genome stability

A family of proteins called heterogenous nuclear ribonucleoproteins (hnRNPS) regulate gene expression by coordinating different features of RNA metabolism, including transcription and mRNA nuclear export (Geuens, Bouhy and Timmerman, 2016). In both yeast and humans, it has been shown that some hnRNPs and hnRNP-like proteins remove nascent RNA transcripts from their DNA template and prevent unscheduled R-loop formation (Chan, Hieter and Stirling, 2014; José M. Santos-Pereira and Aguilera, 2015). By doing so, these hnRNPs prevent genome instability. On the other hand, recent studies suggest that R-loops are essential mediators of DNA repair. These suggests that hnRNPs and other R-loop regulatory proteins directly mediate DNA repair mechanisms and further contribute to genome integrity. Mutations in hnRNP factors are prevalent in human cancers, as they likely increase genome instability (Chan, Hieter and Stirling, 2014).

In addition to their global regulation of genome stability, certain hnRNP proteins like hnRNPA1 promote telomere function through different mechanisms. Mammalian hnRNPs, for example, regulate telomere length maintenance (LaBranche *et al.*, 1998; Moran-Jones *et al.*, 2005; Huang, Hung and Wang, 2010), at least partially by stimulating telomerase activity (Zhang *et al.*, 2006). Additionally, hnRNPs associate with TERRA and regulate TERRA levels to prevent telomere dysfunction (De Silanes, D'Alcontres and Blasco, 2010). In particular, hnRNPA1 may prevent telomere dysfunction by associating TERRA and coordinating the localization of POT1 to telomeres (Flynn *et al.*, 2011). This and other functions of hnRNPA1 facilitate telomere replication and telomerase-mediated telomere elongation (Zhang *et al.*, 2006; Flynn *et al.*, 2011; Redon, Zemp and Lingner, 2013; Sui *et al.*, 2015). Interestingly, over expression of hnRNPA3 shortens telomeres (Huang, Hung and Wang, 2010), suggesting that telomere function requires a balanced association of hnRNPs to chromosome ends.

Similarly, yeast hnRNP-like proteins promote telomere integrity. For example, the yeast THO complex prevents unscheduled R-loop formation at telomeres and mediates senescence rate (Pfeiffer *et al.*, 2013; Yu, Kao and Lin, 2014). Indeed, deletion of *THO2* or *HPR1* anticipates senescence onset, likely due to increased telomeric R-loop levels (Yu, Kao and Lin, 2014). Deletion of the hnRNP-like *NPL3* increases R-loop levels genome-wide and accelerates senescence rate (Lee-Soety *et al.*, 2012; Santos-Pereira *et al.*, 2013). On the other hand, stabilization of telomeric R-loops by *YRA1* over expression anticipates senescence onset and impairs telomere recombination (Gavaldá *et al.*, 2016; García-Rubio *et al.*, 2018). These studies demonstrate that also in yeast, balanced hnRNP activity at telomeres coordinates telomere integrity in the presence and absence of telomerase.

Npl3

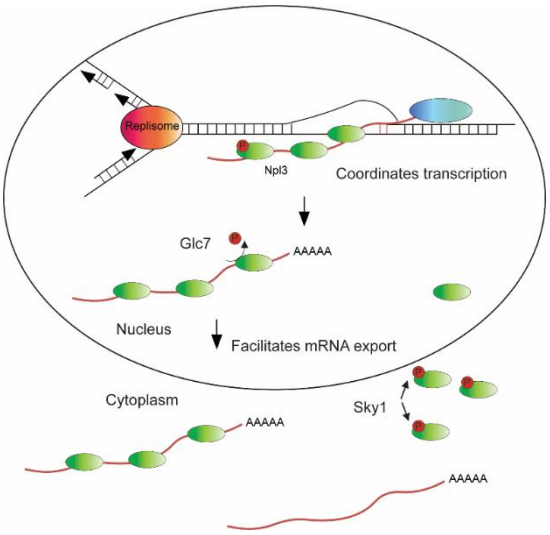


Figure 11 *Npl3 coordinates transcription, RNA export and genome stability*

Npl3 coordinates transcription and prevents unscheduled R-loop formation. Glc7-mediated de-phosphorylation facilitates translocation to cytoplasm and mRNA export. Npl3 return to nucleus is mediated by Sky1 phosphorylation. AAAAA represents polyA tails on messenger RNAs. –P represents phosphorylation of Npl3. Reviewed by Santos-Pereira et al., 2014.

Npl3 is a yeast RNA binding protein that coordinates a diverse number of RNA regulatory processes including transcription, splicing, mRNA export translation and genome integrity (Santos-Pereira *et al.*, 2014). Balanced levels of Npl3 are required for proper gene expression and genome maintenance, as deletion of *NPL3* increases transcription-replication conflicts and over expression of *NPL3* increases recombination rates (Lund, Kress and Guthrie, 2008; Santos-Pereira *et al.*, 2013).

The activities and localization of Npl3 are tightly regulated by posttranslational modifications to ensure proper mRNA processing and export (Figure 11). Indeed, Npl3 interacts with the C-terminal domain of RNA polymerase II and promotes transcription elongation (Lei, Krebber and Silver, 2001; Dermody *et al.*, 2008). Co-transcriptional phosphorylation of Npl3 by Casein Kinase Cka1 promotes the association of Npl3 to the nascent transcript, releasing Npl3 from transcriptional machinery and thus facilitating transcription termination (Bucheli and Buratowski, 2005). As maturation and processing of RNA proceeds, Npl3 is dephosphorylated by Glc7, a nuclear phosphatase that promotes mRNA export. Unphosphorylated Npl3 tethers the export factor Mex67 to mRNPs, facilitating nuclear export of mRNAs after processing (Gilbert and Guthrie, 2004). Finally, Sky1-mediated Npl3 phosphorylation in the cytoplasm promotes the dissociation between Npl3 and cytoplasmic mRNA, to facilitate the nuclear re-localization of Npl3 through an interaction with Mtr10 (Gilbert, Siebel and Guthrie, 2001).

Importantly, Npl3 participates in DNA repair and genome stability maintenance. First, by promoting the proper formation of mRNPs, Npl3 has been proposed to prevent unscheduled R-loop formation at transcribed regions (Santos-Pereira *et al.*, 2013, 2014). This hypothesis is supported by the increased replication stress observed in *npl3* cells, which is partially reduced by *RNH1* over expression (Santos-Pereira *et al.*, 2013). Further, deletion of *NPL3* increases sensitivity to genotoxic agents, suggesting that Npl3 prevents DNA damage accumulation (Santos-Pereira *et al.*, 2013). One possibility is that *NPL3* promotes DSB resection by ensuring proper *EXO1* biogenesis (Colombo *et al.*, 2017). However, additional mechanisms may participate in genome maintenance. For example, Npl3 phosphorylation by Mec1/Tel1/Rad53 kinases may re-localize it to sites of damage to promote DNA repair (Smolka *et al.*, 2007). Future research will elucidate the specific role and regulation of Npl3 upon genome instability.

Deletion of *NPL3* results in a premature senescence onset in telomerase negative cells (Lee-Soety *et al.*, 2012). Given that other yeast hnRNP-like proteins coordinate telomere transcription and telomeric R-loops (Pfeiffer *et al.*, 2013; Yu, Kao and Lin, 2014; García-Rubio *et al.*, 2018), it would be interesting

to elucidate if the accelerate senescence observed in *npl3 tlc1* cells is a consequence of telomeric R-loops mysregulation.

Rationale

Telomeres safeguard genome integrity by protecting chromosome ends. Importantly, chromosome end protection highly relies on telomere associated proteins. Deep characterization of telomere binding proteins is essential to understand telomere biology and dissect regulatory mechanisms that may have implications in human disease. Budding yeast offer good models to study different aspects of telomere biology. In particular, yeast models offer great advantages for the study of replicative senescence and telomere maintenance in the absence of active telomerase. Importantly, yeast studies may provide insights into how ALT cells keep their proliferative capacity as well as how they arise from a population of cells without active telomerase. Therefore, yeast studies may contribute to our understanding of ALT cells and cancer progression.

In this work, we aim to study the molecular mechanisms that coordinate telomere length regulation using budding yeast as a model organism. Yeast telomeres are associated withan array of proteins that ensure their functionality. However, even though some factors remain telomere associated in different conditions, some may transiently bind telomeres to facilitate specific processes. Quantitative interactomics is a powerful tool to identify factors implicated in different biological contexts (Kappei *et al.*, 2013, 2017; Aeby *et al.*, 2016; Jahn *et al.*, 2017; Li *et al.*, 2017) In this study, we used quantitative interactomics to identify novel telomere associated proteins in *S. cerevisiae*. In particular, we were interested in characterizing factors that associate to short telomeres and may regulate senescence rate and R-loops in yeast. With this approach, we aim to expand our understanding of telomere associated proteins and how TERRA R-loops regulate senescence rate in yeast. Our data may provide mechanistic insights into how R-loops coordinate telomere maintenance and HDR. Further, our results may facilitate understanding of how R-loops are regulated to promote repair at DSBs.

Results

Identification of telomere associated proteins in *S.cerevisiae*

To identify proteins associated to telomeres in *S.cerevisiae*, we performed quantitative label-free interactomics. Briefly, we synthesized biotinylated DNA baits with a telomeric sequence and performed affinity purification using protein extracts from wild type cells (WT). We controlled for sequence binding specificity of the proteins by using a different DNA sequence (Figure 12A). With this approach, we identified 69 proteins that specifically associated to telomeric sequences *in vitro* (Figure 12, 13). Importantly, among them we identified Rap1, the well-characterized telomeric protein in *S.cerevisiae*, validating our experimental approach (Figure 12). Additionally, we identified the RNA binding protein Npl3, which we further characterized at yeast telomeres (see below).

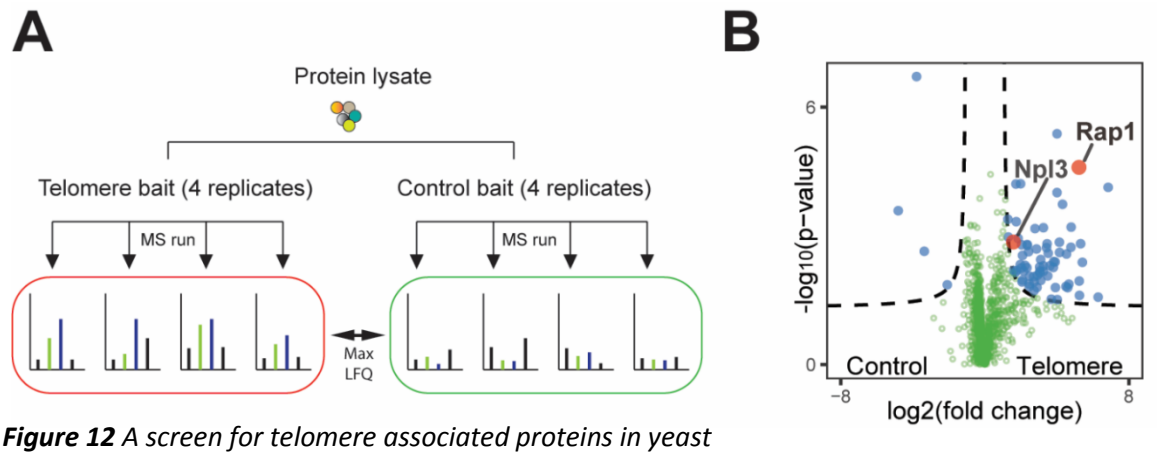


Figure 12 *A screen for telomere associated proteins in yeast*

A) Schematic representation of the quantitative interactomics approach to identify proteins binding to the *S. cerevisiae* telomeric sequence. B) Volcano plot of quantified proteins using WT protein lysates. Log_2 fold change was determined as the difference between the mean LFQ intensity of the four replicates of telomeric to control sequence and p-values were calculated with a Welch t-test. Proteins above the threshold p=0.05, s0=1, c=0.5 (dashed line) are considered enriched. Abbreviations: MS: mass spectrometry-based; LFQ: label free quantitation.

Gene Ontology (GO) analysis shows that the identified telomere interactors are enriched for metabolic processes, gene expression, nucleic acid binding, RNA binding and DNA binding (Figure 13B). In fact, 32 proteins were annotated as RNA binding proteins (RBPs) (Figure 13A) and some of the candidates with the highest log2 LFQ intensity were annotated as DNA/RNA helicases (Figure 13A). Interestingly,

some of the identified RBPs interact with each other (Figure 13C), which suggests that several RBPs may cooperate to regulate telomeres.

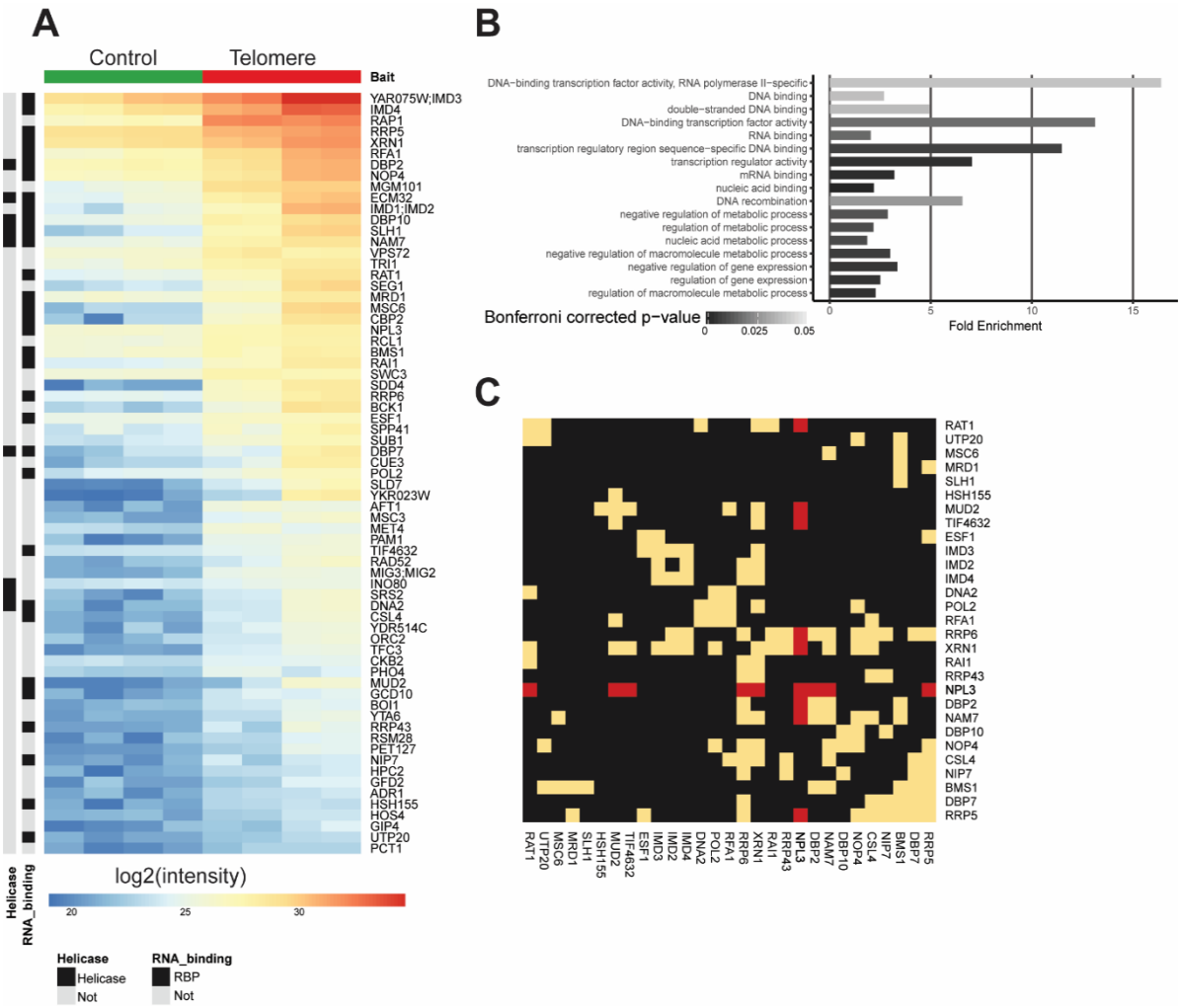


Figure 13 *Identification of telomere associated proteins in S. cerevisiae*

A) Heatmap for enriched proteins at the telomere bait in WT protein lysates. Color code indicates measured log2 LFQ intensities in the telomere pull-down (Figure 12B). Heatmap was constructed using the “pheatmap” package in R, where clustering is performed using complete data based on the Euclidean distance. RNA binding proteins and helicases are indicated with a black box. B) Gene ontology enrichment analysis of the telomere-sequence associated interactors. Analysis was performed using PantherDB.org. Bonferroni correction was applied. C) Telomere interactors in WT cell lysates with RNA-binding function form interaction clusters. Biogrid protein interactions are represented as a heatmap. Yellow is used for presence and black is used for absence of an annotated interaction. Clustering is performed using the complete data based on binary distance. Npl3 interactors are highlighted in red. Abbreviations: RBP: RNA binding protein.

To get functional insights into the roles of these candidates at telomeres, we checked for overlap between our telomere-associated proteins and factors that have been previously implicated in the regulation of telomere length and senescence rate (Askree *et al.*, 2004; Gatbonton *et al.*, 2006; Ungar *et al.*, 2009; Chang *et al.*, 2011). Surprisingly, there is little overlap between our telomere associated candidates and proteins identified in other studies as regulators of telomere length (Appendix table 2, see discussion).

Npl3 associates to yeast telomeres in vivo

We decided to characterize one of our telomere binders and hence focused on the hnRNP-like protein Npl3. Npl3 has been proposed to regulate transcription and R-loops genome wide (Santos-Pereira *et al.*, 2014) and, in addition, deletion of *NPL3* anticipates senescence onset in telomerase negative cells (Lee-Soety *et al.*, 2012), which combined with our interaction data is suggestive of a direct effect. Npl3 contains two RNA binding motifs (Figure 14A) (Santos-Pereira *et al.*, 2014), which raises the interesting possibility of Npl3 regulating TERRA transcription/localization. To characterize the *in vivo* binding of Npl3 to telomeres, we employed a TAP-tagged variant of Npl3, which is regulated under the *NPL3* endogenous promoter (Figure 14B). We verified that the TAP-tagged Npl3 variant is fully functional, as cells expressing the TAP-tag variant do not increase the sensitivity to high temperatures, like the *npl3* mutants do (Figure 14C).

Using the Npl3 TAP-tagged variant, we first validated the association of Npl3 to telomeres *in vivo* by performing Chromatin Immunoprecipitation (ChIP) (Figure 14D-F). As *NPL3* regulates senescence rate in telomerase negative cells, we asked whether Npl3 also associates to short telomeres. To test this possibility, we propagated telomerase negative cells (*tlc1*) for 90 populations to shorten their telomeres and performed ChIP-qPCR (Figure 14 D-F). In our conditions, *tlc1* cells at population doubling 90 have short telomeres and experience crisis (Figure 14 D-E). In this scenario, we performed ChIP-qPCR to test binding of Npl3 to critically short telomeres. Surprisingly, our data shows that Npl3 associates more strongly to short telomeres (Figure 14F). Interestingly, there is no increase in protein levels of Npl3 in *tlc1* cells, excluding that changes in Npl3 protein levels are the cause for the stronger association of Npl3 to short telomeres (Figure 14G). Therefore, we conclude that Npl3 localizes to telomeres *in vivo* and this association increases as telomeres shorten.

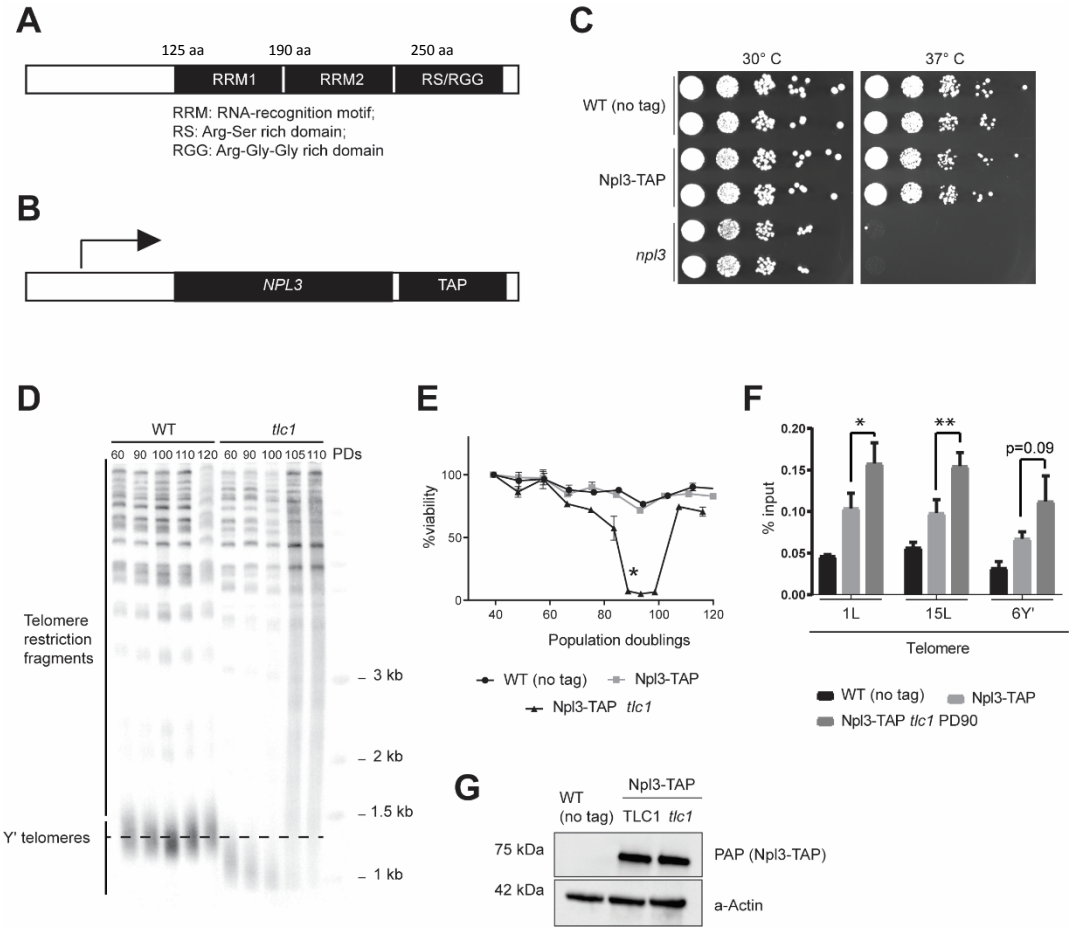


Figure 14 *Functional Npl3-TAP associates strongly to short telomeres*

A) Annotated RNA interaction motifs for Npl3. B-C) Npl3-TAP is functional. B) Npl3-TAP tagged variant representation. Arrow indicates endogenous *NPL3* promoter. C) *npl3* cells are temperature sensitive at 37°C. This sensitivity is not observed in WT or Npl3-TAP cells. Serial dilutions of indicated strains were assayed on YPD. Cells were plated at indicated temperatures and grown for 48h. D) Critically short telomeres arise after 90 populations in telomerase negative cells (*tlc1*). DNA from WT and *tlc1* cells at different population doublings was extracted, digested with XhoI and used for Southern blot. A radioactive probe was used to recognize telomeric DNA. E) Npl3-TAP *tlc1* cells at population doubling 90 are at crisis state due to telomere shortening. Daily cell viability was measured and plotted. %Viability is calculated based on optical densities (OD_{600nm}), normalizing daily values to first time point (referred to as 100%). Asterisk indicates time point of collection for ChIP in Figure 14F. F) Npl3-TAP associates strongly to short telomeres. Cross-linked samples from indicated strains were used in a TAP-ChIP. Enrichment at telomeres was determined by quantitative PCR on indicated telomeres. Data represents mean % input +/- SEM n=3 (paired t-test one tailed *p<0.05, **p<0.01). PD90 refers to 90 population doublings in the absence of *TLC1* (telomerase RNA subunit). G) Npl3-TAP protein levels do not change in *tlc1* cells. Protein levels are determined using PAP (to detect the TAP tag) and anti-actin antibodies for loading comparison. Abbreviations: PAP: peroxidase anti peroxidase; TAP: tandem affinity purification tag.

TERRA recruits Npl3 to telomeres

Npl3 associates strongly to short telomeres *in vivo (*Figure 14F*)*. It is also known that short telomeres accumulate TERRA and telomeric R-loops (Graf *et al.*, 2017). Given that Npl3 is an RNA-regulatory protein, we hypothesized that TERRA may mediate the association of Npl3 to short telomeres. To investigate this, we tested if the association of Npl3 to telomeres is RNA-mediated using quantitative interactomics. We propagated telomerase negative cells for 90 populations and collected protein extracts from cells with critically short telomeres (Figure 15). Subsequently, we performed telomere pull downs in the presence or absence of RNase A and RNase H, which degrade RNA and R-loops, respectively. With this approach, we show that Npl3 associates to telomeric baits when using *tlc1* extracts (Figure 15A). Interestingly, this association is lost when the protein extracts are incubated with RNase A and RNase H, suggesting that the binding of Npl3 to telomeric baits is RNA-mediated (Figure 15B).

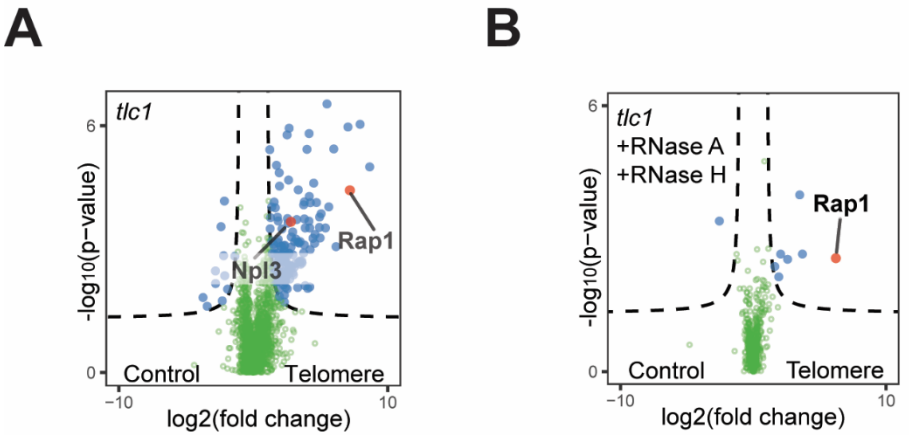


Figure 15 *Identification of telomere associated proteins in telomerase negative cells*

Telomere interactors in *tlc1* cells. Telomere interactors were identified and plotted as a volcano plot as in figure 12 but using *tlc1* protein extracts. Interactors were identified using untreated protein extracts from *tlc1* cells (A) or *tlc1* extracts digested with RNase A and RNase H (B).

Similarly, 95% of the proteins identified in the *tlc1* telomere pull-down no longer associate to the telomere baits upon treatment with RNase A and RNase H (Figure 16A). Thus, our experiment provides an interesting dataset of RNA-dependent telomere binders in yeast. Gene Ontology (GO) analysis shows that the telomere interactors in *tlc1* cells are enriched for homologous recombination pathways (Figure 16 A, B), highlighting the importance of these processes for the survival of cells with short telomeres. Additionally, we identified a subset of helicases and other interactors enriched for RNA binding and RNA processing GOs (Figure 16A, B). Moreover, among the proteins identified at telomeres in *tlc1* cells, we observed some that form functional interactions with each other (Figure 16C). These results suggests that several proteins associate to short telomeres in an RNA-dependent manner and may coordinate telomere recombination. Importantly, we did not detect changes in the association of Rap1 to the telomeric bait, further validating our approach (Figure 16A). To get functional insights into the putative role of these telomere binders, we compared our telomere candidate list with factors that have been described previously as regulators of telomere length and senescence rate (Askree *et al.*, 2004; Gatbonton *et al.*, 2006; Ungar *et al.*, 2009; Chang *et al.*, 2011). Similar to our WT telomere interactors, there is little overlap between our identified telomere binders in *tlc1* cells and proteins known to regulate telomere length or senescence rate (Appendix table 3, see discussion).

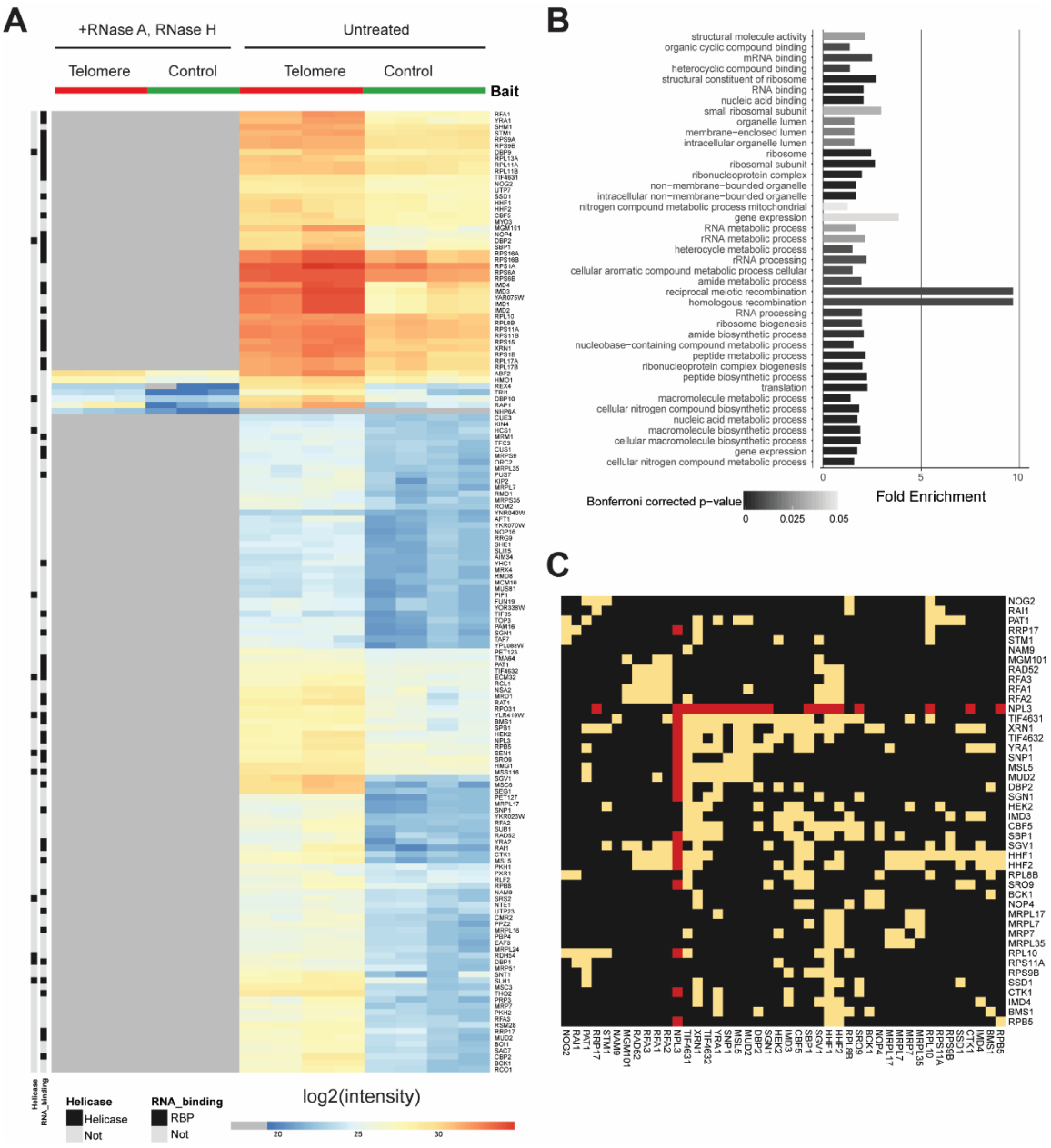


Figure 16 *Telomere interactors in telomerase negative cells (tlc1)*

A) Heatmap for enriched proteins at the telomere bait in *tlc1* cells and *tlc1* cells treated with RNase A and RNase H. Color code indicates measured log2 LFQ intensities in the telomere pull-down. Heatmap was constructed using the "pheatmap" package in R, where clustering is performed using complete data based on the Euclidean distance. Grey indicates not detected proteins. RNA binding proteins and helicases are indicated with a black box. B) Gene ontology enrichment analysis of the telomere-sequence associated interactors. Analysis was performed using the PantherDB.org overrepresentation test. Fisher's exact test was subjected to Bonferroni correction. C) RNA-dependent telomere interactors in *tlc1* cell lysates form interaction clusters. Proteins identified exclusively in *tlc1* non-treated pull down when compared to *tlc1* RNAse A and RNase H treated extracts were used for the analysis (i.e. the RNase sensitive interactors). Biogrid protein interactions are represented as heatmap. Proteins with less than 5 physical interactions were filtered out. Yellow is used for presence and black for absence of interaction. Clustering was performed using the complete data based on binary distance. Npl3 interactors are highlighted in red.

Our results propose that Npl3 associates to telomeres in an RNA-dependent manner and that the elevated TERRA levels in *tlc1* cells promote the association of Npl3 to short telomeres. We hypothesized that, if TERRA promotes the association of Npl3 to telomeres, TERRA should interact with Npl3. Npl3 has been shown to associate to different mRNAs as well as to bind telomeric RNAs *in vitro* (Lee-Soety *et al.*, 2012; Santos-Pereira *et al.*, 2014). Therefore, we tested the association of Npl3 to TERRA *in vivo* using RNA Immunoprecipitation (RIP) followed by qPCR. Using the functional TAP-tagged Npl3 variant, we pulled down soluble associated RNAs after protein-RNA crosslink. To quantify for the RNA binding, we reverse transcribed associated RNAs and performed qPCR on specific loci. Our results show that Npl3 associates to TERRA *in vivo*, as well as to actin *(ACT1)* messenger RNAs (Figure 17A).The observed Npl3 binding to actin RNA serves as positive control, as Npl3 associates transcripts derived from RNA polymerase II transcription. Importantly, Npl3 associates very strongly to TERRA, suggesting that the majority of TERRA is bound by Npl3 *in vivo*.

As TERRA may associate to telomeric DNA forming a functionally relevant telomeric R-loop (Balk *et al.*, 2013; Graf *et al.*, 2017), we tested if Npl3 may also associate to chromatin-bound TERRA. To do this, we applied a Chromatin-associated RNA-IP approach (ChRIP) using our TAP-tagged Npl3 variant. Briefly, we first pulled down Npl3 associated RNAs in the chromatin fraction and digested associated DNA. Lastly, we purified Npl3 associated RNAs prior to RT-qPCR. To confirm that the pulled down chromatin-bound RNAs were forming R-loops, a fraction of the chromatin extract was digested with RNase H. Our data show that Npl3 may associate to TERRA RNAs in the chromatin fraction (Figure 17B). Interestingly, the RT-telomere signal decrease in RNase H treated samples at several telomeres tested, which highly suggests that Npl3 associates to TERRA R-loops (Figure 17B). The different amplification signals detected after RNase H treatment may be explained by different degradation efficiencies at the telomeres tested. Altogether, our data suggest that Npl3 may associate TERRA molecules both in their soluble and chromatin associated form (Figure 17).

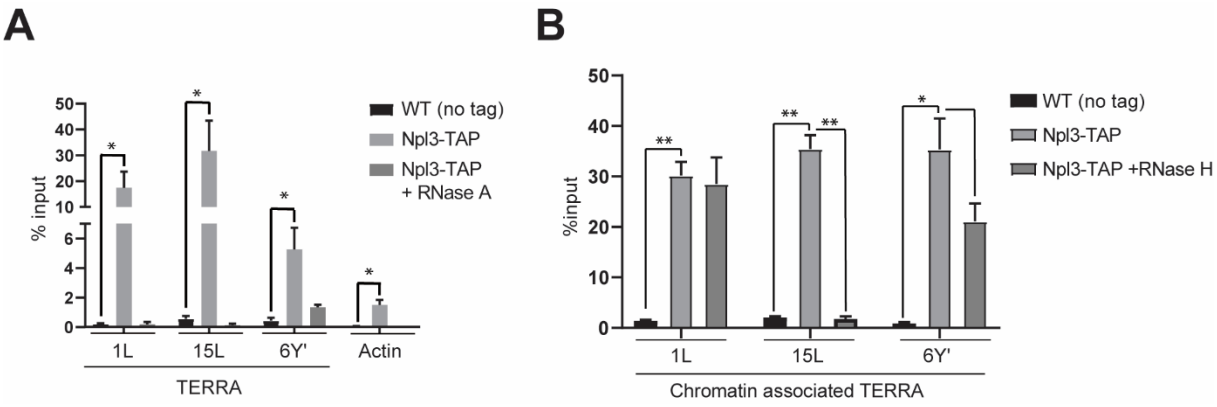


Figure 17 *Npl3 associates to TERRA*

A) Npl3 associates to soluble TERRA. Cross-linked samples from the indicated strains were subjected to Npl3-TAP RNA IP (RIP). Enrichment at telomeric RNAs and actin RNA was determined by quantitative PCR on pulled-down reverse-transcribed RNAs. Data represents mean % input +/- SEM n=3 (unpaired t-test two tailed *p<0.05, **p<0.01).B) Npl3 associates to chromatin associated TERRA. Cross-linked samples from the indicated strains were subjected to Npl3-TAP Chromatin-RNA IP (ChRIP). Enrichment at different chromatin associated RNAs was determined by quantitative PCR on pulled-down reverse-transcribed RNAs. Data represents mean % input +/- SEM n=3 (paired t-test two tailed *p<0.05, **p<0.01).

We hypothesized that the association between Npl3 and TERRA mediate the localization of Npl3 to telomeres. To characterize this regulation, we studied the binding of Npl3 to telomeres upon different TERRA and TERRA R-loop levels. First, we tested the binding of Npl3 to telomeres upon increased TERRA levels. Thus, we performed Npl3-TAP ChIP-qPCR in a Rat1^{AID} (auxin inducible degron) mutant strain, as Rat1 is a negative regulator of TERRA (Luke *et al.*, 2008; Graf *et al.*, 2017). Upon addition of 1mM auxin, the Rat1^{AID} variant is degraded and TERRA levels increase (Figure 18A, (Graf *et al.*, 2017)). In this condition, Npl3 association to telomeres increases at all telomeres tested (Figure 18B), suggesting that TERRA levels regulate the recruitment of Npl3 to the telomeres. To complement this experiment, we tested if Npl3 association to telomeres was abolished upon degradation of TERRA. To degrade TERRA, we overexpressed *RAT1* (Figure 18C). Indeed, cells over expressing *RAT1* did not show association of Npl3 to several telomeres, further suggesting that TERRA mediates the recruitment of Npl3 to telomeres (Figure 18C, D).

As mentioned above, TERRA may associate to telomeres forming a telomeric R-loop (Balk *et al.*, 2013; Graf *et al.*, 2017). We therefore asked if telomeric R-loops may recruit Npl3 to telomeres and whether degradation of TERRA R-loops affect Npl3 association to telomeres. Thus, we measured Npl3 binding

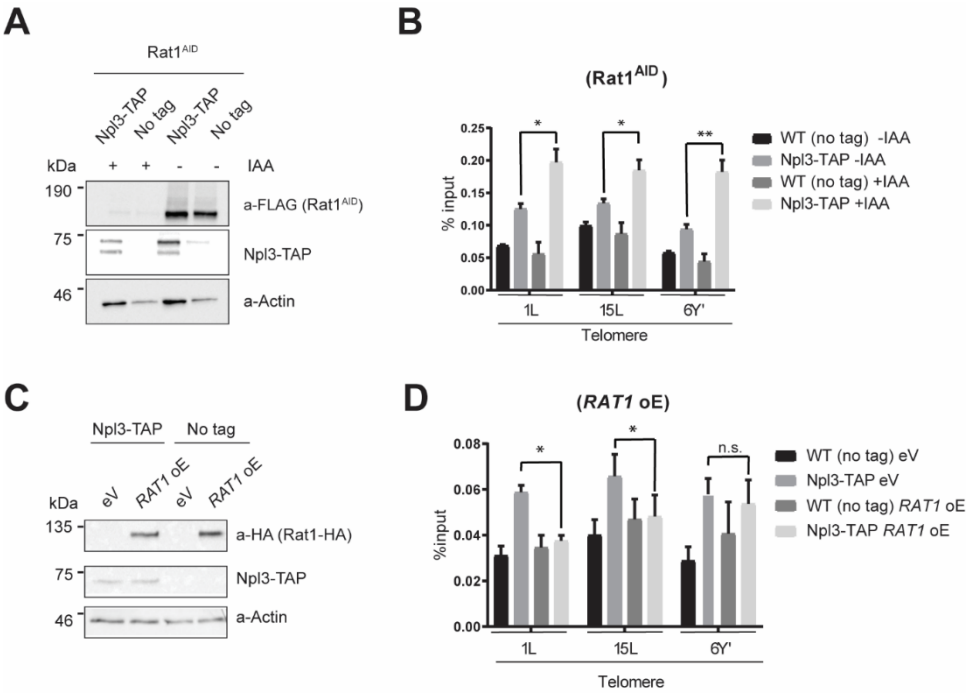


Figure 18 *Npl3 associates to telomeres in a TERRA-dependent manner*

A-B) Npl3 associates strongly to telomeres in the absence of Rat1. A) Rat1-AID variant protein levels decrease after 1h 1mM IAA treatment. Protein levels are determined using anti-FLAG and anti-actin antibodies. B) Functional Npl3-TAP associates strongly to telomeres after 1mM IAA treatment in cells with Rat1-AID variants. Cross-linked samples from indicated strains were used in a TAP-ChIP. Enrichment at telomeres was determined by quantitative PCR on indicated telomeres. Data represents mean % input +/- SEM n=3 (paired t-test one tailed *p<0.05, **p<0.01).C-D) Npl3 association to telomeres decreases upon *RAT1* over expression. C) *RAT1* was over expressed from a galactose-inducible promoter. Protein levels are determined using anti-HA and anti-actin antibodies D) Functional Npl3-TAP does not associate to telomeres upon *RAT1* over expression. Cross-linked samples from indicated strains were used in a TAP-ChIP. Enrichment at telomeres was determined by quantitative PCR on indicated telomeres. Data represents mean % input +/- SEM n=3 (paired t-test one tailed *p<0.05, **p<0.01).

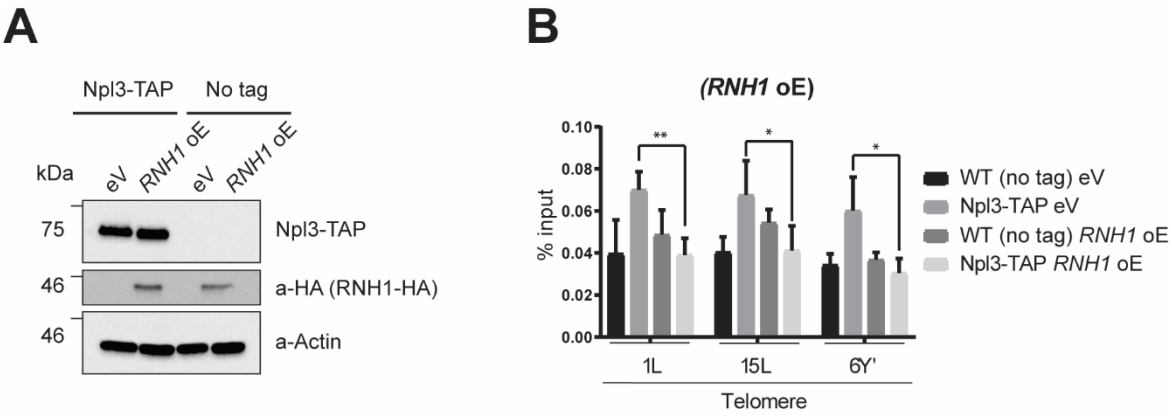


Figure 19 *Npl3 associates to telomeres in an R-loop-dependent manner*

A) *RNH1* was over expressed from a galactose-inducible promoter. Protein levels are determined using anti-HA and anti-actin antibodies. B) Functional Npl3-TAP associates to telomeres in an R-loop-dependent manner. Cross-linked samples from indicated strains were used in a TAP-ChIP. Enrichment at telomeres was determined by quantitative PCR on indicated telomeres. Data represents mean % input +/- SEM n=3 (paired t-test one tailed *p<0.05, **p<0.01).

to telomeres using ChIP-qPCR in cells overexpressing *RNH1*, which degrades R-loops (Figure 19). In this scenario, the association of Npl3 to telomeres is significantly reduced, supporting that TERRA R-loops promote the recruitment of Npl3 to telomeres (Figure 19).

TERRA levels are regulated during the cell cycle (Porro *et al.*, 2010; Graf *et al.*, 2017). As we have observed that TERRA and TERRA R-loops mediate the binding of Npl3 to telomeres, we wondered if Npl3 associates to telomeres in a cell-cycle regulated manner. To test this, we measured the binding of Npl3 to telomeres by ChIP-qPCR in different cell cycle phases. We arrested cells in G1 with alpha factor pheromone and released them into medium containing 250mM HU and 75mM HU to arrest the cells in early and late S phase, respectively (Figure 20A). We subsequently performed ChIP-qPCR on the different cell cycle stages. Interestingly, we detected an increased association of Npl3 to telomeres in early S (250mM HU) which declined in late S (75mM HU) (Figure 20B). Importantly, we did not detect changes in Npl3 protein levels throughout the cell cycle (Figures 20C). As the TERRA and telomeric R-loop levels increase in early S and decline in late S (Graf *et al.*, 2017), we conclude that the Npl3 association to long telomeres requires TERRA and R-loops. As a result, the binding of Npl3 to telomeres is cell cycle regulated.

Altogether, our data suggests that Npl3 associates to telomeres in a TERRA-dependent manner. Therefore, Npl3 localizes to long telomeres in early S phase and it strongly associates to short telomeres, when TERRA and R-loops accumulate.

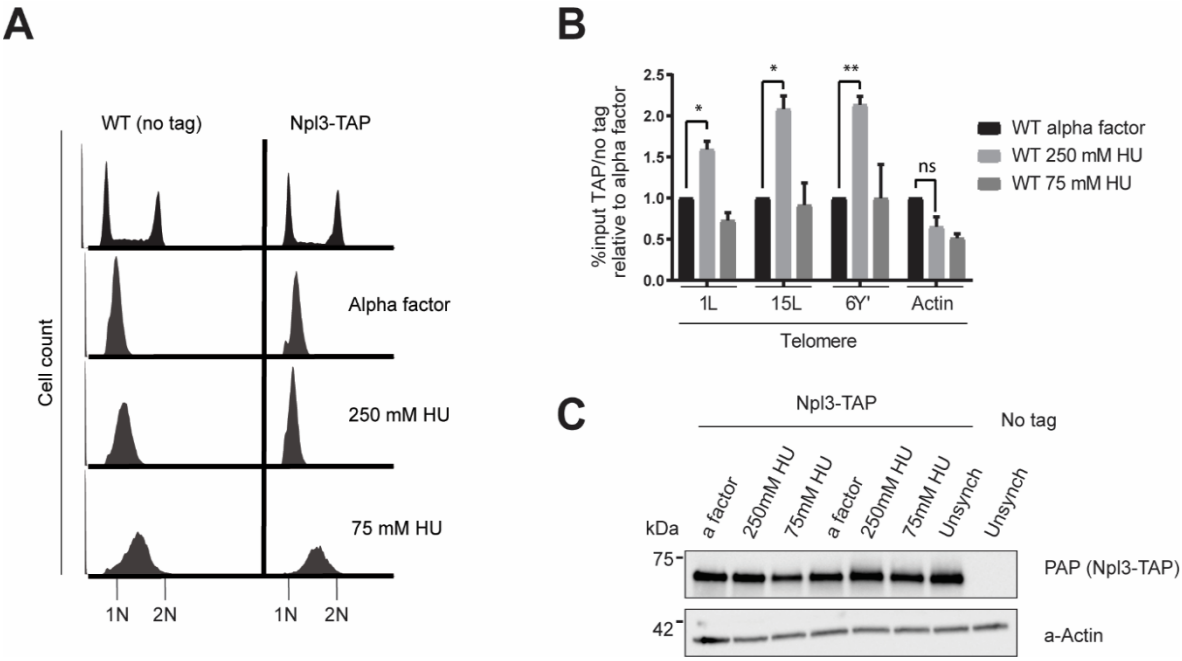


Figure 20 *Npl3 associates to telomeres in early S*

A) Cell cycle profiles of indicated strains. Cells were synchronized in G1 with alpha-factor for 2,5h and released into the cell cycle at 30°C in pre-warmed medium supplemented with the indicated HU concentrations. B) Npl3-TAP associates strongly to telomeres in early S (250 mM HU condition). Cross-linked samples from indicated strains were used in a TAP-ChIP. Enrichment at telomeres was determined by quantitative PCR on indicated telomeres. Data represents mean % input +/- SEM relative to cells arrested in alpha factor n=3 (paired t-test two tailed *p<0.05, **p<0.01). C) Npl3-TAP protein levels are constant throughout the cell cycle. Protein levels are determined using PAP and anti-actin antibodies.

NPL3 stabilizes telomeric R-loops

NPL3 has been proposed to prevent unscheduled R-loop formation genome-wide, as its deletion causes transcription replication conflicts that are sensitive to *RNH1* overexpression (Santos-Pereira *et al.*, 2013). Additionally, other yeast hnRNP-like proteins such as Yra1, associate to R-loops and stabilize them (García-Rubio *et al.*, 2018). Because our data suggests that Npl3 associates to telomeres in a TERRA and R-loop dependent manner, we hypothesized that *NPL3* may stabilize telomeric R-loops as well. To test this possibility, we developed an inducible overexpression system for *NPL3*. In this system, the expression of *NPL3* is controlled under a strong galactose-regulated promoter, which allows for high protein expression upon growing the cells in 2% galactose containing medium (Figure 21A). Using this system, we tested if the overexpression of *NPL3* stabilizes telomeric R-loops by using DRIP-qPCR. We hypothesized that *NPL3* stabilizes pre-existing R-loops, rather than promoting R-loop formation. Therefore, we compared the telomeric R-loop levels in early and late S phase in cells overexpressing *NPL3*, to measure the R-loop stability after their formation in early S phase (Graf *et al.*, 2017).

We observed that in control cells, the telomeric R-loop levels decrease in late S compared to early S phase as it has been previously reported (Figure 21B, (Graf *et al.*, 2017)). However, upon overexpression of *NPL3* the telomeric R-loops remain stable in late S phase, suggesting that the Npl3 association to telomeres in early S promotes R-loop stability (Figure 21B). Altogether, our data support

a role of *NPL3* in telomeric R-loop stabilization. In cells with long telomeres, the *NPL3*-mediated R-loop stabilization is likely cell cycle regulated (Figure 20B), possibly to prevent collisions with the replication machinery (discussed below).

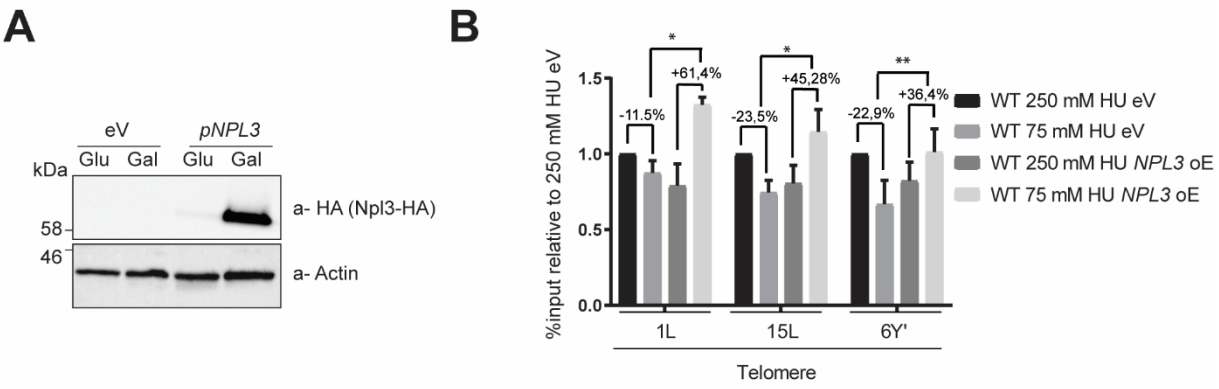


Figure 21 *NPL3 stabilizes R-loops*

A) *NPL3* over expression system. *NPL3* is over expressed when cells are grown in 2% galactose medium. Protein levels are determined using anti-HA and anti-actin antibodies. B) The indicated strains were grown on 1% raffinose 2% galactose for *NPL3* over expression. Cross-linked samples were subjected to DRIP. DNA-RNA hybrids were immunoprecipitated using the S9.6 antibody. R-loop levels were determined by quantitative PCR on indicated telomeres. Data represents mean % input +/- SEM relative to 250 mM HU eV n=3 (paired t-test two tailed *p<0.05, **p<0.01).

We wondered if the *NPL3*-mediated R-loop stabilization that we have characterized at the telomeres is a genome-wide phenomenon. To investigate this, we measured R-loop levels genome-wide using Southwestern blotting (dot blot) with the S9.6 antibody in cells overexpressing *NPL3* and a vector control. Briefly, we spotted serial dilutions of genomic DNA (gDNA) onto a positively charged nylon membrane that we incubated with the anti R-loop antibody S9.6. To exclude unspecific binding of the S9.6 antibody to dsRNA accumulated in our mutants, we treated our gDNA with RNase III and T1. Additionally, we tested the specificity of the antibody towards R-loops by treating the samples with RNase H. Finally, we used an anti-double strand DNA (dsDNA) antibody for normalization. Our dot blot results show that *NPL3* overexpression increases R-loops genome wide, as we detect increased S9.6 antibody signal that is completely sensitive to RNase H treatment (Figure 22 A, B).

Our dot blot data suggests that *NPL3* stabilizes R-loops genome-wide when overexpressed (Figure 22 A, B). We hypothesized that, if *NPL3* stabilizes R-loops, it would impair viability of cells as a consequence of transcription replication conflicts and genome instability. Therefore, we used a spotting assay to compare the viability of cells overexpressing *NPL3* to an empty vector control. We could not detect decreased viability of WT cells overexpressing *NPL3* (Figure 22 C), likely because WT cells are proficient in R-loop degradation. However, the viability of *sen1-1* mutants (which are not proficient in R-loop degradation) was severely impaired (Figure 22 C). *sen1-1* mutants accumulate R-loops genome-wide (Mischo *et al.*, 2011) and show severe growth defects when *NPL3* is overexpressed (Figure 22 C). This result suggests that *NPL3* stabilizes R-loops and causes genome instability when R-loops accumulate genome-wide.

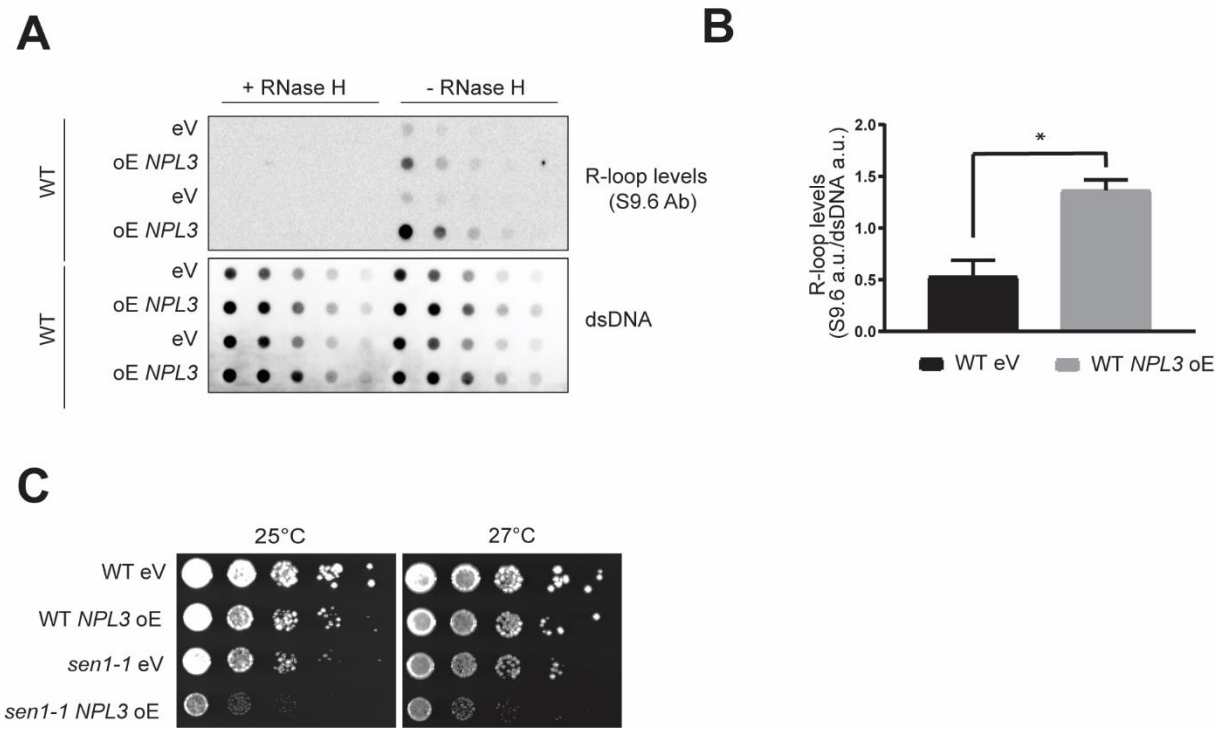


Figure 22 *NPL3 over expression increases R-loop levels genome-wide*

A) R-loop dot blot. The indicated strains were grown on 1% raffinose 2% galactose. R-loop levels and dsDNA levels were determined using the S9.6 antibody and anti-dsDNA antibody. Specificity of the S9.6 antibody was confirmed by treatment with RNase H. B) R-loop dot blot quantification. Data represents R-loop signal as the S9.6 antibody signal relative to corresponding dsDNA signal n=3 (unpaired t-test two tailed *p<0.05). C) Serial dilutions of indicated strains were assayed on 1% raffinose 2% galactose media. Plates were imaged after 72h incubation at indicated temperatures. Abbreviations: eV: empty vector; oE: over expression.

NPL3 regulates telomeric R-loops to prevent fast senescence

Previous studies have reported that deletion of *NPL3* accelerates senescence rate (Lee-Soety *et al.*, 2012). Here, we have shown that Npl3 associates strongly to short telomeres and that *NPL3* stabilizes telomeric R-loops when overexpressed. We hypothesize that the Npl3 localization to short telomeres may promote telomeric R-loop stabilization, drive telomere recombination and prevent anticipated senescence onset. To investigate this possibility, we performed a series of senescence assays. First, as the overexpression of *RNH1* in telomerase negative cells accelerates senescence onset due to the degradation of TERRA R-loops (Balk *et al.*, 2013) (Figure 23A, rows 4 and 5), we tested the effect of *RNH1* overexpression on *tlc1 npl3* cells. Interestingly, over expression of *RNH1* is epistatic to *NPL3* deletion in telomerase negative cells (Figure 23A, bottom two rows). This result suggest that indeed, Npl3 promotes R-loop stabilization in *tlc1* cells to prevent fast senescence.

On the other hand, deletion of *RNH201* in telomerase negative cells delays senescence onset, as telomeric R-loops accumulate in the cells ((Balk *et al.*, 2013), Figure 23B, rows 4 and 5). Therefore, if deletion of *NPL3* in telomerase negative cells anticipates senescence onset due to lack of R-loop stability, one should be able to rescue this rapid senescence by deleting *RNH201,* as it would compensate for the telomeric R-loop loss. Indeed, deletion of *RNH201* rescues the accelerated senescence rate of *tlc1 npl3* cells (Figure 23B, bottom rows), suggesting that *NPL3* stabilizes telomeric R-loops to prevent early senescence onset.

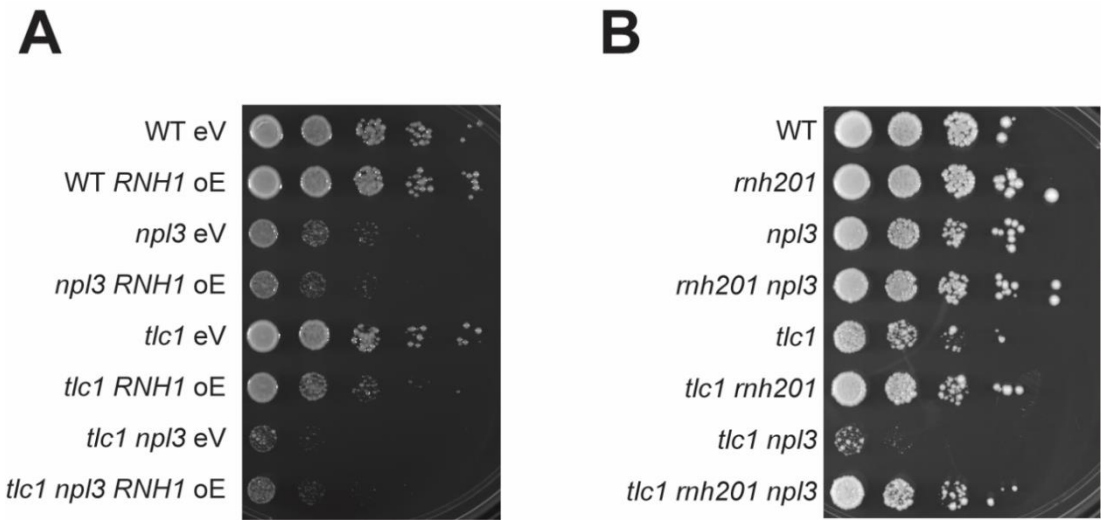


Figure 23 *NPL3 stabilizes telomeric R-loops to prevent accelerated senescence*

A) Serial dilutions of indicated strains were assayed on SC-His media. Cells were plated after 30-50 generations propagated on SC-His agar media. Plates were imaged after 72h growth at 30°C. B) Serial dilutions of indicated strains were assayed on YPD media. Cells were plated after 30-50 generations propagated on YPD agar media. Plates were imaged after 72h growth at 30°C.

Altogether, our data supports a model in which TERRA mediates the recruitment of Npl3 to telomeres to stabilize telomeric R-loops. When telomeres are long, Npl3 is recruited in early S phase to promote a transient R-loop stabilization. When telomeres shorten, TERRA and TERRA R-loops accumulate, increasing the recruitment of Npl3 to short telomeres. This recruitment mediates R-loop stabilization and possibly facilitates homology-directed repair, thus preventing anticipated senescence onset.

Discussion

Quantitative interactomics identifies telomere interactors

Quantitative interactomics has been extensively used to identify telomere associated factors. Indeed, several factors that coordinate telomere trimming, telomerase recruitment and oxidative stress at mammalian telomeres have been identified using this method (Kappei *et al.*, 2013; Aeby *et al.*, 2016; Jahn *et al.*, 2017). Additionally, quantitative telomere interactomics has facilitated the study of conserved telomere interactors in vertebrates (Kappei *et al.*, 2017). Therefore, telomere interactomics is a powerful tool to identify telomere associated proteins in different organisms and can also be employed to monitor telomere associated proteins at different states of telomere function.

Validation of the screening and overlap with previous studies

In this study, we have used an unbiased mass spectrometry-based quantitative interactomics approach to identify telomere associated proteins in *S. cerevisiae*. Briefly, we synthesized DNA baits harboring telomeric sequences and pulled down associated proteins (Figure 12). Furthermore, we performed two separate experiments to identify potential telomere binders in telomerase positive and telomerase negative cells (Figure 12, 15). Our experiments provide an extensive list of protein candidates that may regulate telomere integrity through binding to telomeres in both telomerase positive and negative cells. The identification of the well-characterized telomere binding protein Rap1 in our telomere pull-downs validates our experimental approach. Interestingly, 34 additional candidates were commonly identified as telomere interactors in both WT and telomerase negative cells (Appendix table 1). This suggests that these common factors may be telomere binders *in vivo*, independently of telomere length. Additionally, we identified candidates that were exclusively associating with telomeric baits in one of the pull-down experiments, either in WT or telomerase negative cells (*tlc1)* (Appendix table 1). Thus, these different proteins may be recruited to telomeres depending on telomere length or the state of replicative senescence.

Previous studies in yeast have screened for factors that regulate telomere length. To identify these factors, telomere length phenotypes were studied in a collection of viable *S. cerevisiae* knock-out strains (Askree *et al.*, 2004; Gatbonton *et al.*, 2006; Ungar *et al.*, 2009). To get insights into the functional relevance of our telomere-binding candidates, we compared the identified candidates in our telomere pull-downs (using WT and *tlc1* protein extracts) to those that may regulate telomere length according to published literature. We detected very little overlap between published factors whose deletion affects telomere length telomere length regulators and our telomere associated candidates (Appendix table 2). Two possible explanations may account for these differences. On the one hand, it is likely that the previously published genes regulate telomere length through both direct and indirect mechanisms. Alternatively, it is possible that characterization of the telomere function of our candidates may not be possible in telomerase positive cells, as telomerase rapidly extends short telomeres (Marcand, Brevet and Gilson, 1999; Teixeira *et al.*, 2004; Bianchi and Shore, 2007b; Jacobs, 2013). Indeed, we do not know if our DNA bait would actually get elongated following incubation with our extracts. Deletion of certain factors may affect telomere length if it impacts the protein expression of a true telomere length regulator. Indeed, many of the factors implicated in telomere length regulation in previous studies, participate in transcription coordination and gene expression (Askree *et al.*, 2004; Gatbonton *et al.*, 2006; Ungar *et al.*, 2009). It is therefore possible that deletion of those genes misregulates the expression of telomere associated proteins and consequently affects telomere length regulation. On the other hand, it is possible that the effects of our candidates on telomere biology cannot be detected in telomerase positive cells, but only when the telomerase function is compromised.

Despite the little overlap between previous studies and our telomere-associated candidates, we did identify one exciting overlapping candidate, Nam7, which may regulate telomere length by associating to telomeres (Figure 25, Appendix tables 2 and 3). Interestingly, Nam7 is the yeast homolog of human UPF1, which removes TERRA from telomeres to coordinate telomere replication on the leading strand (Chawla *et al.*, 2011)(Figure 25). A similar Nam7-mediated function at yeast telomeres may explain why deletion of *NAM7* shortens telomeres, as accumulated TERRA may interfere with telomere replication. It has previously been argued that the effects of telomere shortening in the *upf1* mutants are due to misregulated Ten1 and Stn1 mRNA levels (Enomoto *et al.*, 2004), however our data and data in human cells suggests that there may indeed be a direct role.

In 2011, the Lydall group identified genes that regulate senescence in yeast (Chang *et al.*, 2011). Those factors may better represent some of our candidates, as the experiment tested telomere phenotypes in telomerase negative cells. Under these conditions, telomere length phenotypes become more apparent, as short telomeres are not rapidly elongated by telomerase (Marcand, Brevet and Gilson, 1999; Teixeira *et al.*, 2004; Bianchi and Shore, 2007b; Jacobs, 2013). Unfortunately, there is little overlap between our telomere associated candidates and those identified to regulate senescence in yeast (Appendix tables 2 and 3). In particular, the common factors identified in both studies are Rad52, Swc3 and Sdd4 (Appendix tables 2 and 3). Rad52 has been extensively studied at yeast telomeres and coordinates telomere recombination in telomerase negative cells (Le *et al.*, 1999; Abdallah *et al.*, 2009). On the other hand, little is known about the functions of Swc3 and Sdd4, which opens up new possibilities for telomere maintenance functions.

Finally, we wondered if the levels of some of our telomere-binding candidates change during senescence in *S. cerevisiae*. These protein level changes may indicate a specific role of some of our candidates at short telomeres. To investigate this possibility, we compared our telomere interactors list from both WT and *tlc1* experiments with the dataset of proteins that significantly change protein levels during senescence in yeast (Wagner et al.,2019, in preparation). With this analysis, we identified two interesting overlapping factors: Dbp2 and Orc2, whose protein levels decrease in *tlc1* cells (Appendix tables 1,2,3). Orc2 regulates DNA replication (Bell, Kobayashi and Stillman, 1993; Foss *et al.*, 1993), while Dbp2 is an RNA helicase involved in transcription coordination and genome stability (Bond *et al.*, 2001; Cloutier *et al.*, 2012; Ma, Cloutier and Tran, 2013). In particular, Orc2 is an interesting candidate as human ORC proteins may coordinate telomere replication together with TERRA (Deng *et al.*, 2009; Takahama *et al.*, 2013). Dbp2 on the other hand, may have a role in TERRA and telomeric R-loops regulation, similar to its genome-wide function in R-loop regulation (Cloutier *et al.*, 2016). As these factors may potentially facilitate telomere replication, decreased levels of both factors in telomerase negative cells may therefore increase replication stress at short telomeres (Wagner, 2019 in preparation). This may facilitate subsequent homology-directed repair (HDR) at short telomeres and promote telomere maintenance.

Possible function of RNA binding proteins at telomeres

Our data show a strong association of RNA binding proteins to the telomeric baits, suggesting that RBPs may be functionally relevant at yeast telomeres (Figure 13, 16). Indeed, 32 out of the 69 of identified candidates in our WT telomere pull-down are RBPs. Similarly, when pulling down telomere associated proteins in *tlc1* cells, we could identify 61 RBPs out of the 152 proteins enriched at telomeric baits (Figure 13, 16). These results highlight the importance of RNA regulatory proteins in telomere maintenance. Indeed, RBPs may be implicated in TERRA transcription regulation and function. Further, they may participate in telomeric R-loop regulation.

hnRNPs in telomere biology, R-loop regulation and senescence

HnRNPs coordinate gene expression in both mammalian cells and yeast. In yeast, several hnRNP-like proteins have been implicated in telomere biology already. These include members of the THO complex and Yra1, which regulate telomeric transcription, telomeric R-loop levels and senescence rate (Pfeiffer *et al.*, 2013; Yu, Kao and Lin, 2014; Gavaldá *et al.*, 2016; García-Rubio *et al.*, 2018). It has been proposed that several of these factors regulate transcription to prevent unscheduled R-loop formation genome-wide and at telomeres. In addition, Yra1 may regulate R-loop levels in two distinct mechanisms, as it also may associate to R-loops and stabilize them (Gavaldá *et al.*, 2016; García-Rubio *et al.*, 2018). The coordinated action of different hnRNP-like factors at telomeres may regulate telomere integrity both in the presence and absence of telomerase. At long telomeres, hnRNP-like proteins may be required to prevent toxic accumulation of R-loops, which may lead to replication stress and telomere loss (Pfeiffer *et al.*, 2013). At short telomeres, certain hnRNP-like proteins may be required to promote R-loop stabilization and telomere recombination (García-Rubio *et al.*, 2018). Consequently, deletion of hnRNP-related genes in telomerase negative cells accelerates senescence rate and have imbalanced telomeric R-loop levels (Lee-Soety *et al.*, 2012; Yu, Kao and Lin, 2014; García-Rubio *et al.*, 2018).

Our screening identified different factors and RBPs that physically interact with each other and with telomeric sequences (Figure 13, 16). This suggests that certain factors may work together at both long and short telomeres to promote telomere integrity. In particular, several RBPs have been identified as telomere interactors in *tlc1* cells (Figure 16). As some RBPs can regulate R-loop levels (Pfeiffer *et al.*, 2013; Yu, Kao and Lin, 2014; Gavaldá *et al.*, 2016; García-Rubio *et al.*, 2018) and R-loops are essential for short telomere maintenance (Balk *et al.*, 2013; Graf *et al.*, 2017), it is tempting to speculate that certain RBPs may form a protein network that regulates R-loop stabilization at short telomeres. By stabilizing R-loops, different RBPs may promote telomere recombination and short telomere maintenance. In support of this, several studies have described increased recombination rates in cells overexpressing RBPs in yeast. This overexpression phenotypes may mirror a situation when RBPs locally accumulate at short telomeres. Even though the mechanism regulating increased recombination rates in RBP mutants has not been explored in detail, it remains possible that they are a consequence of R-loop stabilization, similar to what has been reported for Yra1 (Gavaldá *et al.*, 2016). Altogether, the recruitment of RBPs or hnRNP-like factors to short telomeres may promote R-loop stabilization and promote HDR-mediated telomere maintenance.

Yeast hnRNP-like proteins and RBPs may have additional roles at telomeres, as homologs in other species play different roles in telomere maintenance. In human cells, for example, some hnRNPs regulate telomerase-mediated telomere maintenance and end protection (Zhang *et al.*, 2006; Flynn *et al.*, 2011; Redon, Zemp and Lingner, 2013). Even though our screening mainly identified yeast hnRNP-like proteins associating to telomeric baits in *tlc1* protein extracts *in vitro* (Figure 16), it is possible that yeast hnRNPs may function *in vivo* at long telomeres as well. In support of this, members of the THO complex have been identified at yeast wild type length telomeres to regulate R-loop levels (Pfeiffer *et al.*, 2013). As telomeric R-loops may form G-quadruplexes (G4s) on the displaced single-strand DNA and G4s may promote telomere capping in yeast (Smith *et al.*, 2011), it is tempting to hypothesize that yeast hnRNP-like proteins may also play a role in telomere protection, possibly when capping is compromised. Indeed, hnRNP-like proteins may be essential to promote telomere capping through the regulation of R-loops, as both R-loops and G4s prevent excessive resection (Smith *et al.*, 2011; Ohle *et al.*, 2016). Future studies may perhaps determine the role of yeast hnRNP-like proteins at telomeres, in conditions when end protection is compromised, such as in *cdc13-1* mutants. The possible implication of yeast hnRNP-like proteins in telomerase regulation remains to be elucidated.

hnRNPs may not only regulate telomeric R-loops but additionally regulate TERRA levels and localization. In fact, hnRNPs participate in mRNA nuclear export. Even though TERRA mainly localizes in nuclear compartments, it is possible that under certain conditions TERRA is exported to the cytoplasm (Wang *et al.*, 2015; Perez-Romero *et al.*, 2018). This relocalization may be an active mechanism in response to, for example, diauxic shift in yeast or inflammation in human cells (Wang *et al.*, 2015; Perez-Romero *et al.*, 2018). Therefore, it is possible that hnRNP-like proteins in yeast assist TERRA nuclear export upon specific stress conditions. More research is required to validate this hypothesis.

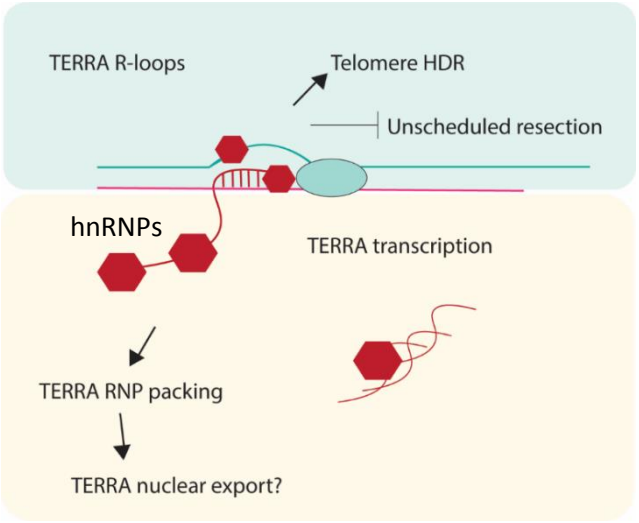


Figure 24: *Proposed functions of yeast hnRNP-like proteins at telomeres*

hnRNP-like proteins may regulate TERRA (lower panel) and TERRA R-loops (upper panel). By associating nascent TERRA transcripts, hnRNP-like proteins may prevent unscheduled R-loop formation at telomeres and proper TERRA packing into functional RNPs. Presumably, they may also participate in TERRA nuclear export upon certain conditions. By associating and stabilizing telomeric R-loops, hnRNP-like proteins may promote telomere HDR in cells without active telomerase or prevent unscheduled resection at telomeres, thus promoting telomere capping. Red polygons represent hnRNP-like proteins. Abbreviations: RNP: ribonucleoprotein complex; TERRA: Telomeric

RNA and DNA helicases in telomere biology and R-loop regulation

Our telomere pull-downs identified several RNA and DNA helicases associated to telomeric baits in both WT and *tlc1* cells (Figure 13, 16). This suggests that different helicases may function at telomeres, perhaps depending on telomere length.

RNA and DNA helicases may have different functions at telomeres. First, they may safe guard telomere replication, which is subject to a large variety of structural obstacles including G4s and R-loops (Maestroni, Matmati and Coulon, 2017b)). Second, helicases may indirectly regulate telomere elongation by mediating the folding of telomerase RNA (Chen and Greider, 2003; Lattmann *et al.*, 2011; Booy *et al.*, 2012).Third, yeast helicases may participate in telomere replication by promoting end-resection (Bonetti *et al.*, 2009). Finally, helicases may participate in ALT by promoting accurate telomere recombination. In particular, helicases may resolve holiday junctions generated from telomere recombination or resolve toxic recombination intermediates (Johnson *et al.*, 2001; Azam *et al.*, 2006; Lee *et al.*, 2007).

Examples of functional helicases at telomeres include mammalian factors RTEL1, UPF1, SMARCAL1, BLM or WRN (Maestroni, Matmati and Coulon, 2017b) and yeast Pif1 and Rrm3 (Pfeiffer and Lingner,

2013; Carly L Geronimo and Zakian, 2016). These factors may coordinate telomere replication and stability through different mechanisms (Maestroni, Matmati and Coulon, 2017a). Additionally, yeast helicases like Sgs1 may participate in the resolution of toxic recombination intermediates and, by doing so, coordinate senescence rate (Johnson *et al.*, 2001; Azam *et al.*, 2006; Lee *et al.*, 2007). Our screening in *S. cerevisiae* may complement the current knowledge on DNA and RNA helicases at yeast telomeres and future experiments may validate their role in telomere maintenance. Characterization of yeast helicases function at telomeres may provide insights into mammalian telomere maintenance mechanisms.

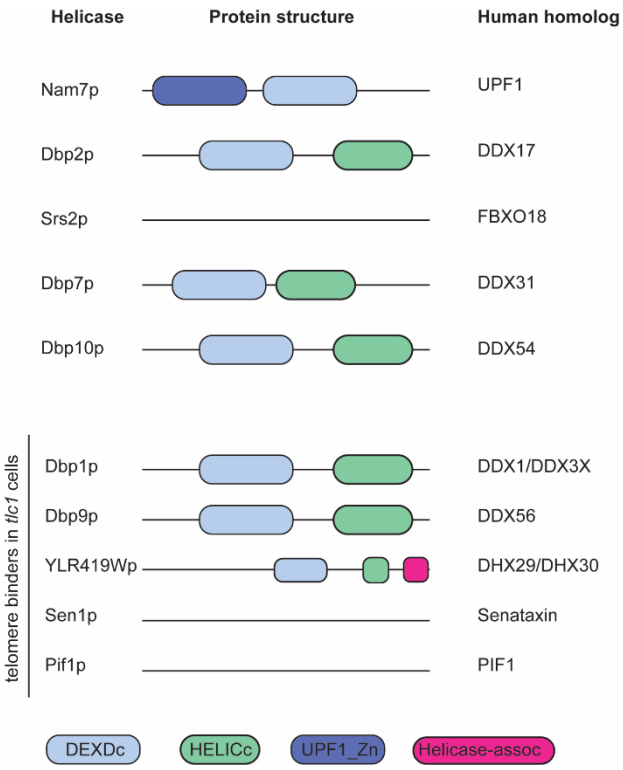


Figure 25: *Yeast helicases identified in this study as telomere interactors*

Yeast helicases identified in this study at telomeric baits are indicated. Corresponding protein structure and human homologs are indicated. Legend for protein domains is indicated at the bottom of the table. Lower panel shows RNA helicases identified exclusively at telomeric baits when assaying telomerase negative protein lysates.

It is particularly interesting that several helicases identified as telomere interactors in both WT and *tlc1* cells are DEAD-box helicases (Figure 25), as recent studies suggest that the latter may be implicated in R-loop regulation genome-wide (Song *et al.*, 2017; Almeida *et al.*, 2018; Chakraborty, Huang and Hiom, 2018; Tedeschi *et al.*, 2018; Mersaoui *et al.*, 2019). Moreover, some human homologs of the identified yeast DEAD-box helicases participate in telomere maintenance and genome integrity, sometimes through the regulation of R-loop levels. For example, human UPF1 (homolog of Nam7) coordinates R-loop levels at telomeres, likely by unwinding TERRA associated with the telomeric leading strand (Chawla *et al.*, 2011). Similarly, other human and yeast DEAD-box helicases have been implicated in R-loop regulation genome-wide. In yeast, for example, Dbp2 regulates R-loop levels, possibly in coordination with yeast Sen1 (Cloutier *et al.*, 2016; Tedeschi *et al.*, 2018). Both proteins were identified as telomere interactors in this study (Figure 25) and may also regulate R-loops at telomeres. However, the exact Dbp2-mediated mechanism that regulates R-loop levels is not clear, as it has been proposed that Dbp2 facilitates R-loop formation at certain loci (Cloutier *et al.*, 2016) while it may also coordinate R-loop degradation through interaction with Sen1 (Tedeschi *et al.*, 2018). Therefore, it would be interesting to determine the role of Dbp2 and other yeast DEAD-box helicases at telomeres, in the context of telomeric R-loop levels. As mentioned above, it is possible that at long telomeres Dbp2 resolves R-loops to facilitate telomere replication (Figure 24). In cells with short telomeres, decreased levels of Dbp2 (Wagner 2019, in preparation) may increase replication stress at telomeres and facilitate telomere recombination.

Other DEAD-box helicases like Dbp1, Dbp9 or YLR419Wp are interesting candidates to regulate R-loop levels at short telomeres, as they were identified as telomere binders exclusively in telomerase negative protein extracts (Figure 16A). Whether these factors promote R-loop formation, stability or degradation is currently unknown. However, they may be required to sustain R-loop-mediated telomeric HDR exclusively at short telomeres (Figure 26). As R-loop levels at telomeres must be balanced to promote telomere recombination, other helicases including our candidates Sen1 or Pif1 (Figure 26) may be recruited to telomeres to facilitate R-loop resolution (Mischo *et al.*, 2011; Pohl and Zakian, 2019). The specific recruitment of different factors in a timely manner may depend, among others, in the structure acquired on the displaced DNA strand in the R-loop (Carrasco-Salas *et al.*, 2019), which may differ between stable R-loops or recombining R-loops.

It is likely that helicases do not function at telomeres on their own, but rather interact with additional factors. At yeast telomeres, for example, Sgs1 may cooperate with Top3 (identified as a potential telomere interactor in *tlc1* cells in this study, Figure 26) to resolve recombination intermediates and promote HDR (Tsai *et al.*, 2006). This mechanism seems conserved, as human Topoisomerase III participates in telomere maintenance in ALT cells (Tsai *et al.*, 2006). Similarly, yeast Pif1 (identified in this study as telomere interactor in *tlc1* cells, Figure 26) may promote R-loop resolution at short telomeres, perhaps through interaction with RNase H enzymes (Pohl and Zakian, 2019). In summary, the interaction of different factors with helicases may promote balanced telomeric R-loop levels at both long and short telomeres. Our study provides an extensive candidate list to investigate this hypothesis.

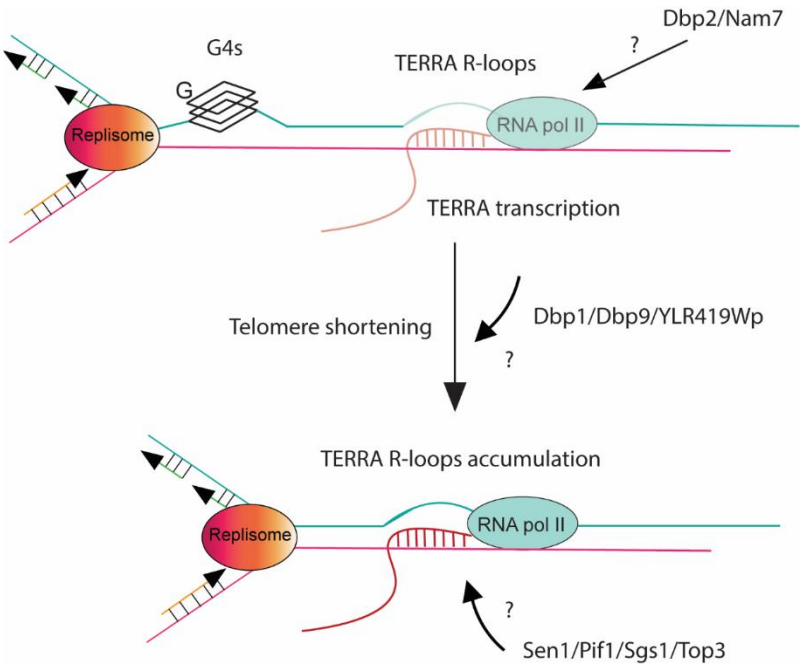


Figure 26: *Proposed roles of different helicases at yeast telomeres*

At yeast long telomeres, Nam7 and Dbp2 may resolve telomeric R-loops and other complex structures like G4s to facilitate telomere replication. Upon telomere shortening, other helicases including Dbp1, Dbp9 and YLR419Wp may be recruited to telomeres. To maintain balanced R-loop levels at short telomeres, factors like Sen1, Pif1, Sgs1 and Top3 may be recruited to short telomeres. Abbreviations: G4: G quadruplexes; TERRA: telomeric repeat containing RNA.

One interesting possibility is that certain helicases are recruited to stalled replication forks at short telomeres through interaction with replisome components such as PCNA. In fact, telomere-associated helicases like RTEL1 in humans and Srs2 in yeast interact with PCNA (yeast Pol30) (Armstrong, Mohideen and Lima, 2012; Vannier *et al.*, 2013). This mechanism may ensure the recruitment of specific helicases to telomeres after fork stalling. Indeed, this could be beneficial for recombining telomeres, as replication stress may be a pre-requisite to initiate HDR. This way, R-loops accumulated at short telomeres (Graf *et al.*, 2017) may promote replication stress and fork stalling, initiate HDR and only later be resolved by helicases to ensure completion of recombination. The proposed mechanism may only be applicable to yeast telomeres, as senescent mammalian cells may have no functional PCNA (Chang *et al.*, 1991). Alternatively, helicases may be recruited to telomeres upon R-loop accumulation. The mechanistic details of helicase recruitment to short telomeres require further investigation.

Possible implication of other identified factors in telomere biology

Our screening identified recombination proteins such as Rad52 and Mgm101 as possible telomere interactors in both WT and *tlc1* cells (Figure 13, 16, Appendix table 1). Even though Mgm101 has been traditionally associated to mitochondrial DNA repair (Chen, Guan and Clark-Walker, 1993), recent studies suggest that it may have a more general role in nuclear DNA damage response (Rendeková *et al.*, 2016). In particular, it appears that Mgm101 participates in the repair of DNA inter-strand crosslinks (ICL) independently of Rad52 and perhaps through the interaction with Mph1 (Rendeková *et al.*, 2016; Silva *et al.*, 2016). Given that Mph1 participates in telomeric R-loop regulation in yeast (Lafuente-Barquero *et al.*, 2017), it is likely that Mgm101, together with Mph1, is also involved in R-loop regulation. More specifically, it is possible that Mph1 and Mgm101 cooperate to resolve R-loop-derived recombination intermediates similarly to their putative role in resolving ICL recombination intermediates (Rendeková *et al.*, 2016). Interestingly, Mgm101 may facilitate telomere elongation in cells without active recombination (Rendeková *et al.*, 2016), suggesting that Mgm101 may also coordinate senescence rate in yeast. Future studies may validate these exciting possibilities.

Npl3 is a functionally relevant telomere associated protein

Of our telomere binding candidates, Npl3, was particularly interesting. Npl3 is an RBP that coordinates transcription and gene expression and prevents unscheduled R-loop formation genome-wide (Santos-Pereira *et al.*, 2014). Furthermore, *NPL3* was especially interesting as its deletion accelerates senescence onset in telomerase negative cells (Lee-Soety *et al.*, 2012). Therefore, we investigated the function of *NPL3* in R-loop regulation and at telomeres.

Npl3 regulates R-loop levels in two different ways

NPL3 participates in transcription regulation and gene expression genome-wide (Santos-Pereira *et al.*, 2014). In particular, it has been proposed that Npl3 is recruited to transcribed regions through its interaction with RNA polymerase II and it is required for proper packaging of nascent transcripts into functional RNPs (Santos-Pereira *et al.*, 2014). The described role of Npl3 in transcription led to the hypothesis that Npl3 may prevent R-loop formation by associating nascent RNAs. Indeed, deletion of *NPL3* causes transcription-replication conflicts that are alleviated by *RNH1* overexpression (Santos-Pereira *et al.*, 2013). These data suggest that genome-wide, Npl3 prevents unscheduled R-loop formation of highly transcribed genes to facilitate gene expression.

Interestingly, some yeast hnRNP-like proteins regulate R-loop levels through two distinct mechanisms (Gavaldá *et al.*, 2016). For example, Yra1 prevents unscheduled R-loop formation through binding nascent RNAs but can also associate to pre-existing R-loops and stabilize them (Gavaldá *et al.*, 2016; García-Rubio *et al.*, 2018). We therefore wondered if similar mechanisms may apply to Npl3-mediated R-loop regulation. Similar to Yra1, we hypothesize that Npl3 may prevent unscheduled R-loop formation by binding nascent RNAs (Santos-Pereira *et al.*, 2013), but may also promote R-loop

stabilization. Our data suggest that Npl3 indeed does stabilize R-loops, as we detected increased R-loop levels in cells overexpressing *NPL3* and decreased viability of *sen1-1* mutants upon *NPL3* over expression (Figure 22). Further, we hypothesized that the increased R-loop levels observed upon *NPL3* overexpression may be explained by an Npl3-mediated R-loop stabilization, rather than an active Npl3-mediated R-loop formation. Two different experiments support this hypothesis. On the one hand, the association of Npl3 to chromatin-bound RNAs strongly suggests that Npl3 may associate to R-loops (Figure 17). On the other hand, the R-loop-dependent recruitment of Npl3 to telomeres (Figure 19), supports that Npl3 associates to R-loops at specific loci. Finally, the stable telomeric R-loops observed upon *NPL3* overexpression (Figure 21) suggests that Npl3 stabilizes R-loops in the cell cycle after their formation in early S phase (Graf *et al.*, 2017) (Figure 20B, 21). Altogether, our data suggests that Npl3 stabilizes R-loops, as well as prevents unscheduled R-loop formation (Santos-Pereira *et al.*, 2013).

The dual Npl3-mediated R-loop stabilization may be biologically relevant for gene expression. On the one hand, Npl3 may prevent unscheduled R-loop formation and ensure efficient transcription, nuclear export and protein expression (Figure 27) (Santos-Pereira *et al.*, 2013). On the other hand, Npl3 may stabilize R-loops at promoter regions and terminators to coordinate gene expression (Figure 27) (Skourti-stathaki and Proudfoot, 2014) . Indeed, deletion of *NPL3* causes transcription read-through (Holmes *et al.*, 2015), suggesting that Npl3 participates in transcription termination. Further, Npl3 associates towards the 3' end of transcribed genes (Santos-Pereira *et al.*, 2013), which supports its implication in transcription termination. Our data raises the interesting possibility that Npl3 coordinates transcription termination through R-loop stabilization. Altogether, Npl3 regulates both stabilization and formation of R-loops genome-wide, which may be important for proper gene expression (Figure 27).

Additionally, the Npl3-mediated R-loop stabilization may allow a dynamic regulation of R-loop levels at specific loci. For example, at chromosome ends, Npl3 may stabilize R-loops exclusively at short telomeres to prevent premature senescence (Figure 27). Similar mechanisms may operate at different loci to allow a transiently regulated R-loop stabilization, depending on the biological context.

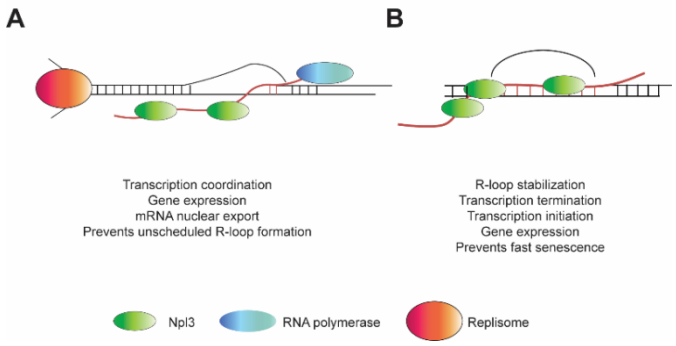


Figure 27: *Two functions of Npl3 in R-loop levels regulation*

A) Npl3 associates nascent RNAs and prevents unscheduled R-loop formation. This Npl3-mediated function coordinates transcription, gene expression and mRNA nuclear export. B) Npl3 stabilizes R-loops. This function may facilitate transcription initiation and termination and coordinate gene expression. Further, Npl3-mediated R-loop stabilization may prevent anticipated senescence onset in telomerase negative cells. Symbol legends are indicated at the bottom of the figure.

Since over stabilization of R-loops may increase replication stress and genome instability genome-wide, balanced regulation of hnRNP-like protein activity may be required for genome integrity. Indeed, both *YRA1* and *NPL3* regulate their own expression (Lund, Kress and Guthrie, 2008; Gavaldá *et al.*, 2016), highlighting the importance of balanced hnRNP-like protein levels in yeast. Similarly, human cancer cells increase protein levels of ALY, the human homolog of Yra1, and increase genome instability (Domínguez-Sánchez *et al.*, 2011). Therefore, analogous hnRNP-mediated R-loop regulatory functions may also exist in human cells and may be important for health and disease.

Possible mechanisms for NPL3-mediated R-loop stabilization

The data presented in this study supports a role of *NPL3* in R-loop stabilization. However, how does Npl3 stabilize R-loops? One possibility is that Npl3 stabilizes R-loops indirectly, through the interaction with other R-loop regulatory factors. Indeed, studies from the Aguilera lab have shown that the Npl3 interactor Yra1 can directly associate to R-loops and stabilize them (García-Rubio *et al.*, 2018). Therefore, it is possible that Npl3 associates to R-loops through its interaction with Yra1 (Tardiff, Abruzzi and Rosbash, 2007; Erce *et al.*, 2013).The interaction between Yra1 and Npl3 may subsequently stabilize R-loops by physically protecting them from degradation or from an approaching replication fork (Hamperl *et al.*, 2017).

Another interesting possibility is that Npl3 stabilizes R-loops by directly associating them, which may protect them from degradation or unwinding. For example, Npl3 may associate through its RRM domains to the intercalated RNA in the R-loop and protect it from degradation (Santos-Pereira *et al.*, 2014) (Figure 14,28A). Indeed, our ChRIP data suggest that, at least at telomeres, Npl3 may associate to chromatin intercalated TERRA (Figure 17B). By physically associating chromatin intercalated RNAs, Npl3 may mask them from R-loop degrading enzymes including RNase H enzymes (Figure 28A). Similarly, Npl3 may protect R-loops from replisome-associated helicases if the RNA associates with the leading strand (Figure 28B). Certainly, this mechanism would be relevant at telomeres, as TERRA associates with the leading strand template. Another possibility is that Npl3 interferes with the RNase H enzymes recognition of R-loops (Figure 28C). Indeed, the Zou lab has demonstrated that RNase H1-mediated R-loop degradation may be enhanced by R-loop associated RPA (H. D. Nguyen *et al.*, 2017).

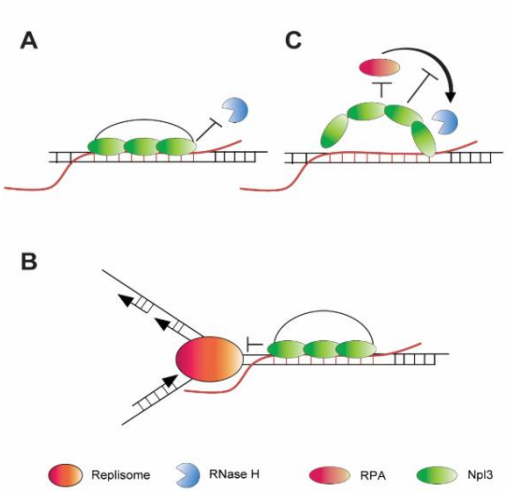


Figure 28: *Proposed Npl3-mediated R-loop stabilization*

A) Npl3 may associate chromatin intercalated RNAs and prevent RNase H enzymes-mediated R-loop degradation. B) Npl3 may associate chromatin intercalated RNAs and prevent R-loop unwinding by replisome associated helicases C) Npl3 may associate displaced telomeric single-strand and exclude RPA binding. This mechanism would prevent RPA-mediated RNase H1 recruitment and activation at telomeres. Symbol legends is indicated at the bottom of the figure.

Although Npl3 has high affinity for RNA molecules, it may also associate to DNA (Lee-Soety *et al.*, 2012) and to G4 structures (Gao *et al.*, 2019). These properties may be particularly important for telomere R-loop stabilization as Npl3 associates *in vitro* to single-strand telomeric sequences (Lee-Soety *et al.*, 2012), which may form G4 quadruplexes (Duquette *et al.*, 2004). By associating to the single DNA strand displaced in the telomeric R-loop, Npl3 may interfere with the RPA recognition of R-loops and reduce the RNase H1-mediated processing (Figure 28C). The details of this mechanism require further investigation.

Npl3 stabilizes telomeric R-loops to prevent anticipated senescence onset

We have identified Npl3 as a telomere binding protein *in* vivo and show that Npl3 associates to telomeres in a TERRA-dependent manner (Figures 13, 16). The TERRA-mediated regulation of Npl3 binding to telomeres results in a strong association of Npl3 to long telomeres in early S phase and to short telomeres (Figure 14, 20D). Further, our data suggests that *NPL3* stabilizes R-loops genome-wide and at telomeres. (Figure 21.22).

At telomeres, Npl3 may participate in the stabilization of R-loops accumulated in early S phase (Figure 20D,22, (Graf *et al.*, 2017), although this stabilization may be regulated depending on telomere length. At long telomeres, RNase H2-mediated R-loop degradation may promote a transient recruitment of Npl3 restricted to early S phase (Figure 20D). On the other hand, the accumulation of R-loops at short telomeres would promote Npl3 recruitment and R-loop stabilization (Figure 29)(Graf *et al.*, 2017). Strong association of Npl3 to short telomeres (Figure 14) may stabilize telomeric R-loops in the cell cycle, similar to what we have observed with our *NPL3* overexpression system (Figure 22). As a consequence, stable telomeric R-loops may increase replication stress and likely regulate telomere HDR and senescence rate in cells with short telomeres (McEachern and Haber, 2006; Balk *et al.*, 2013; Roumelioti *et al.*, 2016b; Graf *et al.*, 2017) (Figure 29). This model would explain the accelerated senescence rate observed in *tlc1 npl3* cells (Lee-Soety *et al.*, 2012), as short telomeres would lack stable R-loops that promote telomere recombination in the absence of *NPL3*. Since deletion of *NPL3* and overexpression of *RNH1* are epistatic in *tlc1* cells (Figure 23A), we conclude that Npl3 indeed stabilizes telomeric R-loops to prevent fast senescence (Figure 29). In support of this, the accelerated senescence rate in *tlc1 npl3* can be rescued by increasing R-loop levels through deletion of *RNH201* (Figure 23B).

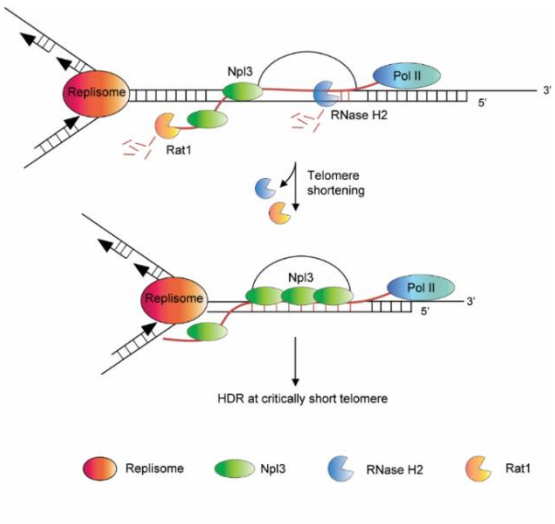


Figure 29: *Proposed model for Npl3 function at telomeres*

At long telomeres, Npl3 associates transiently to telomeres in early S, promoting a transient R-loop stabilization. When telomeres shorten, Npl3 associates strongly to short telomeres to stabilize telomeric R-loops. This may promote HDR at short telomeres and prevent anticipated senescence onset. Symbol legend is indicated at the bottom of the figure.

These results suggest that accumulation of telomeric R-loops alone may not be sufficient to promote HDR at short telomeres. Instead, telomeric R-loops accumulated at short telomeres require Npl3 to prevent accelerated senescence onset. However, why would telomeric R-loops accumulated at short telomeres require additional protein-mediated stabilization? The

answer to this question is not trivial and may depend of several phenomena. On the one hand, R-loops at telomeres may be exposed to degradation by RNase H enzymes. As a consequence, only protected or stable R-loops may be functional at short telomeres. On the other hand, telomeric R-loops may be displaced by incoming replication machineries and require stabilization to promote HDR. Indeed, replisome machineries encountering co-directional R-loops with RNAs associated to the leading strand may displace the R-loops through replisome-associated helicases (Hamperl *et al.*, 2017). Therefore, as TERRA associates with the leading strand at telomeres, TERRA R-loops may be displaced during telomere replication. As a consequence, HDR may be impaired at short telomeres that do not recruit Npl3 (Figure 30). However, data from Garcia-Rubio *et al.* demonstrated that protein-mediated R-loop stabilization prevents replisome-mediated R-loop displacement and increases genome instability (García-Rubio *et al.*, 2018). These results imply that R-loop-associated proteins may prevent displacement of RNAs associated with the leading strand, even when replication machineries encounter R-loops in a co-directional collision. Here, we show that Npl3 strongly associates to short telomeres and stabilizes telomeric R-loops. Further, we show that Npl3 likely associates to telomere associated TERRA (Figure 17B). In conclusion, the binding of Npl3 to short telomeres and TERRA may be required to protect R-loop integrity from approaching replication forks. This protection may subsequently promote replication stress, DSB generation and BIR. In the absence of Npl3, telomeric R-loops may be displaced and HDR may be impaired, resulting in accelerated senescence onset (Figure 30).

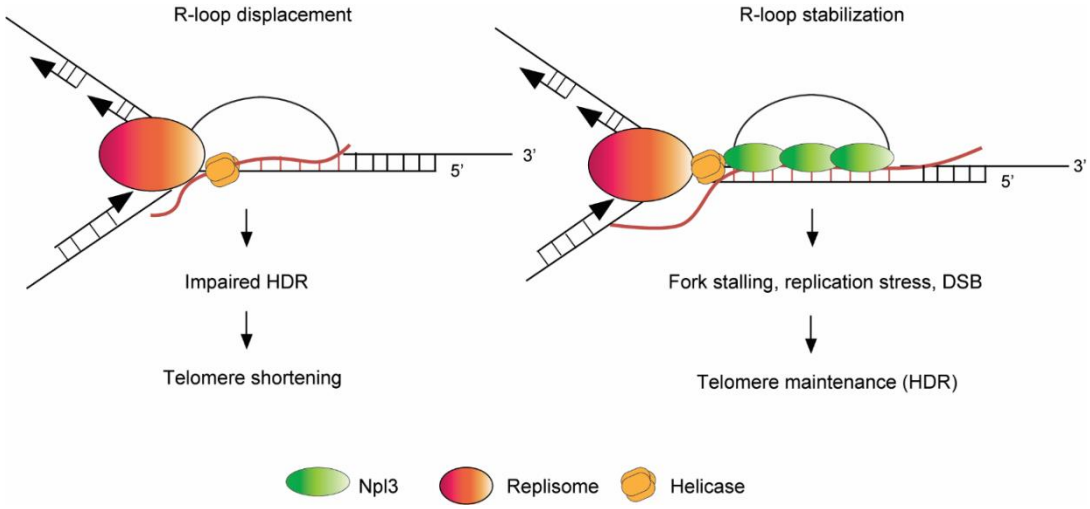


Figure 30: *Proposed mediated mechanism to promote R-loop stabilization at short telomeres*

TERRA R-loops at short telomeres may be displaced by helicases associated with an incoming replisome, as TERRA associates with the leading strand. In the absence of Npl3 (left), replisome-associated helicases may unwind RNAs associated with the leading strand and displace R-loops. This may impair HDR at short telomeres. When Npl3 associates to short telomeres (right), telomeric R-loops may be protected from replisome-associated helicases, which may result in replication stress and HDR-mediated telomere maintenance. Symbol legends are indicated at the bottom of the figure.

Balanced telomeric R-loop regulation

In cells without active telomerase, telomeric R-loops must be balanced to promote telomeric HDR without compromising telomere replication (Balk *et al.*, 2013; Arora *et al.*, 2014; Graf *et al.*, 2017). Therefore, over stabilization of telomeric R-loops may accelerate senescence rate in yeast (Yu, Kao and

Lin, 2014; García-Rubio *et al.*, 2018) and increase replication stress in human cells (Arora *et al.*, 2014; Silva *et al.*, 2019).

To promote HDR-mediated telomere maintenance, stable R-loops accumulate at short telomeres in yeast (Graf *et al.*, 2017). This is facilitated by several mechanisms, including decreased recruitment of RNase H2 (Graf *et al.*, 2017) and perhaps recruitment of hnRNP-like proteins including Npl3 (this study). Interestingly, initial accumulation of R-loops may facilitate Npl3 recruitment to stabilize telomeric R-loops. Subsequently, stable R-loops may increase replication stress and initiate HDR (Figure 31). It is worth mentioning that stable R-loops may directly participate in telomere HDR, as transient formation of R-loops may recruit repair factors and facilitate DNA repair in both yeast and humans (Ohle *et al.*, 2016; D'Alessandro *et al.*, 2018; Teng *et al.*, 2018). In support of this hypothesis, telomeric R-loops recruit the recombination factor Rad51 (Graf *et al.*, 2017). Whether this recruitment is a direct consequence of R-loop accumulation is currently unknown, but it clearly shows the implication of R-loops in telomeric recombination (Graf *et al.*, 2017). Additionally, stable R-loops may be necessary to prevent excessive resection during HDR (Ohle *et al.*, 2016). In summary, Npl3 and other factors such as Yra1 may directly stabilize R-loops to facilitate HDR at yeast short telomeres (Figure 31). Whether this is an active or passive mechanisms it is currently unknown, although Npl3 may only stabilize telomeric R-loops as a consequence of R-loop accumulation in the absence of RNase H2.

However, to successfully complete telomere recombination, the R-loops may need to be removed at a later stage as they may interfere with DNA repair processes (Ohle *et al.*, 2016; D'Alessandro *et al.*, 2018) and generate toxic recombination intermediates . To maintain balanced telomeric R-loop levels, different factors may be required. On the one hand, transient association of R-loop stabilizing factors may be necessary to ensure R-loops removal after initiation of recombination. On the other hand, R-loop regulatory factors such as RNase H enzymes, RNA helicases and DEAD-box helicases or other helicases (like Pif1 or Sen1), may degrade or resolve R-loops once recombination has initiated (Figure 31). To ensure appropriate R-loop removal, Npl3 may need to dissociate from short telomeres. This may require post-translational modifications, which may be regulated by checkpoint kinases. Indeed, checkpoint kinases target Npl3 and post-translational modifications on Npl3 mediate its localization and function (Smolka *et al.*, 2007) (Figure 31). Once Npl3 has dissociated from the telomeres, RNase H enzymes and possibly other R-loop regulatory factors may promote R-loop degradation to complete HDR. As mentioned above, one possibility is that yeast helicases participate in telomeric R-loop removal at short telomeres, as certain helicases regulate R-loop levels and coordinate recombination. For example, the yeast RecQ helicase Sgs1 prevents the accumulation of toxic recombination intermediates, thus facilitating telomere recombination and preventing accelerated senescence (Lee *et al.*, 2007). Similarly, in plants, RTEL1 helicase may facilitate telomere recombination in the absence of telomerase (Olivier *et al.*, 2018). In addition, mammalian RTEL1 regulates homologous recombination and telomere maintenance (Uringa *et al.*, 2011; Vannier *et al.*, 2012, 2013; Margalef *et al.*, 2018). It is therefore possible that certain helicases promote successful HDR by assisting the resolution R-loop-derived recombination intermediates. Interestingly, our screening for telomere associated proteins identified several RNA and DNA helicases (Figure 13, 16), which may additionally participate in R-loop resolution to complete HDR.

Once telomeres have recombined, elongated telomeres may regulate R-loop levels differently to short telomeres to prevent replication stress. For example in yeast, the Rif1-mediated recruitment of RNase H2 to long telomeres would decrease R-loop levels (Graf *et al.*, 2017). On top of that, decreased R-loop levels would regulate a transient association of Npl3 and other R-loop stabilization factors. Further, additional factors including helicases may prevent R-loop accumulation at long telomeres. Altogether, in yeast, telomere length, RNase H2 and other factors including Npl3 mediate R-loop stabilization exclusively at short telomeres ((Graf *et al.*, 2017), Figure 31). Our screening may provide further

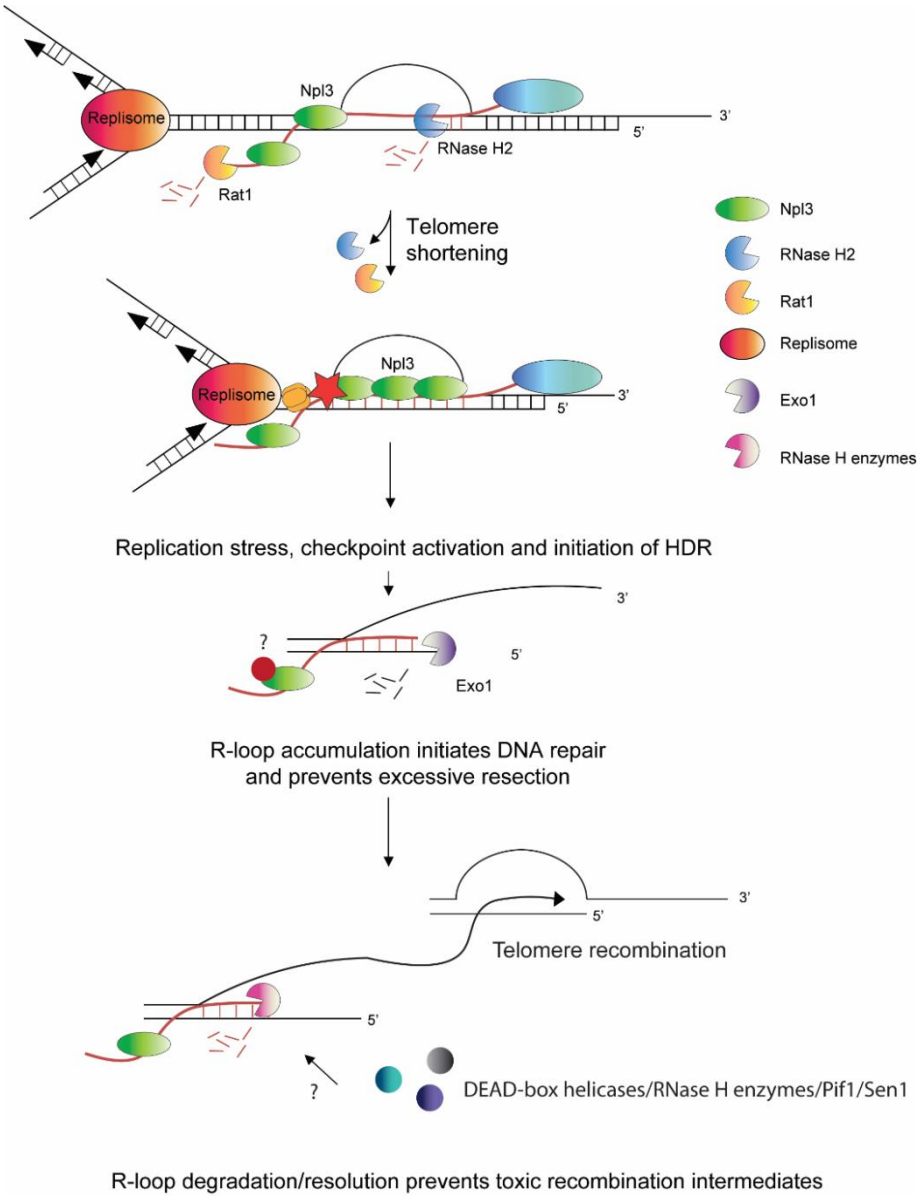


Figure 31: *Proposed model for short telomere maintenance.*

TERRA and R-loop levels are regulated at long telomeres by Rat1, RNase H2 and Npl3. Possibly other hnRNP-like proteins are involved in these process. When telomeres shorten, R-loops accumulate due to decreased association of RNase H2 to telomeres. This results in increased telomeric R-loop levels at short telomeres, which recruit Npl3. Npl3 association to short telomeres stabilizes R-loops, which may protect them from RNase H-mediated degradation and replisome-associated helicases. As a result, stable R-loops generate replication stress, checkpoint activation and initiation of HDR. Accumulated R-loops may prevent excessive resection at telomeres once HDR has initiated. Presumably, checkpoint activation may result in post-translational modifications on Npl3, which may release it from associated R-loops. This would facilitate telomere recombination (see text). Additionally, R-loop regulatory proteins such as RNase H enzymes, DEAD-box helicases, Pif1 or Sen1 may be recruited to recombining telomeres to promote successful HDR-mediated telomere elongation. Symbol legends are indicated on the right side of the figure. Red star represents replication stress; Red circle represents post-translational modifications on Npl3. Abbreviations: HDR: homology-directed repair.

insights into the different factors recruited to telomeres to regulate balanced R-loop levels in telomerase negative cells.

Additional roles of NPL3 in senescence

In addition to R-loop stabilization, Npl3 may regulate senescence onset through different mechanisms. Indirectly, deletion of *NPL3* may negatively affect the expression of specific genes that may impact senescence rate (Nautiyal, DeRisi and Blackburn, 2002; Platt *et al.*, 2013; Santos-Pereira *et al.*, 2014). For example, Npl3 may regulate resection by promoting the biogenesis of *EXO1* mRNA (Colombo *et al.*, 2017). By coordinating the expression of *EXO1* and possibly other genes implicated in DNA and DSB repair, Npl3 may prevent anticipated senescence onset. Another possibility is that Npl3-mediated transcription regulation coordinates histone levels and senescence rate. Histone levels may impact the efficiency of DSB repair and possibly HR-mediated telomere maintenance, as histone levels decrease in senescent cells and determine senescence rate (Nautiyal, DeRisi and Blackburn, 2002; Platt *et al.*, 2013). Deletion of *NPL3* may result in different histone levels regulation in telomerase negative cells, which may affect senescence onset. Finally, as Npl3 coordinates the expression of genes transcribed by RNA polymerase II, it is possible that Npl3 regulates TERRA expression. The Johnson lab has proposed that TERRA levels affect senescence onset (Wanat *et al.*, 2018), raising the question if Npl3 may mediate senescence rate through TERRA regulation. Altogether, it is possible that Npl3-mediated transcription regulation coordinates gene expression and, as a result, impacts senescence rate.

Npl3 directs mRNAs to nuclear pores and facilitates mRNA nuclear export (Santos-Pereira *et al.*, 2014). Interestingly, short telomeres and DSBs are transcribed and relocate to nuclear pores in yeast, likely to facilitate homology-directed repair and short telomere maintenance (Taddei and Gasser, 2006; Therizols *et al.*, 2006; Khadaroo *et al.*, 2009; Churikov *et al.*, 2016; Freudenreich and Su, 2016; Ohle *et al.*, 2016).The fact that TERRA in yeast may localize to the cytoplasm upon stress conditions such as diauxic shift suggests that yeast TERRA may also relocate to nuclear pores under other conditions including telomere shortening (Perez-Romero *et al.*, 2018). Indeed, TERRA localization to nuclear pores may derive from active RBP-mediated transport, which may also tether telomeres to nuclear pores if TERRA remains associated to chromatin. In support of this model, studies in human cells have shown that imbalanced transcription termination may tether chromatin to nuclear pores and increase replication stress (Teloni *et al.*, 2019). In particular, the Altmeyer lab proposes that DNA tethering to nuclear pores is promoted by export factors that relocate to nuclear pores, whilst nascent transcripts still are associated to chromatin (Teloni *et al.*, 2019). A similar mechanism may be functional at yeast short telomeres, where TERRA transcription termination may be impaired due to decreased localization of Rat1 factor (Graf *et al.*, 2017). Under these conditions, Npl3 may associate to TERRA and target it to nuclear pores. If TERRA remains chromatin-associated, short telomeres would also be relocated to nuclear pores. Altogether, these studies raise the interesting possibility that the association of TERRA with export factors such as Npl3 tether short telomeres to nuclear pores and facilitate HR-mediated telomere elongation.

In human ALT cells, telomeres localize to APBs likely to facilitate HR-mediated telomere recombination (Sobinoff and Pickett, 2017). Further, human TERRA has been found outside the nucleus, in exosome compartments (Wang *et al.*, 2015; Wang and Lieberman, 2016). This suggests that TERRA localization is dynamic. It is therefore possible that TERRA can mediate localization of telomeres to different cellular compartments, to promote telomere maintenance. Further, this may be mediated by a very complex network of TERRA interactors (Scheibe *et al.*, 2013; Chu *et al.*, 2017).

Possible conservation of hnRNP-mediated R-loop stabilization

Our data suggest that *NPL3* stabilizes R-loops when overexpressed (Figure 21, 22). In addition, our data suggest that balanced Npl3-mediated R-loop stabilization at telomeres preserves telomere integrity and prevents premature senescence onset (Figure 29).

Yeast Npl3 functions may be conserved in hnRNPs in human cells to promote balanced telomere replication and R-loop regulation. For example, hnRNPA1 and hnRNPA3 associate TERRA (Flynn *et al.*, 2011; Chu *et al.*, 2017). Interestingly, hnRNPA3 also associates to telomeres through its RRM domains (Huang, Hung and Wang, 2010). Further, overexpression of hnRNPA3 shortens telomeres in human cells (Huang, Hung and Wang, 2010). Even though the molecular mechanism for this phenotype has not been explored in detail, it remains possible that hnRNPA3, similarly to yeast Npl3, stabilizes telomeric R-loops. Unscheduled R-loop stabilization upon hnRNPA3 overexpression may therefore shorten telomeres as a consequence of R-loop-mediated replication stress. Further, it may compromise cell viability especially in ALT cells (Arora *et al.*, 2014; Silva *et al.*, 2019).

Balanced telomeric R-loop regulation in human cells may therefore require a series of different proteins including RNase H enzymes, Falconi Anemia enzymes and hnRNPs (Huang, Hung and Wang, 2010; Arora *et al.*, 2014; Pan *et al.*, 2017; Silva *et al.*, 2019). The coordinated activity of these enzymes at telomeres may have important consequences in human syndromes, as stable R-loops may increase genome instability, cancer progression and expansion of repetitive sequences (José M Santos-Pereira and Aguilera, 2015; Neil *et al.*, 2018). In particular, ALT cancer cells may regulate these enzymes differently to sustain increased R-loop levels that promote HR-dependent telomere maintenance.

Conclusions

This study provides an extensive dataset of telomere interacting proteins in wild type and *tlc1* cells that may increase our understanding of telomere maintenance in *S. cerevisiae*. Functional characterization of these factors may provide insights into mammalian telomere length regulation.

From our telomere interactors screening, we characterized the function of Npl3 at telomeres. We described the role of Npl3 in the regulation of R-loop levels genome-wide and regulation of telomeric R-loop in a functional manner. The results of this study expand our understanding of TERRA R-loop regulation, in particular during senescence in budding yeast. Our data provide a basis for further studies and insight into R-loop regulation during DNA repair and DSB repair. Furthermore, our data may provide further insights and research options into mechanistic regulation of telomeric R-loops in ALT cancer cells.

Appendix

Table 1 *Telomere binding candidates identified in WT cells and tlc1 cells*

"Common WT-*tlc1"*displays factor identified as telomere interactors in both WT and *tlc1* cells. "Unique WT" shows the telomere interacting factors identified exclusively in WT cells. "Unique *tlc1"* indicates the telomere associated factors identified exclusively in *tlc1* cells.

Common WT-tlc1	**Unique WT**	**Unique *tlc1***			
RAP1	MIG2	REX4	SBP1	DBP9	RDH54
RCL1	SWC3	SGV1	CUS1	RPL10	MRP51
TFC3	SDD4	SHM1	YPL088W	PKH1	SPB1
MGM101	CKB2	MRP7	RFA2	MRPL35	BFR1
AFT1	ESF1	HMG1	NOG2	TIF35	OTU1
BOI1	NAM7	MSL5	RMD8	AIM34	ACC1
NPL3	INO80	UTP7	RPS15	RRP17	PPZ2
MSC3	RRP5	MYO3	SEN1	NSA2	GCD11
SLH1	GCD10	RPS11A	MRM1	MCM10	ATG20
TRI1	MET4	MSS116	NTE1	RPS1A	PYC1
CBP2	VPS72	RPS9A	PRP3	MRPL16	POM33
RFA1	PCT1	SNP1	RPS16A	TOP3	PYC2
SUB1	PAM1	SAC7	SLI15	PET123	PXR1
ECM32	YDR514C	TIF4631	MRPS9	FUN19	GIS2
DBP10	NIP7	CTK1	YLR419W	RPS1B	FMP52
SRS2	CSL4	RCO1	RPL17A	EAF3	MDV1
IMD4	SLD7	THO2	DPI29	KIP2	
RAI1	PHO4	TMA64	TAF7	MRX4	
RAD52	UTP20	IMD2	RPB5	RPB8	
XRN1	DNA2	SRO9	RMD1	SHE1	
BMS1	ADR1	PAT1	UTP23	NAM9	
ORC2	HOS4	ROM2	RPL13A	YKR070W	
RAT1	YTA6	YRA1	PBP4	MUS81	
TIF4632	RRP6	RFA3	RPS6A	HHF2	
RSM28	IMD3	KIN4	YRA2	RLF2	
PET127	HSH155	MRPL17	YHC1	HMO1	
MSC6	HPC2	MRPL7	DBP1	CBF5	
MRD1	POL2	IMD3	HEK2	SSD1	
SEG1	SPP41	STM1	PAM16	RPL11A	
DBP2	GFD2	PKH2	SGN1	RPL11B	
BCK1	IMD2	CMR2	PIF1	MRPL24	
NOP4	GIP4	ABF2	PUS7	RRG9	
MUD2	RRP43	HCS1	RPO31	SNT1	
RQT4	DBP7	RPL8B	MRPS35	NOP16	
CUE3					

Table 2 *Overlap between telomere interactors in WT cells and previously published telomere length regulatory factors*

Telomere interactors identified when using WT protein extracts are indicated. Overlap with published studies is indicated at the bottom. Black box indicates overlap, whereas white box indicates no overlap. RBPs and helicases are highlighted in black. Abbreviations: RBP: RNA binding protein.

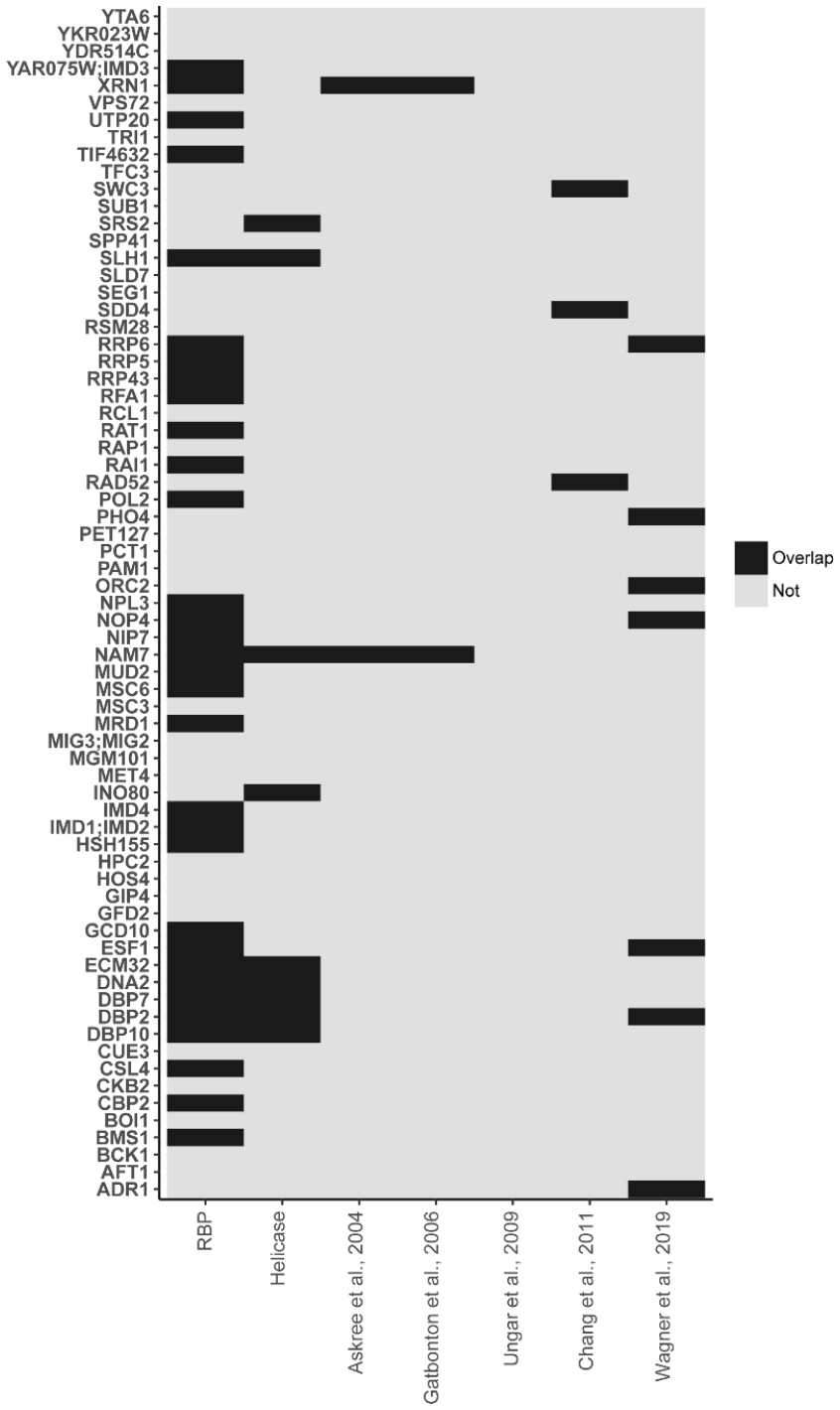
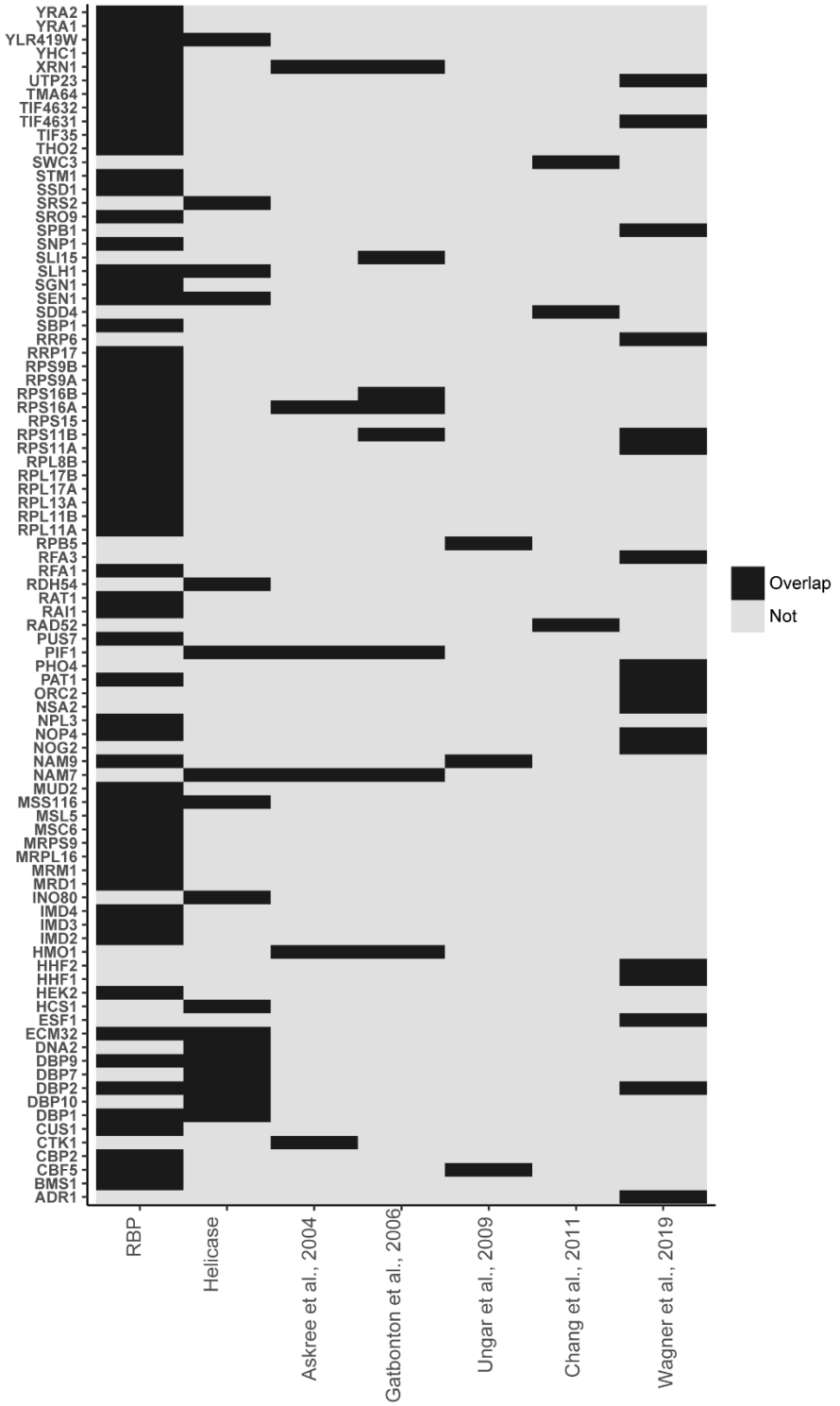


Table 3 *Overlap between telomere interactors in tlc1 cells and previously published telomere length regulatory factors*

Telomere interactors identified when using *tlc1* protein extracts are indicated. Overlap with published studies is indicated at the bottom. Only proteins showing overlap with at least one study are represented. Black box indicates overlap, whereas white box indicates no overlap. RBPs and helicases are highlighted in black. Abbreviations: RBP: RNA binding protein.



Materials and methods

Materials

Yeast strains

The strains used on this study are listed below. Yeast culture was conducted at 30°C in YPD medium unless otherwise indicated.

All yeast strains used in this study derive from *S. cerevisiae* parental strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) (Winston, Dollard and Ricupero-Hovasse, 1995). Strains were generated using standard yeast protocols (Guthrie, C., and Fink, 1991).

Strain n.	Name	Genotype	Source
yBL7	wildtype S288C	MATa *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Euroscarf
yLP10	*TLC1/tlc1*	S288C Mat a/Mat α *TLC1/tlc1::Nat*	This study
	NPL3/NPL3-TAP	*NPL3/NPL3-TAP-HIS3MX6*	
yLP130	*tlc1*	S288C Mat a/Mat α *TLC1/tlc1::Nat*	This study
	npl3 +pBL335 (eV)	*NPL3/npl3* +pBL335	
yLP131	*tlc1*	S288C Mat a/Mat α *TLC1/tlc1::Nat*	This study
	npl3 +pBL336 (*RNH1* oE)	*NPL3/npl3* +pBL336 (*RNH1* oE)	
yLP16	*NPL3-TAP*	S288C Mat a *NPL3-TAP-HIS3MX6*	Dharmacon
yLP193	*NPL3-TAP*	S288C Mat α *NPL3-TAP-HIS3MX6*	This study
	RAT1-AID	*RAT1-AID-HYG*; *bar1::KAN; afb2::LEU2*	
	bar1		
	afb2		
yLP202	*RAT1-AID*	S288C Mat α	This study
	bar1	*RAT1-AID-HYG*; *bar1::KAN; afb2::LEU2*	
	afb2		
yLP303	*NPL3-TAP* +eV	S288C Mat a *NPL3-TAP-HIS3MX6* +pBL211(eV)	This study
yLP305	*NPL3-TAP* + *RNH1* oE	S288C Mat a *NPL3-TAP-HIS3MX6* +pBL352 (*RNH1* oE)	This study
yLP307	wildtype S288C +eV	S288C Mat a +pBL211(eV)	This study
yLP309	wildtype S288C +*RNH1* oE	S288C Mat a +pBL352(*RNH1* oE)	This study
yLP368	*NPL3-TAP* +*RAT1* oE	S288C Mat a *NPL3-TAP-HIS3MX6* +pBL566 (pRD54_*RAT1* oE)	This study

yLP371	*NPL3-TAP* +eV	S288C Mat a *NPL3-TAP-HIS3MX6* +pBL19(eV)	This study
yLP374	wildtype +*RAT1* oE	S288C Mat a+pBL566 (pRD54_*RAT1* oE)	This study
yLP377	wildtype +eV	S288C Mat a +pBL19(eV)	This study
yLP380	*rnh201* *tlc1* *npl3*	S288C Mat a/Mat α *TLC1/tlc1::Nat RNH201/rnh201::HYG; NPL3/npl3::KAN*	This study
yLP400	*npl3* *tlc1* +*NPL3* oE	S288C Mat a/Mat α *TLC1/tlc1::Nat* *NPL3/npl3::KAN* +pBL565 (pRD54_*NPL3* oE)	This study
yLP550	wildtype S288C +eV pRD54	S288C Mat a +pBL19(pRD54_eV)	This study
yLP551	wildtype S288C +*NPL3* oE	S288C Mat a +pBL565 (pRD54_*NPL3* oE)	This study
yLP679	*sen1-1* +eV pRD54	S288C Mat a *sen1-1::KAN* +pBL19(pRD54_eV)	This study
yLP681	*sen1-1* +*NPL3* oE	S288C Mat a *sen1-1::KAN* +pBL565 (pRD54_*NPL3* oE)	This study
yMD1245	*TLC1*/*tlc1*	S288C Mat a/Mat α Tlc1/tlc1::his	(Balk et al., 2013)

Plasmids

Plasmid and recombinant DNA used on this study are listed below. Plasmid name, DNA backbone and source are indicated.

Recombinant DNA	**Source**	**Internal number**
Plasmid: pRS425- GAL, 2u, *LEU2*	Matthias Peter	pBL211
Plasmid: pRS425- *RNH1-HA,* GAL, 2u, *LEU2*,	(Balk et al., 2013)	pBL352
Plasmid: pRD54 GAL-HA, CEN, *URA3*	Matthias Peter	pBL19
Plasmid: pRD54 *NPL3-HA*, GAL, CEN, *URA3*	This study	pBL565
Plasmid: pRD54 *RAT1-HA*, GAL, CEN, *URA3*	This study	pBL566
Plasmid: pT316 GPD, CEN, *HIS3*	Teixiera lab	pBL335
Plasmid: pT316 RNH1 GPD, CEN, *HIS3*	Teixiera lab	pBL335

Oligonucleotides

Oligonucleotides used in this study are listed below. The following oligonucleotides were used for PCR amplification, reverse transcription, qPCR, telomere bait polymerization for pull-down experiment and cloning.

Oligo n.	Use	Sequence (5'-3')
oBL295	qPCR -1L telomere	CGGTGGGTGAGTGGTAGTAAGTAGA
oBL296	qPCR -1L telomere	ACCCTGTCCCATTCAACCATAC
oBL292	qPCR- actin	CCCAGGTATTGCCGAAAGAATGC
oBL293	RT and qPCR-actin	TTTGTTGGAAGGTAGTCAAAGAAGCC
oLK57	qPCR-15L	GGGTAACGAGTGGGGAGGTAA
oLK58	qPCR-15L	CAACACTACCCTAATCTAACCCTGT
oLK49	qPCR- 6Y'	GGCTTGGAGGAGACGTACATG
oLK50	qPCR- 6Y'	CTCGCTGTCACTCCTTACCCG
oBL207	TERRA RT	CACCACACCCACACACCACACCCACA
oBL29	Confirm *NPL3* KO	CTGCAGCGAGGAGCCGTAAT
oLP8	Confirm *NPL3* KO	GGCTTATTGATTACAATTGCTTGTT
oLP115	Telomere bait	GTGGGTGTGTGGTGTGGGTGTGTGGGTGTGTG TGGTGTGGGTGTGTGTGGGTGTGTGTGTGGGT GTGGGTGTGGTGT
oLP116	Telomere bait	ACACACCACACCCACACCCACACACACACCCAC ACACACCCACACCACACACACCCACACACCCAC ACCACACACCC
oLP117	Control bait	GTGAGTGTGAGTGTGAGTGTGAGTGTGAGTGTG AGTGTGAGTGTGAGTGTGAGTGTGAGT
oLP118	Control bait	ACACTCACACTCACACTCACACTCACACTCACAC TCACACTCACACTCACACTCACACTC
oLP134	Cloning *NPL3* into pRD54	AGGGCTGCAGGAATTCTCTGAAGCTCAAGAAAC TCACGT
oLP135	Cloning *NPL3* into pRD54	GCTTGATATCGAATTCAAACTTATTTTTGTTAATT TTGCAGCACAT
oLP136	Cloning *RAT1* into pRD54	AGGGCTGCAGGAATTCGGTGTTCCGTCATTTTT CAGATGG
oLP137	Cloning *RAT1* into pRD54	GCTTGATATCGAATTCTTTTCCCTAATACTTGGTTCC TCGC

Liquid media

Medium	Composition
Lysogeny broth medium (LB)	1% (w/v) NaCl 1% (w/v) Bacto tryptone 0.5% (w/v) Bacto yeast extract Supplemented with 100 ug/ml carbenicillin
Yeast peptone dextrose (YPD)	10 % (w/v) peptone 5% (w/v) Bacto-yeast extract 2% (w/v) glucose
Synthetic complete medium without amino acids (SC-)	0.192% (w/v) yeast synthetic dropout medium without amino acids (of choice) 0.67% (w/v) yeast nitrogen base without amino acids 2% (w/v) glucose OR 1%(w/v) raffinose 2% (w/v) galactose
Sporulation medium (SPO)	0.005% zinc acetate 1% potassium acetate

Agar plates

Type of plate	Composition
LB plates	1% (w/v) tryptone 0.5% (w/v) Bacto yeast extract 1% (w/v) NaCl 1.5% (w/v) agar Supplemented with 100 ug/ml carbenicillin
YPD plates	6.5% (w/v) YPD agar 10 % (w/v) peptone 5% (w/v) Bacto-yeast extract 2% (w/v) glucose

Synthetic complete medium without amino acids plates (SC- plates)	0.192% (w/v) yeast synthetic dropout medium without amino acids (of choice) 0.67% (w/v) yeast nitrogen base without amino acids 2.4% (w/v) agar 2% (w/v) glucose OR 1%(w/v) raffinose 2% (w/v) galactose
Pre-sporulation plates (PRE-SPO)	3% (w/v) nutrient broth 1% (w/v) Bacto yeast extract 2% (w/v) agar 5% (w/v) glucose

YPD plates and SC- plates were supplemented (when required) with the following antibiotic concentrations:

Antibiotic	**Final concentration**
G418 disulfate solution (Kanamycin)	250 ug/ml
Hygromycin B	300 ug/ml
Nourseothricin-dihydrogen sulfate (clonNat)	100 ug/ml

Solutions and buffers

Solution or buffer	**Composition**
LiAc mix	100mM lithium acetate in 1x TE
PEG mix	40% (w/v) PEG 400 in LiAc mix Sterile filtered
Lysis Buffer - IGEPAL	50 mM Tris-HCl pH 7.5 150 mM NaCl 5 mM MgCl2 1 mM PMSF Supplemented with complete protease inhibitor cocktail [Roche]
Lysis Buffer + IGEPAL	50 mM Tris-HCl pH 7.5 150 mM NaCl 5 mM MgCl2 1 mM PMSF

	Supplemented with complete protease inhibitor cocktail [Roche] and 0.2 % IGEPAL CA-630
Annealing buffer	20 mM Tris-HCl
	10 mM MgCl2
	100 mM KCl
PBB buffer	150 mM NaCl
	50 mM Tris-HCl pH 7.5
	0.5% IGEPAL CA-630
	5 mM MgCl2
	Supplemented with fresh DTT (1mM final concentration)
ABC buffer	50 mM ammonium bicarbonate
Buffer A	0.1% formic acid
Buffer B	80% ACN, 0.1% formic acid
Reduction buffer	10 mM DTT in 50 mM ABC buffer
Alkylation buffer	50 mM iodoacetamide (IAA) in 50 mM ABC buffer
FA buffer – SOD	50 mM HEPES pH 7,5
	140mM NaCl
	1mM EDTA pH8
	0,1% Triton X-100
FA buffer + SOD	50 mM HEPES pH 7,5
	140mM NaCl
	1mM EDTA pH8
	0,1% Triton X-100
	0.1 % sodium deoxycholate (SOD)
FA 500	50 mM HEPES pH 7,5
	500 mM NaCl
	1mM EDTA pH8
	0,1% Triton X-100
	0.1 % sodium deoxycholate (SOD)
Buffer III	10 mM Tris-HCl pH8
	1 mM EDTA
	250 mM LiCl
	1% NP-40

	1% sodium deoxycholate (SOD)
10X TE	100 mM Tris-HCl pH7,5
	1% SDS
	10mM EDTA pH8
Elution buffer B	50 mM Tris-HCl pH7,5
	1% SDS
	10mM EDTA pH8
Solution 1 (protein extraction)	1.85 M NaOH supplemented with 1.09 M 2-mercaptoethanol
Solution 2 (protein extraction)	50% Trichloroacetic acid (TCA) in H2O
Urea buffer (protein extraction)	120 mM Tris-HCl pH 6,8
	5% glycerol
	8M Urea
	143 mM 2-mercaptoethanol
	8% SDS
	Bromophenol blue
10X SDS running buffer	25mM Tris-HCl
	192 mM glycine
	0,1% SDS
	Final pH adjusted to 8,3
10X blotting buffer	25mM Tris-HCl
	192 mM glycine
	Sterile filtered
10X PBS	1,37 M NaCl
	0,03 M Kcl
	0.08 M Na2HPO4 x2 H2O
	0.02 M KH2PO4
	Final pH adjusted to 7.4
1X PBST	1X PBS
	0.1% Tween-20
Blocking buffer	1X PBST
	5% (w/v) skim milk powder
10X TBE	890 mM Tris base
	890 mM boric acid

	20 mM EDTA pH8
6X DNA loading dye	15% Ficoll
	10mM EDTA pH8
	Orange G
EDTA pH8	500 mM disodium EDTA x2 H2O
	pH adjusted with NaOH
SSC 20X pH7	3M NaCl
	0.3M Na3 citrate x2 H2O
Denaturing solution (for Southern Blot)	400 mM NaOH
	600 mM NaCl
Neutralizing solution (for Southern Blot) pH7.4	1M Trizma base
	1.5 M NaCl
Washing solution I (for Southern Blot)	2X SSC
	0.1% SDS
Washing solution II (for Southern Blot)	0.5X SSC
	0.1% SDS

Reagents

Reagent	Supplier	Identifier
Antibodies		
Mouse anti-FLAG M2	Sigma-Aldrich	Cat. Number F3165
		RRID:AB_259529
Rabbit PAP (Peroxidase Anti-peroxidase soluble complex)	Sigma-Aldrich	Cat. number
		RRID:AB_1079562
Mouse monoclonal anti-Actin Clone C4	Millipore	Cat. number MAB1501R
		RRID:AB_2223041
Mouse monoclonal anti-HA.11 Clone 16B12	Covance	Cat. number MMS-101P
		RRID:AB_2314672
Mouse monoclonal anti-DNA-RNA Hybrid (S9.6)	Kerafast	Cat. number ENH001; RRID:AB_2687463

Mouse monoclonal anti-ds DNA (35I9 DNA)	Abcam	Cat. number ab27156; RRID:AB_470907
Goat Immun-Star anti-mouse (GAM)-HRP conjugate	Bio-Rad	Cat. number 170-5047; RRID: AB_11125753
Chemicals, Peptides, and Recombinant Proteins		
alpha-factor mating pheromone	Zymo Research	Cat. number Y1001
Hydroxyurea	Sigma-Aldrich	Cat. number H8627-25G
RNase H	NEB	Cat. number M0297S
RNase III	Invitrogen	Cat. number 10301375
RNase T1	Thermo Scientific	Cat. number EN0541
SYBR Gold	Invitrogen	Cat. number S11494
SYTOX Green nucleic acid stain	Thermo Scientific	Cat. number S7020
2-mercaptoethanol	Sigma-Aldrich	Cat. number M6250
cOmplete EDTA-free Protease Inhibitor Cocktail	Roche	Cat. number 4693159001
RNase A	Thermo Scientific	Cat. number EN0531
Proteinase K	Qiagen	Cat. number 19133
SuperSignal West Pico PLUS Chemiluminescent Substrate	Thermo Scientific	Cat. number 34578
GE Healthcare IgG Sepharose™ 6 Fast-Flow-Medium	Fisher Scientific	Cat. number 11574955
GE Healthcare nProtein A Sepharose™ 4 Fast Flow Affinity Media	Fisher Scientific	Cat. number 11359931
PMSF BioChemica	Applichem	Cat. number A0999,0005
Igepal CA-630	Sigma-Aldrich	Cat. number I8896-50ML

T4 Polynucleotide Kinase	NEB	Cat. number M0201S
T4 DNA ligase	(Thermo Scientific)	Cat. number M0202S
Phenol:Chloroform:Isoamyl Alcohol 25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA.	Sigma-Aldrich	Cat. number P2069-100mL
N6-(6-Amino)hexyl-dATP - Biotin (Biotin-7-dATP	(Jena Bioscience)	Cat. number NU-835-BIO
Klenow fragment exo	(Thermo Scientific).	Cat. number EP0422
Dynabeads® MyOne™ Streptavidin C1	Invitrogen	Cat. number 65002
DTT (dithiothreitol)	Invitrogen	Cat. number Y00147
Salmon Sperm DNA (sheared, 10 mg/ml) (1 ml Tube) (Invitrogen™)	Invitrogen	Cat. number AM9680
NuPAGE® LDS Sample Buffer (4X) (250 ml)	Life Technologies	Cat. number NP0008
MOPS buffer	Applichem	Cat. number A1076,0250
Ammonium bicarbonate	Sigma Aldrich	Cat. number A6141-500G

Iodoacetamide (IAA)	Sigma Aldrich	Cat. number I6125-10G
Lysyl Endopeptidase, Protease LysC (10AU)	Wako Chemicals USA, Inc.	Cat. number 129-02541
CAN-acetonitrile	VWR	Cat. number 20048.320
Formic acid 98-100%	Supelco	Cat. number 5.33002
Formaldehyde solution 37 % for molecular biology	Applichem	Cat. number A0877,0250
Glycine		
3-Indoleacetic acid (IAA) plant cell culture tested, crystalline	Sigma-Aldrich Chemie GmbH	Cat. number I2886-5G
HEPES Puffer 1M pH 7,5	Applichem	Cat. number A6916,0250
Triton X-100	Sigma Aldrich	Cat. number **X100-1L**
Sodium deoxycholate (SOD)	Sigma Aldrich	Cat. number D6750-100G
BSA, Molecular Biology Grade, 20 mg/ml	New England Biolabs (NEB)	Cat. number B9000S
Lithium Chloride (LiCl)	Applichem	Cat. number A6286,0250
Nonylphenylpolyethyleneglycol N4, Nonidet P40	AppliChem	Cat. number A1694, 0500
SDS 20%	Applichem	Cat. number A0675,1000
RNase-Free DNase Set (DNase I)	QIAGEN	Cat. number 79254

SuperScript III reverse transcriptase	Fisher Scientific	Cat. number 10368252
Dynameo Flash SYBR Green qPCR kit	Fisher Scientific	Cat. number 10334009
Urea	Sigma Aldrich	Cat. number U5378
Bromophenol blue	Sigma Aldrich	Cat. number B0126-250
Nylon membrane, positively charged	Roche	Cat. number 11417240001
Hybridization solution- Perfect Hyb-Buffer	Sigma Aldrich	Cat. number H7033
D-Sorbitol	Sigma Aldrich	Cat. number 51876
Lyticase (lyticase crude from arthrobacter luteus)	Sigma Aldrich	Cat. number L4025-25KU
XhoI	NEB	Cat. number R0146S
PerfectHyb™ Plus Hybridization Buffer	Sigma Aldrich	Cat. number H7033
dATP alpha-P32	Perkin Elmer	Cat. number NEG512H250UC
Microspin Sephadex G50 columns	GE Healthcare	Cat. number 27-5330-02
NuPAGE 4-12% BT Gel 1.0MM10W	Life Technologies	Cat. number NP0321BOX
NuPAGE LDS loading buffer	Fisher Scientific	Cat. number NP0007
C18 Extraktionsdisk, 47 mm, 60 Disks pro VE (3 Pack à 20 Disk)	3M	Cat. number 2215

Instruments

Instrument	Supplier
EASY-nLC 1000 system	Thermo
Q Exactive Plus mass spectrometer	Thermo
Electrospray ion source (Nanospray Flex™)	Thermo
BD FACSVerse™ flow cytometer	BD
BRANSON sonifier 450	BRANSON Ultrasonics corporation
BioRuptor Pico	Diagenode
Typhoon™ FLA 9500	GE Healthcare

Software

Software and Algorithms	Provider
FACSuite V1.0.5	Becton Dickinson
FlowJo V10.5.3.	FlowJo
Image Lab 5.2	Bio-Rad
MaxQuant software V1.5.2.8	MaxQuant
R	© The R Foundation
thePantherDB.org	PantherDB.org
CFX Manager (qPCR data)	BioRad
CFX384 Touch Real-Time PCR Detection System	BioRad
STRING DB V11	STRING
Excel 2013	Microsoft
Word 2013	Microsoft
PowerPoint 2013	Microsoft
Adobe Illustrator CC2018	Adobe
Prism 8	GraphPad
Mendeley Desktop	Elsevier
Image Lab V5	BioRad

SnapGene Viewer V5	SnapGene (R)

Commercial assays

Critical commercial assays	**Supplier**
Gentra Puregene Yeast/Bact. Kit B	QIAGEN
QIAprep Spin Miniprep Kit (50)	QIAGEN
PCR purification kit	QIAGEN
RNeasy MinElute Cleanup kit	QIAGEN

Methods

Yeast strains, culture and manipulation

Heterozygous diploid strains were generated by crossing Mat a and Mat alpha strains with different genetic backgrounds. Cells were patched together on YPD plates and grown together for 12-24h at appropriate temperature. Selection of heterozygous diploids was based on growth on double selection. Haploid yeast strains obtained by tetrad dissection were generated as follows: heterozygous diploid strains were patched on pre-sporulation plates and grown for 12-24h at 30°C and sporulated on sporulation medium at 23°C for at least 72h. 7,5ul of cells resuspended on sporulation medium were mixed 1:1 with lyticase and incubated at room temperature for 10minutes. Tetrads were picked by micromanipulation and grown on appropriate agar plates for 24h-72h before scoring genotypes.

Yeast cells were transformed with different plasmids as follows. First, competent yeast cells were generated by incubation of exponentially growing cells with 5ml LiAc-mix. 100ul of competent cells were incubated at room temperature for 30minutes with 10ul Yeast Marker Carrier DNA , 700ul PEG-mix and corresponding plasmid. Cells were heat shocked for 15min at 37°C, harvested by centrifugation and resuspended in non-selective YPD medium. After 30minutes incubation at 30°C, cells were plated on appropriate selective medium.

Bacterial transformation

Competent DH5alpha *E. coli* cells were incubated with 100ng of plasmid and incubated for 30minutes on ice. Subsequently, cells were heat shocked for 1minute at 42°C and incubated on ice for another 2 minutes. 300ul of non-selective LB medium was added to cells followed by a 30minute at 37°C recover period. Finally, bacterial cells were plates on selective LB plates and grown over night at 37°C.

Protein extraction for MS/MS

100 ml of exponentially growing cultures were collected by centrifugation for 3 min at 1731 rcf. Cell pellets were lysed in lysis buffer by two rounds of 30s at 6.5 M/s on a FastPrep machine (MP Biomedical). Cell extracts were resuspended in lysis buffer supplemented with 0.2% IGEPAL CA-630 and centrifuged at 13.000 rcf for 15 min at 4° C. The soluble fraction was subjected to a second round of centrifugation recovering the supernatant (soluble protein extract). 500 µg of protein extract was used for each telomere pull-down.

Polymerization of DNA baits

25 µg of forward and reverse oligonucleotides harboring telomere and control sequence were diluted in annealing buffer and denatured at 95° C for 10 min. Oligos were annealed by cooling down to room temperature and polymerized with 50 units of T4 Polynucleotide Kinase (NEB) for 2h at 37° C. Fragments were ligated with 80 units of T4 DNA ligase (Thermo Scientific) at RT overnight. Ligated DNA baits were purified with Phenol/chloroform extraction and biotinylated with N6-(6-Amino)hexyl-dATP - Biotin (Biotin-7-dATP)(Jena Bioscience) and 30 units of DNA polymerase Klenow fragment exo- (Thermo Scientific). DNA baits were purified using Microspin Sephadex G50 columns (GE Healthcare) before performing the telomere pull-down.

Telomere pull-down

Biotinylated DNA baits were immobilized on magnetic streptavidin beads (MyOne C1 Streptavidin Dynabeads, Thermo) for 30 min at room temperature on a rotation wheel. DNA baits were incubated

with 500 µg of protein extracts diluted in PBB Buffer using 20 µg sheared salmon sperm DNA as a competitor (Thermo). Protein extracts were incubated with DNA baits for 1h at 4° C on a rotation wheel. A fraction of the samples was treated with 5 units of RNase H (NEB) and 20 µg of RNase A (Thermo Scientific) during incubation. DNA baits were washed three times with PBB buffer and bound proteins were eluted by heating for 10 min at 75° C in 1x NuPAGE LDS buffer (Thermo) supplemented with 100 mM DTT.

MS sample processing

Eluted proteins from telomere pull-down were separated on a 4-12% NuPAGE Bis –Tris precasted PAGE gel (Thermo). Eluates were run at 180V for 10 min and processed by in-gel digestion (Shevchenko *et al.*, 2006). Briefly, samples were reduced in reduction buffer for 1h at 56° C and alkylated in alkylation buffer for 45 min in the dark. Proteins were digested with 2 µg Protease LysC (Wako Chemicals) overnight at 37° C in 50 mM ABC buffer. Digested peptides were desalted on a C18 StageTip as described (Rappsilber, Mann and Ishihama, 2007) and analyzed by nanoflow liquid chromatography on an EASY-nLC 1000 system (Thermo) coupled to a Q Exactive Plus mass spectrometer (Thermo). The peptides were separarated on a self-packed reverse phase capillary (75 µm diameter, 25 cm length packed with C18 beads of 1.9 µm (Dr Maisch GmbH). The capillary was clamped on an electrospray ion source (Nanospray Flex™, Thermo). A 90 min gradient starting from 2%-60% gradient acetonitrile in 0.1% formic acid was used at a flow of 225 nl/min. Data was collected in data-dependent acquisition mode with one MS full scan followed by up to 10 MS/MS scan with HCD fragmentation.

MS data processing and bioinformatic analysis

MS raw files were processed using the MaxQuant software (version 1.5.2.8) and the ENSEMBL *S.cerevisiae* protein database (Saccharomyces_cerevisiae.R64-1-1.24). LFQ quantitation and match between run options were activated. MaxQuant output files were analyzed using an in-house R script. Briefly, known contaminants, reverse hits and protein groups only identified by site were removed. Identified protein groups (minimum 2 peptides, 1 of them unique) were further filtered to a minimum of 2 quantification events per experiment. Missing values were imputed using a downshifted and compressed beta distribution within the 0.001 and 0.015 percentile of the measured values for each replicate individually. The LFQ intensities were $\log_2$ transformed and a two sample Welch t-test was performed. Volcano plots were generated by plotting $-\log_{10}$(p-values) and fold changes. The threshold line for enriched proteins is defined empirically with p-value = 0.05, s0=1 and c=0.5. Gene ontology analysis for telomere associated candidates was performed with thePantherDB.org overrepresentation Test (Release 20190701) with the annotation database released on 20190202. Fisher's exact test followed by Bonferroni correction was applied. Heatmaps for enriched proteins were generated using the "pheatmap" package in R with clustering the complete data based on the Euclidean distance. Biogrid protein interaction were clustered using the complete data based on binary distance.

TAP-ChIP, TAP-RIP, TAP ChRIP and DRIP

100-150 ml of exponentially growing cultures were cross-linked for 10 min with 1.2% formaldehyde (Applichem) after equal normalization of cell number. Samples were quenched with glycine (360 mM, Applichem) for 5 min at room temperature. After cooling down to 4° C on ice for 15 min, cells were pelleted at 4° C by centrifugation (1731 rcf, 3 min), washed twice with ice-cold PBS and stored at -80° C until processing. Cell pellets were lysed in FA buffer via 2x30s rounds of 6.5 M/s FastPrep (MP Biomedical). Samples were diluted in FA buffer supplemented with 0.1% sodium-deoxycholate (SOD). Chromatin extracts were separated by centrifugation (7 min at 17949 rcf) and then sonicated in 2 rounds of 10 cycles (30sec on, 30sec off) using the Bioruptor Pico (Diagenode). Sonication was verified by running 50 µl of extracts on 1% agarose gel after de cross-linking overnight at 65° C, and digestion with Proteinase K (0.75mg/ml, QIAGEN) and 20µg of RNase A (Thermo Scientific). For TAP ChIP and

ChRIP, 1 mg and 2mg (respectively) of sonicated chromatin extracts were incubated overnight at 4° C with 50 µl of pre-washed IgG Sepharose Beads (GE Healthcare) with 5% BSA. 50 µl of extracts was separated as an input control. For DRIP 1 mg of sonicated chromatin extracts were pre-cleared with 30 µl of pre-washed Protein A Sepharose Beads (GEHealthcare) for 1h at 4°C and subsequently incubated with 2 mg S9.6 antibody (Kerafast) at 4° C for 1h. After incubation, 50 µl of pre-washed Protein A Sepharose beads (GE Healthcare) were added to chromatin extracts and incubated overnight at 4° C. For TAP-RIP, 2mg of soluble extracts were incubated overnight at 4° C with 75 µl of pre-washed IgG Beads (GE Healthcare) with 5% BSA. 50 µl of extracts was separated as an input control. Beads were washed with 1ml of FA buffer, Buffer 500, Buffer III and TE buffer at 4° C with 5 min incubation times between washes. For ChIP, ChRIP and DRIP, proteins were eluted in Elution Buffer in 2x8 min denaturation runs at 65° C. Proteins were digested overnight at 65° C with 0.75mg/ml proteinase K in Elution buffer. DNA was purified using the PCR purification kit (QIAquick, QIAGEN) and eluted in 50 µl ddH2O. 2µl of purified DNA were used for ChIP quantification by qPCR. For RIP, proteins were eluted in Elution Buffer in 2x8 min denaturation runs at 65° C. DNA was decroslinked for 2h at 65° C and subsequently digested with 3 units of DNAse I (QIAGEN) for 2h at 37° C. After digestion, eluted samples were digested with proteinase K (0.75 mg/ml) for 2h at 65° C. RNA samples were purified using the RNeasy MinElute Cleanup kit (QIAGEN). Purified RNA samples were digested once more with 3 units of DNAse I (QIAGEN) and purified. RNA samples were subjected to reverse-transcription before quantification by qPCR for different loci. To test for IP specificity, one third of the eluted samples were digested with 20 µg of RNase A at 37° C for 30 min before reverse transcription. For reverse transcription, RNA samples were split into 3 reactions. One reaction contained the RIP eluted RNA, another contained the eluted RNA digested with RNase A. The last reaction was used as a negative control of reverse transcription. The RNA was incubated at 90° C for 1 min with 0,4 µl 25 mM dNTPs, 1 µl 10 µM oBL207, 0.4 µl 10 µM oBL293 in 10 µl final volume reaction. The RNA was then cooled-down to 55° C at a 0.8C/s temperature rate. A mix of 1 µl 100 mM DTT, 1 µl SuperScript III in 1x FS-buffer (Invitrogen) was added to the reactions. Negative control sample did not contain SuperScript III reverse-transcriptase. The RNA was reverse transcribed for 60 min at 55° C. The enzyme was inactivated at 70° C for 15 min. RNA samples were diluted with 30 µl H2O and subjected to qPCR.

Dot blot

Cells were grown in 25 ml cultures and collected at exponential growth in appropriate medium. Genomic DNA was extracted using the Gentra Puregene Yeast/Bacteria Extraction kit (QIAGEN). 4.8 µg of DNA were digested for 2.5 h at 37° C with 5 units of RNase III (Ambion, Thermo Scientific) and 1 unit of RNAse T1 (Thermo Fisher). A fraction of the samples were additionally treated with 10 units of RNase H (NEB) for 2.5 h at 37° C. Digested DNA was split in two and spotted onto positively a charged nylon membrane (Roche) in SSC 2X serial dilutions (1:2). Once dried, the membranes were cross-linked with UV 30 sec on auto cross-link (1200u Joules) and blocked with 5% Milk in PBS-0.1% Tween for 1h at room temperature. Blocked membranes were incubated over night at 4° C in agitation with S9.6 antibody (Kerafast ENH001, 2µg diluted in 3% BSA) and anti-dsDNA antibody (Abcam ab27156, 1:1000). Membranes were washed 3 times with PBS-Tween 0.1% at room temperature and incubated with secondary antibody (Goat anti-mouse, BioRad 170-5047 1:3000 in 5% Milk in PBS-0.1% Tween) for 1h at room temperature. Membranes were developed using 10 ml of Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) in ChemiDoc Touch Imaging System (BioRad). Spot signal was quantified using ImageJ Software. Background was subtracted from S9.6 and dsDNA spots. S9.6 signal was normalized to dsDNA signal.

Western Blot

1-2 OD_{600} units of exponentially growing cells were pelleted and resuspended in 150µl of solution 1. After 10 min incubation on ice, 150µl solution 2 were added and further incubated 10 min on ice.

Proteins were pelleted by centrifugation at 17949 rcf for 2 min at 4° C and washed with 100% Acetone. Proteins were resuspended in 100-150 µl Urea Buffer and denatured for 10 min at 65° C. Samples were loaded on 4-15% gradient pre-casted polyacrylamide gels (BioRad) for 30 min at 200V and transferred to a nitrocellulose membrane using the Trans-Blot Turbo (BioRad) on High molecular Weight Program. Membranes were blocked with 5% Milk for 1h at room temperature and incubated with the following antibodies: anti-FLAG (Sigma Aldrich, F3165, mouse, 1:1000), PAP (Sigma Aldrich, P1291, rabbit, 1:200), anti-actin (Millipore, MAB1501R, mouse, 1:2000), anti-HA (Covance, MMS-101P, mouse, 1:2000). After incubation with corresponding secondary antibody (Goat anti-mouse, BioRad, 170-5047, 1:3000) proteins were imaged using Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher) and ChemiDoc Touch Imaging System (BioRad).

Flow Cytometry

0.2 units of exponentially growing cells were collected for DNA content analysis. Cells were pelleted and fixed in 70% EtOH over night at 4° C. Fixed cells were treated with RNase A (0.25 mg/ml) in 50 mM Tris buffer pH 7.5 for 3h at 37° C and subsequently treated with Proteinase K (1 mg/ml) for 2h at 50° C. Cells were sonicated with BRANSON sonifier 450 for at least 10 seconds (Constant mode) and diluted in 50mM Tris-HCl pH 7.5 supplemented with SYTOX Green (Thermo Fisher Scientific). DNA content of at least 10000 cells was analyzed by flow cytometry using a BD FACS Verse flow cytometer. Data analysis was performed with FlowJo (v10.5.3).

Southern Blot

Exponentially growing cells were collected for genomic DNA extraction. Cells were lysed with a 900 mM sorbitol, 100 mM EDTA pH 8 solution supplemented with 14 mM 2-mercaptoethanol and 5 units of 100T lyticase (Sigma Aldrich). Spheroblasts were pelleted by centrifugation (17949956 rcf 1 min) and resuspended in TE buffer. A solution containing 2.5 mM EDTA pH 8, 222 mM Tris-base and 2.2% SDS was added. Samples were incubated 30 min at 65° C before 80 µl 5 M potassium acetate were added. Samples were cooled down to 4° C for 1h. After centrifugation (20817 rcf 15 min), soluble DNA was precipitated with ice-cold 100% ethanol. DNA pellets were resuspended in TE buffer and remaining RNA was digested with 25 µg RNAse A 60 min at 37° C (Thermo Fisher). DNA was precipitated with ice-cold 100% isopropanol and resuspended in TE buffer. 5-10 µg of extracted DNA were digested with 1 µl XhoI (NEB) for 5h at 37° C and then loaded into a 0.8% agarose gel. DNA fragments were separated by electrophoresis at 50V overnight. The agarose gel was denatured for 1h in denaturing solution and neutralized with neutralizing solution for 1h. After neutralization, the DNA fragments were capillary transfered to a nylon membrane (Roche) in 10X SSC for 72h and cross-linked with UV light 30sec on auto crosslink (1200u Joules). The membrane was then incubated in hybridization solution (Perfect Hyb-Buffer, Sigma Aldrich) for 5h at 55° C on rotation. The membrane was hybridized overnight at 55° C with a telomere specific probe generated by radioactive labelling with dATP alpha-P^{32} (Perkin Elmer). After hybridization, the membrane was washed twice (1h each) in washing solution I and twice with washing solution II both pre-warmed to 55° C. The membrane was dried at room temperature for 30 min and exposed for 2-4 days. Membrane was imaged using Typhoon FLA 9500 (GE Healthcare).

References

Abdallah, P. *et al.* (2009) 'A two-step model for senescence triggered by a single critically short telomere', *Nature Cell Biology*. Nature Publishing Group, 11, p. 988. Available at: https://doi.org/10.1038/ncb1911.

Aeby, E. *et al.* (2016) 'Peroxiredoxin 1 Protects Telomeres from Oxidative Damage and Preserves Telomeric DNA for Extension by Telomerase', *Cell Reports*. ElsevierCompany., 17(12), pp. 3107–3114. doi: 10.1016/j.celrep.2016.11.071.

Aguilera, A. and García-Muse, T. (2012) 'R Loops: From Transcription Byproducts to Threats to Genome Stability', *Molecular Cell*, 46(2), pp. 115–124. doi: 10.1016/j.molcel.2012.04.009.

Aguilera, A. and Gómez-González, B. (2008) 'Genome instability: a mechanistic view of its causes and consequences', *Nature Reviews Genetics*. Nature Publishing Group, 9, p. 204. Available at: https://doi.org/10.1038/nrg2268.

Aguilera, A. and Huertas, P. (2003) 'Cotranscriptionally formed DNA:RNA hybrids mediate transcription elongation impairment and transcription-associated recombination', *Molecular Cell*, 12(3), pp. 711–721. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14527416.

Ai, W. *et al.* (2002) 'Regulation of Subtelomeric Silencing during Stress Response', *Molecular Cell*. Elsevier, 10(6), pp. 1295–1305. doi: 10.1016/S1097-2765(02)00695-0.

Almeida, C. R. De *et al.* (2018) 'RNA Helicase DDX1 Converts RNA G-Quadruplex Structures into R-Loops to Promote IgH Class Switch Recombination', *Molecular Cell*. Elsevier Inc., 70(4), pp. 650-662.e8. doi: 10.1016/j.molcel.2018.04.001.

Apte, M. S. and Cooper, J. P. (2017) 'Life and cancer without telomerase: ALT and other strategies for making sure ends (don't) meet', *Critical Reviews in Biochemistry and Molecular Biology*, 52(1), pp. 57–73. doi: 10.1080/10409238.2016.1260090.

Arab, K. *et al.* (2019) 'GADD45A binds R-loops and recruits TET1 to CpG island promoters', *Nature Genetics*. Springer US, 51(2), pp. 217–223. doi: 10.1038/s41588-018-0306-6.

Armstrong, A. A., Mohideen, F. and Lima, C. D. (2012) 'Recognition of SUMO-modified PCNA requires tandem receptor motifs in Srs2', *Nature*, 483(7387), pp. 59–63. doi: 10.1038/nature10883.

Arnerić, M. and Lingner, J. (2007) 'Tel1 kinase and subtelomere-bound Tbf1 mediate preferential elongation of short telomeres by telomerase in yeast', *EMBO reports*. 2007/10/05. Nature Publishing Group, 8(11), pp. 1080–1085. doi: 10.1038/sj.embor.7401082.

Arnoult, N., Van Beneden, A. and Decottignies, A. (2012) 'Telomere length regulates TERRA levels through increased trimethylation of telomeric H3K9 and HP1α', *Nature Structural and Molecular Biology*, 19(9), pp. 948–956. doi: 10.1038/nsmb.2364.

Arora, R. *et al.* (2014) 'RNaseH1 regulates TERRA-telomeric DNA hybrids and telomere maintenance in ALT tumour cells', *Nature Communications*. Nature Publishing Group, 5, pp. 1–11. doi: 10.1038/ncomms6220.

Arora, R. and Azzalin, C. M. (2015) 'Telomere elongation chooses TERRA ALTernatives', *RNA Biology*, 12(9), pp. 938–941. doi: 10.1080/15476286.2015.1065374.

Artandi, S. E. *et al.* (2000) 'Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice', *Nature*, 406(6796), pp. 641–645. doi: 10.1038/35020592.

Artandi, S. E. and DePinho, R. A. (2009) 'Telomeres and telomerase in cancer', *Carcinogenesis*, 31(1), pp. 9–18. doi: 10.1093/carcin/bgp268.

Askree, S. H. *et al.* (2004) 'A genome-wide screen for Saccharomyces cerevisiae deletion mutants that affect telomere length', *Proceedings of the National Academy of Sciences of the United States of America*, 101(23), pp. 8658–8663. doi: 10.1073/pnas.0401263101.

Azam, M. *et al.* (2006) 'Evidence that the S.cerevisiae Sgs1 protein facilitates recombinational repair of telomeres during senescence', *Nucleic Acids Research*, 34(2), pp. 506–516. doi: 10.1093/nar/gkj452.

Azzalin, C. M. *et al.* (2007) 'Telomeric Repeat Containing RNA and RNA Surveillance Factors at Mammalian Chromosome Ends', *Science*, 318(5851), pp. 798–801. doi: 10.1126/science.1147182.

Azzalin, C. M. and Lingner, J. (2015) 'Telomere functions grounding on TERRA firma', *Trends in Cell Biology*. Elsevier Ltd, 25(1), pp. 29–36. doi: 10.1016/j.tcb.2014.08.007.

Bae, N. S. and Baumann, P. (2007) 'A RAP1/TRF2 Complex Inhibits Nonhomologous End-Joining at Human Telomeric DNA Ends', *Molecular Cell*. Elsevier, 26(3), pp. 323–334. doi: 10.1016/j.molcel.2007.03.023.

Bah, A. *et al.* (2011) 'The telomeric transcriptome of Schizosaccharomyces pombe', *Nucleic Acids Research*, 40(7), pp. 2995–3005. doi: 10.1093/nar/gkr1153.

Bailey, S. M., Brenneman, M. A. and Goodwin, E. H. (2004) 'Frequent recombination in telomeric DNA may extend the proliferative life of telemerase-negative cells', *Nucleic Acids Research*, 32(12), pp. 3743–3751. doi: 10.1093/nar/gkh691.

Baker, D. J. *et al.* (2011) 'Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders', *Nature*, 479(7372), pp. 232–236. doi: 10.1038/nature10600.

Baker, D. J. *et al.* (2016) 'Naturally occurring p16Ink4a-positive cells shorten healthy lifespan', *Nature*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 530, p. 184. Available at: https://doi.org/10.1038/nature16932.

Baldacci, G., Chérif-Zahar, B. and Bernardi, G. (1984) 'The initiation of DNA replication in the mitochondrial genome of yeast.', *The EMBO Journal*, 3(9), pp. 2115–2120. doi: 10.1002/j.1460-2075.1984.tb02099.x.

Balk, B. *et al.* (2013) 'Telomeric RNA-DNA hybrids affect telomere-length dynamics and senescence', *Nature Structural and Molecular Biology*. Nature Publishing Group, 20(10), pp. 1199–1206. doi: 10.1038/nsmb.2662.

Barefield, C. and Karlseder, J. (2012) 'The BLM helicase contributes to telomere maintenance through processing of late-replicating intermediate structures', *Nucleic Acids Research*, 40(15), pp. 7358–7367. doi: 10.1093/nar/gks407.

Baur, J. A. *et al.* (2001) 'Telomere Position Effect in Human Cells', *Science*, 292(5524), pp. 2075 LP – 2077. doi: 10.1126/science.1062329.

Bayona-Feliu, A. *et al.* (2017) 'Linker histone H1 prevents R-loop accumulation and genome instability in heterochromatin', *Nature Communications*. Springer US, 8(1). doi: 10.1038/s41467-017-00338-5.

Beauséjour, C. M. *et al.* (2003) 'Reversal of human cellular senescence: roles of the p53 and p16 pathways', *The EMBO Journal*. John Wiley & Sons, Ltd, 22(16), pp. 4212–4222. doi: 10.1093/emboj/cdg417.

Belen Gomez-Gonzalez and Andres Aguilera (2007) 'Activation-induced cytidine deaminase action is strongly stimulated by mutations of the THO complex', *Proceedings of the National Academy of Sciences*, 104(20), pp. 8409–8414.

Bell, S. P., Kobayashi, R. and Stillman, B. (1993) 'Yeast origin recognition complex functions in transcription silencing and DNA replication', *Science*, 262(5141), pp. 1844–1849. doi:

10.1126/science.8266072.

Benarroch-Popivker, D. *et al.* (2016) 'TRF2-Mediated Control of Telomere DNA Topology as a Mechanism for Chromosome-End Protection', *Molecular cell*. 2016/01/07, 61(2), pp. 274–286. doi: 10.1016/j.molcel.2015.12.009.

Benetti, R. *et al.* (2007) 'Suv4-20h deficiency results in telomere elongation and derepression of telomere recombination', *Journal of Cell Biology*, 178(6), pp. 925–936. doi: 10.1083/jcb.200703081.

Bettin, N., Oss Pegorar, C. and Cusanelli, E. (2019) 'The Emerging Roles of TERRA in Telomere Maintenance and Genome Stability', *Cells*, 8(3), p. 246. doi: 10.3390/cells8030246.

Bhatia, V. *et al.* (2014) 'BRCA2 prevents R-loop accumulation and associates with TREX-2 mRNA export factor PCID2', *Nature*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 511, p. 362. Available at: https://doi.org/10.1038/nature13374.

Bianchi, A. and Shore, D. (2007a) 'Early Replication of Short Telomeres in Budding Yeast', *Cell*, 128(6), pp. 1051–1062. doi: 10.1016/j.cell.2007.01.041.

Bianchi, A. and Shore, D. (2007b) 'Increased association of telomerase with short telomeres in yeast', *Genes & development*. Cold Spring Harbor Laboratory Press, 21(14), pp. 1726–1730. doi: 10.1101/gad.438907.

Blasco, M. A. (2007) 'The epigenetic regulation of mammalian telomeres', *Nature Reviews Genetics*, 8(4), pp. 299–309. doi: 10.1038/nrg2047.

Bodnar, A. G. *et al.* (1998) 'Extension of life-span by introduction of telomerase into normal human cells', *Science*, 279(5349), pp. 349–352. doi: 10.1126/science.279.5349.349.

Bond, A. T. *et al.* (2001) 'Absence of Dbp2p Alters Both Nonsense-Mediated mRNA Decay and rRNA Processing', *Molecular and Cellular Biology*, 21(21), pp. 7366–7379. doi: 10.1128/MCB.21.21.7366-7379.2001.

Bonetti, D. *et al.* (2009) 'Multiple Pathways Regulate 3' Overhang Generation at S. cerevisiae Telomeres', *Molecular Cell*. Elsevier Inc., 35(1), pp. 70–81. doi: 10.1016/j.molcel.2009.05.015.

Bonetti, D., Clerici, M., Anbalagan, S., *et al.* (2010a) 'Shelterin-like proteins and Yku inhibit nucleolytic processing of Saccharomyces cerevisiae telomeres', *PLoS Genetics*, 6(5), p. 1. doi: 10.1371/journal.pgen.1000966.

Bonetti, D., Clerici, M., Anbalagan, S., *et al.* (2010b) 'Shelterin-Like Proteins and Yku Inhibit Nucleolytic Processing of Saccharomyces cerevisiae Telomeres', *PLOS Genetics*. Public Library of Science, 6(5), p. e1000966. Available at: https://doi.org/10.1371/journal.pgen.1000966.

Bonetti, D., Clerici, M., Manfrini, N., *et al.* (2010) 'The MRX complex plays multiple functions in resection of Yku- and Rif2-protected DNA ends', *PLoS ONE*, 5(11). doi: 10.1371/journal.pone.0014142.

Booy, E. P. *et al.* (2012) 'The RNA helicase RHAU (DHX36) unwinds a G4-quadruplex in human telomerase RNA and promotes the formation of the P1 helix template boundary', *Nucleic Acids Research*, 40(9), pp. 4110–4124. doi: 10.1093/nar/gkr1306.

Boulton, S. J. and Jackson, S. P. (1996) 'Identification of a Saccharomyces cerevisiae Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance', *Nucleic acids research*, 24(23), pp. 4639–4648. doi: 10.1093/nar/24.23.4639.

Britton, S. *et al.* (2014) 'DNA damage triggers SAF-A and RNA biogenesis factors exclusion from chromatin coupled to R-loops removal', *Nucleic Acids Research*, 42(14), pp. 9047–9062. doi: 10.1093/nar/gku601.

Bryan, T. M. *et al.* (1997) 'Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines', *Nature Medicine*. Nature Publishing Group, 3, p. 1271. Available at: http://dx.doi.org/10.1038/nm1197-1271.

Bucheli, M. E. and Buratowski, S. (2005) 'Npl3 is an antagonist of mRNA 3' end formation by RNA polymerase II', *The EMBO Journal*. John Wiley & Sons, Ltd, 24(12), pp. 2150–2160. doi: 10.1038/sj.emboj.7600687.

Campisi, J. and D'Adda Di Fagagna, F. (2007) 'Cellular senescence: When bad things happen to good cells', *Nature Reviews Molecular Cell Biology*, 8(9), pp. 729–740. doi: 10.1038/nrm2233.

Capkova Frydrychova, R., Biessmann, H. and Mason, J. M. (2009) 'Regulation of telomere length in Drosophila', *Cytogenetic and Genome Research*, 122(3–4), pp. 356–364. doi: 10.1159/000167823.

Carrasco-Salas, Y. *et al.* (2019) 'The extruded non-template strand determines the architecture of R-loops', *Nucleic Acids Research*, 47(13), pp. 6783–6795. doi: 10.1093/nar/gkz341.

Castellano-Pozo, M. *et al.* (2013) 'R loops are linked to histone H3 S10 phosphorylation and chromatin condensation', *Molecular Cell*, 52(4), pp. 583–590. doi: 10.1016/j.molcel.2013.10.006.

Celli, G. B., Denchi, E. L. and de Lange, T. (2006) 'Ku70 stimulates fusion of dysfunctional telomeres yet protects chromosome ends from homologous recombination', *Nature Cell Biology*, 8(8), pp. 885–890. doi: 10.1038/ncb1444.

Celli, G. B. and de Lange, T. (2005) 'DNA processing is not required for ATM-mediated telomere damage response after TRF2 deletion', *Nature Cell Biology*, 7(7), pp. 712–718. doi: 10.1038/ncb1275.

Cerritelli, S. M. *et al.* (2003) 'Failure to Produce Mitochondrial DNA Results in Embryonic Lethality in Rnaseh1 Null Mice', *Molecular Cell*, 11(3), pp. 807–815. doi: https://doi.org/10.1016/S1097-2765(03)00088-1.

Cerritelli, S. M. and Crouch, R. J. (2009) 'Ribonuclease H: The enzymes in eukaryotes', *FEBS Journal*, 276(6), pp. 1494–1505. doi: 10.1111/j.1742-4658.2009.06908.x.

Cesare, A. J. and Reddel, R. R. (2010) 'Alternative lengthening of telomeres: models, mechanisms and implications', *Nature Reviews Genetics*, 11(5), pp. 319–330. doi: 10.1038/nrg2763.

Chaconas, G. (2005) 'Hairpin telomeres and genome plasticity in Borrelia: All mixed up in the end', *Molecular Microbiology*, 58(3), pp. 625–635. doi: 10.1111/j.1365-2958.2005.04872.x.

Chakraborty, P., Huang, J. T. J. and Hiom, K. (2018) 'DHX9 helicase promotes R-loop formation in cells with impaired RNA splicing', *Nature Communications*. Springer US, 9(4346). doi: 10.1038/s41467-018-06677-1.

Chan, C. S. M. and Tye, B. K. (1983) 'Organization of DNA sequences and replication origins at yeast telomeres', *Cell*, 33(2), pp. 563–573. doi: 10.1016/0092-8674(83)90437-3.

Chan, Y. A., Hieter, P. and Stirling, P. C. (2014) 'Mechanisms of genome instability induced by RNA-processing defects', *Trends in Genetics*. Elsevier Ltd, 30(6), pp. 245–253. doi: 10.1016/j.tig.2014.03.005.

Chang, C. D. *et al.* (1991) 'Senescent human fibroblasts have a post-transcriptional block in the expression of the proliferating cell nuclear antigen gene.', *Journal of Biological Chemistry* , 266(14), pp. 8663–8666. Available at: http://www.jbc.org/content/266/14/8663.abstract.

Chang, H. H. Y. *et al.* (2017) 'Non-homologous DNA end joining and alternative pathways to double-strand break repair', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 18, p. 495. Available at: https://doi.org/10.1038/nrm.2017.48.

Chang, H. Y. *et al.* (2011) 'Genome-wide analysis to identify pathways affecting telomere-initiated senescence in budding yeast', *G3: Genes, Genomes, Genetics*, 1(3), pp. 197–208. doi: 10.1534/g3.111.000216.

Chawla, R. *et al.* (2011) 'Human UPF1 interacts with TPP1 and telomerase and sustains telomere leading-strand replication', *EMBO Journal*. Nature Publishing Group, 30(19), pp. 4047–4058. doi: 10.1038/emboj.2011.280.

Chen, J. L. and Greider, C. W. (2003) 'Template boundary definition in mammalian telomerase', *Genes and Development*, 17(22), pp. 2747–2752. doi: 10.1101/gad.1140303.

Chen, P. B. *et al.* (2015) 'R loops regulate promoter-proximal chromatin architecture and cellular differentiation', *Nature Structural &Amp; Molecular Biology*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 22, p. 999. Available at: https://doi.org/10.1038/nsmb.3122.

Chen, Q., Ijpma, A. and Greider, C. W. (2001) 'Two Survivor Pathways That Allow Growth in the Absence of Telomerase Are Generated by Distinct Telomere Recombination Events', *Molecular and Cellular Biology*, 21(5), pp. 1819–1827. doi: 10.1128/MCB.21.5.1819-1827.2001.

Chen, X.-J., Guan, M.-X. and Clark-Walker, G. D. (1993) 'MGM101 , a nuclear gene involved in maintenance of the mitochondrial genome in Saccharomyces cerevisiae', *Nucleic Acids Research*, 21(15), pp. 3473–3477. doi: 10.1093/nar/21.15.3473.

Chen, Y.-Z. *et al.* (2004) 'DNA/RNA Helicase Gene Mutations in a Form of Juvenile Amyotrophic Lateral Sclerosis (ALS4)', *The American Journal of Human Genetics*, 74(6), pp. 1128–1135. doi: https://doi.org/10.1086/421054.

Chernikova, S. B. *et al.* (2012) 'Deficiency in mammalian histone H2B ubiquitin ligase Bre1 (Rnf20/Rnf40) leads to replication stress and chromosomal instability', *Cancer Research*, 72(8), pp. 2111–2119. doi: 10.1158/0008-5472.CAN-11-2209.

Cho, N. W. *et al.* (2014) 'Interchromosomal homology searches drive directional ALT telomere movement and synapsis', *Cell*. Elsevier Inc., 159(1), pp. 108–121. doi: 10.1016/j.cell.2014.08.030.

Chon, H. *et al.* (2008) 'Contributions of the two accessory subunits, RNASEH2B and RNASEH2C, to the activity and properties of the human RNase H2 complex', *Nucleic Acids Research*, 37(1), pp. 96–110. doi: 10.1093/nar/gkn913.

Chu, H. P. *et al.* (2017) 'TERRA RNA Antagonizes ATRX and Protects Telomeres', *Cell*. Elsevier, 170(1), pp. 86-101.e16. doi: 10.1016/j.cell.2017.06.017.

Chung, I., Leonhardt, H. and Rippe, K. (2011) 'De novo assembly of a PML nuclear subcompartment occurs through multiple pathways and induces telomere elongation', *Journal of Cell Science*, 124(21), pp. 3603–3618. doi: 10.1242/jcs.084681.

Churikov, D. *et al.* (2016) 'SUMO-Dependent Relocalization of Eroded Telomeres to Nuclear Pore Complexes Controls Telomere Recombination', *Cell Reports*, 15(6), pp. 1242–1253. doi: 10.1016/j.celrep.2016.04.008.

Claussin, C. and Chang, M. (2015) 'The many facets of homologous recombination at telomeres', *Microbial Cell*, 2(9), pp. 308–321. doi: 10.15698/mic2015.09.224.

Cloutier, S. C. *et al.* (2012) 'The DEAD-box RNA helicase Dbp2 connects RNA quality control with repression of aberrant transcription', *Journal of Biological Chemistry*, 287(31), pp. 26155–26166. doi: 10.1074/jbc.M112.383075.

Cloutier, S. C. *et al.* (2016) 'Regulated Formation of lncRNA-DNA Hybrids Enables Faster Transcriptional

Induction and Article Regulated Formation of lncRNA-DNA Hybrids Enables Faster Transcriptional Induction and Environmental Adaptation', *Molecular Cell*. Elsevier Inc., 61(3), pp. 393–404. doi: 10.1016/j.molcel.2015.12.024.

Cohen, S. *et al.* (2018) 'Senataxin resolves RNA:DNA hybrids forming at DNA double-strand breaks to prevent translocations', *Nature Communications*. Springer US, 9(1), p. 533. doi: 10.1038/s41467-018-02894-w.

Collado, M. and Serrano, M. (2006) 'The power and the promise of oncogene-induced senescence markers', *Nature Reviews Cancer*, 6(6), pp. 472–476. doi: 10.1038/nrc1884.

Colombo, C. V. *et al.* (2017) 'The RNA binding protein Npl3 promotes resection of DNA double-strand breaks by regulating the levels of Exo1', *Nucleic Acids Research*, 45(11), pp. 6530–6545. doi: 10.1093/nar/gkx347.

Conomos, D. *et al.* (2012) 'Variant repeats are interspersed throughout the telomeres and recruit nuclear receptors in ALT cells', *The Journal of Cell Biology*, 199(6), pp. 893 LP – 906. doi: 10.1083/jcb.201207189.

Conover, H. N. *et al.* (2015) 'Stimulation of Chromosomal Rearrangements by Ribonucleotides', *Genetics*, 201(3), pp. 951–961. doi: 10.1534/genetics.115.181149.

Conrad, M. N. *et al.* (1990) 'RAP1 protein interacts with yeast telomeres in vivo: Overproduction alters telomere structure and decreases chromosome stability', *Cell*. Elsevier, 63(4), pp. 739–750. doi: 10.1016/0092-8674(90)90140-A.

Conticello, S. G. (2008) 'The AID/APOBEC family of nucleic acid mutators', *Genome Biology*, 9(229). doi: 10.1186/gb-2008-9-6-229.

Cooley, C. *et al.* (2014) 'Tel1ATM dictates the replication timing of short yeast telomeres', *EMBO reports*, 15(10), pp. 1093–1101. doi: 10.15252/embr.201439242.

Cosgrove, A. J., Nieduszynski, C. A. and Donaldson, A. D. (2002) 'Ku complex controls the replication time of DNA in telomere regions', *Genes and Development*, 16(19), pp. 2485–2490. doi: 10.1101/gad.231602.

Costantino, L. and Koshland, D. (2015) 'The Yin and Yang of R-loop biology', *Current Opinion in Cell Biology*. Elsevier Ltd, 34, pp. 39–45. doi: 10.1016/j.ceb.2015.04.008.

Costantino, L. and Koshland, D. (2018) 'Genome-wide Map of R-Loop-Induced Damage Reveals How a Subset of R-Loops Contributes to Genomic Instability', *Molecular Cell*. Elsevier Inc., 71(4), pp. 487-497.e3. doi: 10.1016/j.molcel.2018.06.037.

Coutelier, H. *et al.* (2018) 'Adaptation to DNA damage checkpoint in senescent telomerase-negative cells promotes genome instability', *Genes and Development*, 32(23–24), pp. 1499–1513. doi: 10.1101/gad.318485.118.

Coutelier, H. and Xu, Z. (2019) 'Adaptation in replicative senescence : a risky business', *Current Genetics*. Springer Berlin Heidelberg, 0(0), p. 0. doi: 10.1007/s00294-019-00933-7.

Cox, K. E., Maréchal, A. and Flynn, R. L. (2016) 'SMARCAL1 Resolves Replication Stress at ALT Telomeres', *Cell Reports*, 14(5), pp. 1032–1040. doi: 10.1016/j.celrep.2016.01.011.

Crabbe, L. *et al.* (2004) 'Telomere Lagging Strand Synthesis Dysfunction in Cells Lacking WRN Helicase Activity', *Science*, 35(03), pp. 1951–1954. doi: 10.1055/s-2004-832038.

Crossley, M. P., Bocek, M. and Cimprich, K. A. (2019) 'R-Loops as Cellular Regulators and Genomic Threats', *Molecular Cell*. Elsevier Inc., 73(3), pp. 398–411. doi: 10.1016/j.molcel.2019.01.024.

Crow, Y. J. *et al.* (2006) 'Mutations in genes encoding ribonuclease H2 subunits cause Aicardi-Goutières syndrome and mimic congenital viral brain infection', *Nature Genetics*, 38(8), pp. 910–916. doi: 10.1038/ng1842.

Crow, Y. J. and Rehwinkel, J. (2009) 'Aicardi-Goutières syndrome and related phenotypes: linking nucleic acid metabolism with autoimmunity', *Human Molecular Genetics*, 18(R2), pp. R130–R136. doi: 10.1093/hmg/ddp293.

Cubiles, M. D. *et al.* (2018) 'Epigenetic features of human telomeres', *Nucleic Acids Research*, 46(5), pp. 2347–2355. doi: 10.1093/nar/gky006.

Cusanelli, E., Romero, C. and Chartrand, P. (2013) 'Telomeric Noncoding RNA TERRA Is Induced by Telomere Shortening to Nucleate Telomerase Molecules at Short Telomeres', *Molecular Cell*. Elsevier Inc., 51(6), pp. 780–791. doi: 10.1016/j.molcel.2013.08.029.

D'Alessandro, G. *et al.* (2018) 'BRCA2 controls DNA:RNA hybrid level at DSBs by mediating RNase H2 recruitment', *Nature Communications*. Springer US, 9:5376. doi: 10.1038/s41467-018-07799-2.

Denchi, E. L. and de Lange, T. (2007) 'Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1', *Nature*. Nature Publishing Group, 448, p. 1068. Available at: https://doi.org/10.1038/nature06065.

Deng, Z. *et al.* (2009) 'TERRA RNA Binding to TRF2 Facilitates Heterochromatin Formation and ORC Recruitment at Telomeres', *Molecular Cell*. Elsevier Ltd, 35(4), pp. 403–413. doi: 10.1016/j.molcel.2009.06.025.

Deng, Z. *et al.* (2012) 'A role for CTCF and cohesin in subtelomere chromatin organization, TERRA transcription, and telomere end protection', *EMBO Journal*. Nature Publishing Group, 31(21), pp. 4165–4178. doi: 10.1038/emboj.2012.266.

Dermody, J. L. *et al.* (2008) 'Unphosphorylated SR-Like Protein Npl3 Stimulates RNA Polymerase II Elongation', *PLOS ONE*. Public Library of Science, 3(9), p. e3273. Available at: https://doi.org/10.1371/journal.pone.0003273.

Diede, S. J. and Gottschling, D. E. (1999) 'Telomerase-Mediated Telomere Addition In Vivo Requires DNA Primase and DNA Polymerases α and δ', *Cell*, 99(7), pp. 723–733. doi: https://doi.org/10.1016/S0092-8674(00)81670-0.

Dilley, Robert L *et al.* (2016) 'Break-induced telomere synthesis underlies alternative telomere maintenance', *Nature Publishing Group*. Nature Publishing Group, 539(7627), pp. 54–58. doi: 10.1038/nature20099.

Dilley, Robert L. *et al.* (2016) 'Break-induced telomere synthesis underlies alternative telomere maintenance', *Nature*. Nature Publishing Group, 539(7627), pp. 54–58. doi: 10.1038/nature20099.

Dimri, G. P. *et al.* (1995) 'A biomarker that identifies senescent human cells in culture and in aging skin in vivo', *Proceedings of the National Academy of Sciences of the United States of America*, 92(20), pp. 9363–9367. doi: 10.1073/pnas.92.20.9363.

Dionne, I. and Wellinger, R. J. (1998) 'Processing of telomeric DNA ends requires the passage of a replication fork', *Nucleic Acids Research*, 26(23), pp. 5365–5371. doi: 10.1093/nar/26.23.5365.

Doksani, Y. *et al.* (2013) 'Super-resolution fluorescence imaging of telomeres reveals TRF2-dependent T-loop formation', *Cell*, 155(2), pp. 345–356. doi: 10.1016/j.cell.2013.09.048.

Doksani, Y. (2019) 'The response to dna damage at telomeric repeats and its consequences for telomere function', *Genes*, 10(4), pp. 1–17. doi: 10.3390/genes10040318.

Domínguez-Sánchez, M. S. *et al.* (2011) 'Differential expression of THOC1 and ALY mRNP

biogenesis/export factors in human cancers', *BMC cancer*. BioMed Central, 11, p. 77. doi: 10.1186/1471-2407-11-77.

Duquette, M. L. *et al.* (2004) 'Intracellular transcription of G-rich DNAs induces formation of G-loops, novel structures containing G4 DNA', *Genes and Development*, 18(13), pp. 1618–1629. doi: 10.1101/gad.1200804.

Eder, P. S., Walder, R. Y. and Walder, J. A. (1993) 'Substrate specificity of human RNase H1 and its role in excision repair of ribose residues misincorporated in DNA', *Biochimie*, 75(1), pp. 123–126. doi: https://doi.org/10.1016/0300-9084(93)90033-O.

Enomoto, S. *et al.* (2004) 'Telomere Cap Components Influence the Rate of Senescence in Telomerase-Deficient Yeast Cells', *Molecular and Cellular Biology*, 24(2), pp. 837 LP – 845. doi: 10.1128/MCB.24.2.837-845.2004.

Episkopou, H. *et al.* (2014) 'Alternative Lengthening of Telomeres is characterized by reduced compaction of telomeric chromatin', *Nucleic Acids Research*, 42(7), pp. 4391–4405. doi: 10.1093/nar/gku114.

Erce, M. A. *et al.* (2013) 'Interactions Affected by Arginine Methylation in the Yeast Protein–Protein Interaction Network', *Molecular & Cellular Proteomics*, 12(11), pp. 3184–3198. doi: 10.1074/mcp.m113.031500.

Evans, S. K. and Lundblad, V. (1999) 'Est1 and Cdc13 as Comediators of Telomerase Access', *Science*, 286(5437), pp. 117–120. doi: 10.1126/science.286.5437.117.

Fagagna, F. d'Adda di *et al.* (2003) 'A DNA damage checkpoint response in telomere-initiated senescence', *Nature*, 426(6963), pp. 194–198. doi: 10.1038/nature02118.

Fallet, E. *et al.* (2014) 'Length-dependent processing of telomeres in the absence of telomerase', *Nucleic Acids Research*, 42(6), pp. 3648–3665. doi: 10.1093/nar/gkt1328.

Feng, J. *et al.* (1995) 'The RNA component of human telomerase', *Science*, 269(5228), pp. 1236 LP – 1241. doi: 10.1126/science.7544491.

Feretzaki, M., Renck Nunes, P. and Lingner, J. (2019) 'Expression and differential regulation of human TERRA at several chromosome ends', *Rna*, (25), pp. 1470–1480. doi: 10.1261/rna.072322.119.

Ferguson, B. M. and Fangman, W. L. (1992) 'A position effect on the time of replication origin activation in yeast', *Cell*. Elsevier, 68(2), pp. 333–339. doi: 10.1016/0092-8674(92)90474-Q.

Flynn, R. L. *et al.* (2011) 'TERRA and hnRNPA1 orchestrate an RPA-to-POT1 switch on telomeric single-stranded DNA', *Nature*. Nature Publishing Group, 471(7339), pp. 532–538. doi: 10.1038/nature09772.

Flynn, R. L. *et al.* (2015) 'Alternative lengthening of telomeres renders cancer cells hypersensitive to ATR inhibitors', *Science*, 347(6219), pp. 273–277. doi: 10.1126/science.1257216.

Flynn, R. L., Chang, S. and Zou, L. (2012) 'RPA and POT1: Friends or foes at telomeres?', *Cell Cycle*, 11(4), pp. 652–657. doi: 10.4161/cc.11.4.19061.

Foss, M. *et al.* (1993) 'Origin recognition complex (ORC) in transcriptional silencing and DNA replication in S. cerevisiae', *Science*, 262(5141), pp. 1838–1844. doi: 10.1126/science.8266071.

Frank, C. J., Hyde, M. and Greider, C. W. (2006) 'Regulation of Telomere Elongation by the Cyclin-Dependent Kinase CDK1', *Molecular Cell*, 24(3), pp. 423–432. doi: https://doi.org/10.1016/j.molcel.2006.10.020.

Freudenreich, C. H. and Su, X. A. (2016) 'Relocalization of DNA lesions to the nuclear pore complex', *FEMS Yeast Research*, 16(8), pp. 1–9. doi: 10.1093/femsyr/fow095.

Gallardo, F. *et al.* (2011) 'Live Cell Imaging of Telomerase RNA Dynamics Reveals Cell Cycle-Dependent Clustering of Telomerase at Elongating Telomeres', *Molecular Cell*, 44(5), pp. 819–827. doi: https://doi.org/10.1016/j.molcel.2011.09.020.

Gan, W. *et al.* (2011) 'R-loop-mediated genomic instability is caused by impairment of replication fork progression', *Genes and Development*, 25(19), pp. 2041–2056. doi: 10.1101/gad.17010011.

Gao, J. *et al.* (2019) 'DEAD-box RNA helicases Dbp2, Ded1 and Mss116 bind to G-quadruplex nucleic acids and destabilize G-quadruplex RNA', *Chemical Communications*. Royal Society of Chemistry, 55(31), pp. 4467–4470. doi: 10.1039/c8cc10091h.

García-Benítez, F., Gaillard, H. and Aguilera, A. (2017) 'Physical proximity of chromatin to nuclear pores prevents harmful R loop accumulation contributing to maintain genome stability', *Proceedings of the National Academy of Sciences*, 114(41), p. 201707845. doi: 10.1073/pnas.1707845114.

García-Cao, M. *et al.* (2004) 'Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases', *Nature Genetics*, 36(1), pp. 94–99. doi: 10.1038/ng1278.

García-Pichardo, D. *et al.* (2017) 'Histone Mutants Separate R Loop Formation from Genome Instability Induction', *Molecular Cell*, 66(5), pp. 597-609.e5. doi: 10.1016/j.molcel.2017.05.014.

García-Rubio, M. *et al.* (2018) 'Yra1-bound RNA–DNA hybrids cause orientation-independent transcription– replication collisions and telomere instability', *Genes and Development*, 32(13–14), pp. 965–977. doi: 10.1101/gad.311274.117.

Garvik, B., Carson, M. and Hartwell, L. (1995) 'Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint.', *Molecular and Cellular Biology*, 15(11), pp. 6128–6138. doi: 10.1128/MCB.15.11.6128.

Gatbonton, T. *et al.* (2006) 'Telomere length as a quantitative trait: Genome-wide survey and genetic mapping of telomere length-control genes in yeast', *PLoS Genetics*, 2(3), pp. 0304–0315. doi: 10.1371/journal.pgen.0020035.

Gauchier, M. *et al.* (2019) 'SETDB1-dependent heterochromatin stimulates alternative lengthening of telomeres', *Science Advances*, 5(5). doi: 10.1126/sciadv.aav3673.

Gavaldá, S. *et al.* (2016) 'Excess of Yra1 RNA-Binding Factor Causes Transcription-Dependent Genome Instability, Replication Impairment and Telomere Shortening', *PLoS Genetics*, 12(4). doi: 10.1371/journal.pgen.1005966.

Geronimo, Carly L. and Zakian, V. A. (2016) 'Getting it done at the ends: Pif1 family DNA helicases and telomeres', *DNA Repair*. Elsevier B.V., 44, pp. 151–158. doi: 10.1016/j.dnarep.2016.05.021.

Geronimo, Carly L and Zakian, V. A. (2016) 'Getting it done at the ends: Pif1 family DNA helicases and telomeres', *DNA repair*. 2016/05/16, 44, pp. 151–158. doi: 10.1016/j.dnarep.2016.05.021.

Geuens, T., Bouhy, D. and Timmerman, V. (2016) 'The hnRNP family: insights into their role in health and disease', *Human Genetics*. Springer Berlin Heidelberg, 135(8), pp. 851–867. doi: 10.1007/s00439-016-1683-5.

Gilbert, W. and Guthrie, C. (2004) 'The Glc7p Nuclear Phosphatase Promotes mRNA Export by Facilitating Association of Mex67p with mRNA', *Molecular Cell*, 13(2), pp. 201–212. doi: 10.1016/S1097-2765(04)00030-9.

Gilbert, W., Siebel, C. W. and Guthrie, C. (2001) 'Phosphorylation by Sky1p promotes Npl3p shuttling and mRNA dissociation', *Rna*, 7(2), pp. 302–313. doi: 10.1017/S1355838201002369.

Gilson, E. and Géli, V. (2007) 'How telomeres are replicated', *Nature Reviews Molecular Cell Biology*,

8(10), pp. 825–838. doi: 10.1038/nrm2259.

Ginno, P. A. *et al.* (2012) 'R-Loop Formation Is a Distinctive Characteristic of Unmethylated Human CpG Island Promoters', *Molecular Cell*, 45(6), pp. 814–825. doi: 10.1016/j.molcel.2012.01.017.

Ginno, P. A. *et al.* (2013) 'GC skew at the 59 and 39 ends of human genes links R-loop formation to epigenetic regulation and transcription termination', *Genome Research*, 23(10), pp. 1590–1600. doi: 10.1101/gr.158436.113.

Gómez-González, B. *et al.* (2011) 'Genome-wide function of THO/TREX in active genes prevents R-loop-dependent replication obstacles', *EMBO Journal*, 30(15), pp. 3106–3119. doi: 10.1038/emboj.2011.206.

Gonzalo, S. *et al.* (2006) 'DNA methyltransferases control telomere length and telomere recombination in mammalian cells', *Nature Cell Biology*, 8(4), pp. 416–424. doi: 10.1038/ncb1386.

Goudsouzian, L. K., Tuzon, C. T. and Zakian, V. A. (2006) 'S. cerevisiae Tel1p and Mre11p are required for normal levels of Est1p and Est2p telomere association', *Molecular Cell*, 24(4), pp. 603–610. doi: 10.1016/j.molcel.2006.10.005.

Graf, M. *et al.* (2017) 'Telomere Length Determines TERRA and R-Loop Regulation through the Cell Cycle', *Cell*, 170(1), pp. 72-85.e14. doi: 10.1016/j.cell.2017.06.006.

Grandin, N., Damon, C. and Charbonneau, M. (2000) 'Cdc13 cooperates with the yeast Ku proteins and Stn1 to regulate telomerase recruitment', *Molecular and cellular biology*. American Society for Microbiology, 20(22), pp. 8397–8408. doi: 10.1128/mcb.20.22.8397-8408.2000.

Grandin, N., Damon, C. and Charbonneau, M. (2001) 'Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13', *The EMBO journal*. Oxford University Press, 20(5), pp. 1173–1183. doi: 10.1093/emboj/20.5.1173.

Grandin, N., Reed, S. I. and Charbonneau, M. (1997) 'Stn1, a new Saccharomyces cerevisiae protein, is implicated in telomere size regulation in association with Cdc13.', *Genes & Development* , 11(4), pp. 512–527. doi: 10.1101/gad.11.4.512.

Gravel, S. *et al.* (1998) 'Yeast Ku as a Regulator of Chromosomal DNA End Structure', *Science*, 280(5364), pp. 741–744. doi: 10.1126/science.280.5364.741.

Greenwood, J. and Cooper, J. P. (2011) 'Non-coding telomeric and subtelomeric transcripts are differentially regulated by telomeric and heterochromatin assembly factors in fission yeast', *Nucleic Acids Research*, 40(7), pp. 2956–2963. doi: 10.1093/nar/gkr1155.

Greider, C. W. (2016) 'Regulating telomere length from the inside out: The replication fork model', *Genes and Development*, 30(13), pp. 1483–1491. doi: 10.1101/gad.280578.116.

Greider, C. W. and Blackburn, E. H. (1985) 'Identification of a specific telomere terminal transferase activity in tetrahymena extracts', *Cell*, 43(2, Part 1), pp. 405–413. doi: https://doi.org/10.1016/0092-8674(85)90170-9.

Greider, C. W. and Blackburn, E. H. (1987) 'The telomere terminal transferase of tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity', *Cell*. Elsevier, 51(6), pp. 887–898. doi: 10.1016/0092-8674(87)90576-9.

Greider, C. W. and Blackburn, E. H. (1989) 'A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis', *Nature*, 337(6205), pp. 331–337. doi: 10.1038/337331a0.

Griffith, J. D. *et al.* (1999) 'Mammalian Telomeres End in a Large Duplex Loop', *Cell*, 97(4), pp. 503–514. doi: https://doi.org/10.1016/S0092-8674(00)80760-6.

Groh, M. *et al.* (2014) 'R-loops Associated with Triplet Repeat Expansions Promote Gene Silencing in Friedreich Ataxia and Fragile X Syndrome', *PLoS Genetics*, 10(5). doi: 10.1371/journal.pgen.1004318.

Groh, M. and Gromak, N. (2014) 'Out of Balance: R-loops in Human Disease', *PLoS Genetics*, 10(9). doi: 10.1371/journal.pgen.1004630.

Gros, J. *et al.* (2015) 'Post-licensing Specification of Eukaryotic Replication Origins by Facilitated Mcm2-7 Sliding along DNA', *Molecular Cell*. Elsevier Inc., 60(5), pp. 797–807. doi: 10.1016/j.molcel.2015.10.022.

Guthrie, C., and Fink, G. R. (1991) *Guide to Yeast Genetics and Molecular Biology*. San Diego: Academic Press.

El Hage, A. *et al.* (2010) 'Loss of Topoisomerase I leads to R-loop-mediated transcriptional blocks during ribosomal RNA synthesis', *Genes and Development*, 24(14), pp. 1546–1558. doi: 10.1101/gad.573310.

Ten Hagen, K. G. *et al.* (1990) 'Replication timing of DNA sequences associated with human centromeres and telomeres.', *Molecular and Cellular Biology*, 10(12), pp. 6348–6355. doi: 10.1128/MCB.10.12.6348.

Hamperl, S. *et al.* (2017) 'Transcription-Replication Conflict Orientation Modulates R-Loop Levels and Activates Distinct DNA Damage Responses', *Cell*. Elsevier Inc., 170(4), pp. 774-786.e19. doi: 10.1016/j.cell.2017.07.043.

Hamperl, S. and Cimprich, K. A. (2014) 'The contribution of co-transcriptional RNA: DNA hybrid structures to DNA damage and genome instability', *DNA Repair*. Elsevier B.V., 19, pp. 84–94. doi: 10.1016/j.dnarep.2014.03.023.

Hardy, C. F., Balderes, D. and Shore, D. (1992) 'Dissection of a carboxy-terminal region of the yeast regulatory protein RAP1 with effects on both transcriptional activation and silencing.', *Molecular and Cellular Biology*, 12(3), pp. 1209–1217. doi: 10.1128/MCB.12.3.1209.

Hardy, C. F., Sussel, L. and Shore, D. (1992) 'A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation.', *Genes & Development* , 6(5), pp. 801–814. doi: 10.1101/gad.6.5.801.

Hayflick, L. (1965) 'The limited in vitro lifetime of human diploid cell strains', *Experimental Cell Research*, 37(3), pp. 614–636. doi: 10.1016/0014-4827(65)90211-9.

Hayflick, L. and Moorhead, P. S. (1961) 'The serial cultivation of human diploid cell strains', *Experimental Cell Research*, 25(3), pp. 585–621. doi: https://doi.org/10.1016/0014-4827(61)90192-6.

Heaphy, C. M. *et al.* (2011) 'Altered telomeres in tumors with ATRX and DAXX mutations', *Science (New York, N.Y.)*. 2011/06/30, 333(6041), p. 425. doi: 10.1126/science.1207313.

Herbig, U. *et al.* (2004) 'Telomere Shortening Triggers Senescence of Human Cells through a Pathway Involving ATM, p53, and p21CIP1, but Not p16INK4a', *Molecular Cell*, 14(4), pp. 501–513. doi: https://doi.org/10.1016/S1097-2765(04)00256-4.

Hernandez-Segura, A., Nehme, J. and Demaria, M. (2018) 'Hallmarks of Cellular Senescence', *Trends in Cell Biology*, 28(6), pp. 436–453. doi: https://doi.org/10.1016/j.tcb.2018.02.001.

Heyer, W.-D., Ehmsen, K. T. and Liu, J. (2010) 'Regulation of Homologous Recombination in Eukaryotes', *Annual Review of Genetics*. Annual Reviews, 44(1), pp. 113–139. doi: 10.1146/annurev-genet-051710-150955.

Hiraga, S. I. *et al.* (2014) 'Rif1 controls DNA replication by directing Protein Phosphatase 1 to reverse Cdc7- mediated phosphorylation of the MCM complex', *Genes and Development*, 28(4), pp. 372–383. doi: 10.1101/gad.231258.113.

Hirano, Y., Fukunaga, K. and Sugimoto, K. (2009) 'Rif1 and Rif2 Inhibit Localization of Tel1 to DNA Ends', *Molecular Cell*. Elsevier, 33(3), pp. 312–322. doi: 10.1016/j.molcel.2008.12.027.

Holmes, R. K. *et al.* (2015) 'Loss of the Yeast SR Protein Npl3 Alters Gene Expression Due to Transcription Readthrough', *PLOS Genetics*. Public Library of Science, 11(12), p. e1005735. Available at: https://doi.org/10.1371/journal.pgen.1005735.

Horowitz, H., Thorburn, P. and Haber, J. E. (1984) 'Rearrangements of highly polymorphic regions near telomeres of Saccharomyces cerevisiae', *Molecular and cellular biology*, 4(11), pp. 2509–2517. doi: 10.1128/mcb.4.11.2509.

Hu, Y. *et al.* (2019) 'RNA-DNA Hybrids Support Recombination-Based Telomere Maintenance in Fission Yeast', *Genetics*, 213(2), pp. 431–447. doi: 10.1534/genetics.119.302606.

Huang, P. H. *et al.* (2001) 'SGS1 is required for telomere elongation in the absence of telomerase', *Current Biology*, 11(2), pp. 125–129. doi: 10.1016/S0960-9822(01)00021-5.

Huang, P. R., Hung, S. C. and Wang, T. C. V. (2010) 'Telomeric DNA-binding activities of heterogeneous nuclear ribonucleoprotein A3 in vitro and in vivo', *Biochimica et Biophysica Acta - Molecular Cell Research*. Elsevier B.V., 1803(10), pp. 1164–1174. doi: 10.1016/j.bbamcr.2010.06.003.

Iglesias, N. *et al.* (2011) 'Subtelomeric repetitive elements determine TERRA regulation by Rap1/Rif and Rap1/Sir complexes in yeast', *EMBO Reports*. Nature Publishing Group, 12(6), pp. 587–593. doi: 10.1038/embor.2011.73.

Imai, S. *et al.* (2000) 'Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase', *Nature*, 403(6771), pp. 795–800. doi: 10.1038/35001622.

Itoh, T. and Tomizawa, J. (1980) 'Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H.', *Proceedings of the National Academy of Sciences*, 77(5), pp. 2450–2454. doi: 10.1073/pnas.77.5.2450.

Ivessa, A. S. *et al.* (2002) 'Saccharomyces Rrm3p, a 5' to 3' DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA', *Genes and Development*, 16(11), pp. 1383–1396. doi: 10.1101/gad.982902.

Jacobs, J. J. L. (2013) 'Loss of telomere protection: Consequences and opportunities', *Frontiers in Oncology*, 3(88), pp. 1–7. doi: 10.3389/fonc.2013.00088.

Jahn, A. *et al.* (2017) 'ZBTB48 is both a vertebrate telomere-binding protein and a transcriptional activator', *EMBO reports*. John Wiley & Sons, Ltd, 18(6), pp. 929–946. doi: 10.15252/embr.201744095.

Jain, D. *et al.* (2010) 'HAATI survivors replace canonical telomeres with blocks of generic heterochromatin', *Nature*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 467, p. 223. Available at: https://doi.org/10.1038/nature09374.

Jeong, H. *et al.* (2004) 'RNase H2 of Saccharomyces cerevisiae is a complex of three proteins', *Nucleic Acids Research*, 32(2), pp. 407–414. doi: 10.1093/nar/gkh209.

Jiang, J. *et al.* (2018) 'Structure of Telomerase with Telomeric DNA', *Cell*. Elsevier, 173(5), pp. 1179-1190.e13. doi: 10.1016/j.cell.2018.04.038.

Johnson, F. B. *et al.* (2001) 'The Saccharomyces cerevisiae WRN homolog Sgs1p participates in telomere maintenance in cells lacking telomerase', *The EMBO Journal*. John Wiley & Sons, Ltd, 20(4), pp. 905–913. doi: 10.1093/emboj/20.4.905.

Joyce, C. M. (1997) 'Choosing the right sugar: How polymerases select a nucleotide substrate', *Proceedings of the National Academy of Sciences*, 94(5), pp. 1619–1622. doi: 10.1073/pnas.94.5.1619.

Kappei, D. *et al.* (2013) 'HOT1 is a mammalian direct telomere repeat-binding protein contributing to telomerase recruitment', *The EMBO journal*. 2013/05/17. Nature Publishing Group, 32(12), pp. 1681–1701. doi: 10.1038/emboj.2013.105.

Kappei, D. *et al.* (2017) 'Phylointeractomics reconstructs functional evolution of protein binding', *Nature Communications*. The Author(s), 8, p. 14334. Available at: https://doi.org/10.1038/ncomms14334.

Kasahara, M. *et al.* (2000) 'RecA protein-dependent R-loop formation in vitro', *Genes and Development*, 14(3), pp. 360–365. doi: 10.1101/gad.14.3.360.

Kaufer, B. B., Jarosinski, K. W. and Osterrieder, N. (2011) 'Herpesvirus telomeric repeats facilitate genomic integration into host telomeres and mobilization of viral DNA during reactivation', *Journal of Experimental Medicine*, 208(3), pp. 605–615. doi: 10.1084/jem.20101402.

Kaul, Z. *et al.* (2012) 'Five dysfunctional telomeres predict onset of senescence in human cells', *EMBO Reports*. Nature Publishing Group, 13(1), pp. 52–59. doi: 10.1038/embor.2011.227.

Keskin, H. *et al.* (2014) 'Transcript-RNA-templated DNA recombination and repair', *Nature*. Nature Publishing Group, 515(7527), pp. 436–439. doi: 10.1038/nature13682.

Khadaroo, B. *et al.* (2009) 'The DNA damage response at eroded telomeres and tethering to the nuclear pore complex', *Nature Cell Biology*. Nature Publishing Group, 11(8), pp. 980–987. doi: 10.1038/ncb1910.

Kim, N. W. *et al.* (1994) 'Specific association of human telomerase activity with immortal cells and cancer', *Science*, 266(5193), pp. 2011–2015. doi: 10.1126/science.7605428.

Kim, W. and Shay, J. W. (2018) 'Long-range telomere regulation of gene expression: Telomere looping and telomere position effect over long distances (TPE-OLD)', *Differentiation; research in biological diversity*. 2017/11/22, 99, pp. 1–9. doi: 10.1016/j.diff.2017.11.005.

Koering, C. E. *et al.* (2000) 'Identification of high affinity Tbf1p-binding sites within the budding yeast genome', *Nucleic Acids Research*, 28(13), pp. 2519–2526. doi: 10.1093/nar/28.13.2519.

Koering, C. E. *et al.* (2002) 'Human telomeric position effect is determined by chromosomal context and telomeric chromatin integrity', *EMBO reports*. 2002/10/22, 3(11), pp. 1055–1061. doi: 10.1093/embo-reports/kvf215.

Konishi, A. and de Lange, T. (2008) 'Cell cycle control of telomere protection and NHEJ revealed by a ts mutation in the DNA-binding domain of TRF2', *Genes & Development* , 22(9), pp. 1221–1230. doi: 10.1101/gad.1634008.

Koskas, S. *et al.* (2017) 'Heat shock factor 1 promotes TERRA transcription and telomere protection upon heat stress', *Nucleic Acids Research*, 45(11), pp. 6321–6333. doi: 10.1093/nar/gkx208.

Kreuzer, K. N. and Brister, J. R. (2010) 'Initiation of bacteriophage T4 DNA replication and replication fork dynamics: A review in the Virology Journal series on bacteriophage T4 and its relatives', *Virology Journal*. BioMed Central Ltd, 7(1), p. 358. doi: 10.1186/1743-422X-7-358.

Kyrion, G. *et al.* (1993) 'RAP1 and telomere structure regulate telomere position effects in Saccharomyces cerevisiae.', *Genes & Development* , 7(7a), pp. 1146–1159. doi: 10.1101/gad.7.7a.1146.

LaBranche, H. *et al.* (1998) 'Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase', *Nature Genetics*, 19(2), pp. 199–202. doi: 10.1038/575.

Lafuente-Barquero, J. *et al.* (2017) 'The Smc5/6 complex regulates the yeast Mph1 helicase at RNA-DNA hybrid-mediated DNA damage', *PLoS Genetics*, 13(12), pp. 1–25. doi: 10.1371/journal.pgen.1007136.

Lam, Y. C. *et al.* (2010) 'SNMIB/Apollo protects leading-strand telomeres against NHEJ-mediated repair', *The EMBO Journal*. John Wiley & Sons, Ltd, 29(13), pp. 2230–2241. doi: 10.1038/emboj.2010.58.

de Lange T. (2009) 'How Telomeres Solve the End-Protection Problem', *Science*, 326(5955), p. 948. doi: 10.1126/science.1170633.How.

de Lange, T. (2018) 'Shelterin-Mediated Telomere Protection', *Annual Review of Genetics*, 52(1), pp. 223–247. doi: 10.1146/annurev-genet-032918-021921.

Larrivée, M., LeBel, C. and Wellinger, R. J. (2004) 'The generation of proper constitutive G-tails on yeast telomeres is dependent on the MRX complex', *Genes & Development* , 18(12), pp. 1391–1396. doi: 10.1101/gad.1199404.

Larrivée, M. and Wellinger, R. J. (2006) 'Telomerase- and capping-independent yeast survivors with alternate telomere states', *Nature Cell Biology*, 8(7), pp. 741–747. doi: 10.1038/ncb1429.

Lattmann, S. *et al.* (2011) 'The DEAH-box RNA helicase RHAU binds an intramolecular RNA Gquadruplex in TERC and associates with telomerase holoenzyme', *Nucleic Acids Research*, 39(21), pp. 9390–9404. doi: 10.1093/nar/gkr630.

Le, S. *et al.* (1999) 'Rad50 and Rad51 Define Two Pathways That Collaborate to Maintain Telomeres in the Absence of Telomerase', *Genetics*, 152(1), p. 143. Available at: http://www.genetics.org/content/152/1/143.abstract.

Lee-Soety, J. Y. *et al.* (2012) 'Yeast hnRNP-related proteins contribute to the maintenance of telomeres', *Biochemical and biophysical research communications*. 2012/08/10, 426(1), pp. 12–17. doi: 10.1016/j.bbrc.2012.07.144.

Lee, J. Y. *et al.* (2007) 'Evidence that a RecQ helicase slows senescence by resolving recombining telomeres', *PLoS Biology*, 5(6), pp. 1334–1344. doi: 10.1371/journal.pbio.0050160.

Lee, M. *et al.* (2013) 'Telomere extension by telomerase and ALT generates variant repeats by mechanistically distinct processes', *Nucleic Acids Research*, 42(3), pp. 1733–1746. doi: 10.1093/nar/gkt1117.

Lee, Y. W. *et al.* (2018) 'TRF1 participates in chromosome end protection by averting TRF2-dependent telomeric R loops', *Nature Structural & Molecular Biology*. Springer US, 25(2), pp. 147–153. doi: 10.1038/s41594-017-0021-5.

Lei, E. P., Krebber, H. and Silver, P. A. (2001) 'Messenger RNAs are recruited for nuclear export during transcription', *Genes & Development* , 15(14), pp. 1771–1782. doi: 10.1101/gad.892401.

Leman, A. R. *et al.* (2012) 'Timeless preserves telomere length by promoting efficient DNA replication through human telomeres', *Cell Cycle*, 11(12), pp. 2337–2347. doi: 10.4161/cc.20810.

Lendvay, T. S. *et al.* (1996) 'Senescence Mutants of Saccharomyces cerevisiaeWith a Defect in Telomere Replication Identify Three Additional EST Genes', *Genetics*, 144(4), pp. 1399–1412. Available at: http://www.genetics.org/content/144/4/1399.abstract.

Li, J. S. Z. *et al.* (2017) 'TZAP: A telomere-associated protein involved in telomere length control', *Science*, 355(6325), pp. 638–641. doi: 10.1126/science.aah6752.

Li, L. *et al.* (2016) 'DEAD Box 1 Facilitates Removal of RNA and Homologous Recombination at DNA Double-Strand Breaks', *Molecular and Cellular Biology*, 36(22), pp. 2794–2810. doi: 10.1128/mcb.00415-16.

Li, X. and Manley, J. L. (2005) 'Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability', *Cell*, 122(3), pp. 365–378. doi: 10.1016/j.cell.2005.06.008.

Lian, H.-Y. *et al.* (2011) 'The effect of Ku on telomere replication time is mediated by telomere length but is independent of histone tail acetylation', *Molecular Biology of the Cell*, 22(10), pp. 1753–1765. doi: 10.1091/mbc.e10-06-0549.

Lingner, J. *et al.* (1997) 'Three Ever Shorter Telomere (EST) genes are dispensable for in vitro yeast telomerase activity', *Proceedings of the National Academy of Sciences of the United States of America*. The National Academy of Sciences of the USA, 94(21), pp. 11190–11195. doi: 10.1073/pnas.94.21.11190.

Lingner, J, Cooper, J. P. and Cech, T. R. (1995) 'Telomerase and DNA end replication: no longer a lagging strand problem?', *Science*, 269(5230), p. 1533. Available at: http://science.sciencemag.org/content/269/5230/1533.abstract.

Lingner, Joachim, Cooper, J. P. and Cech, T. R. (1995) 'Telomerase and DNA end replication: No longer a lagging strand problem?', *Science*, pp. 1533–1534. doi: 10.1126/science.7545310.

Lockhart, A. *et al.* (2019) 'RNase H1 and H2 are differentially regulated to eliminate RNA-DNA hybrids', *bioRxiv*, p. 593152. doi: 10.1101/593152.

Londoño-Vallejo, J. A. *et al.* (2004) 'Alternative Lengthening of Telomeres Is Characterized by High Rates of Telomeric Exchange', *Cancer Research*, 64(7), pp. 2324–2327. doi: 10.1158/0008-5472.CAN-03-4035.

López de Silanes, I. *et al.* (2014) 'Identification of TERRA locus unveils a telomere protection role through association to nearly all chromosomes', *Nature communications*, 5, p. 4723. doi: 10.1038/ncomms5723.

Lu, W.-T. *et al.* (2018) 'Drosha drives the formation of DNA:RNA hybrids around DNA break sites to facilitate DNA repair', *Nature Communications*. Springer US, 9(1), p. 532. doi: 10.1038/s41467-018-02893-x.

Luke-Glaser, S. and Luke, B. (2012) 'The Mph1 helicase can promote telomere uncapping and premature senescence in budding yeast', *PLoS ONE*, 7(7). doi: 10.1371/journal.pone.0042028.

Luke, B. *et al.* (2008) 'The Rat1p 5' to 3' Exonuclease Degrades Telomeric Repeat-Containing RNA and Promotes Telomere Elongation in Saccharomyces cerevisiae', *Molecular Cell*. Elsevier Inc., 32(4), pp. 465–477. doi: 10.1016/j.molcel.2008.10.019.

Lund, M. K., Kress, T. L. and Guthrie, C. (2008) 'Autoregulation of Npl3, a Yeast SR Protein, Requires a Novel Downstream Region and Serine Phosphorylation', *Molecular and Cellular Biology*, 28(11), pp. 3873–3881. doi: 10.1128/MCB.02153-07.

Lundblad, V. and Blackburn, E. H. (1993) 'An alternative pathway for yeast telomere maintenance rescues est1- senescence.', *Cell*. Elsevier, 73(2), pp. 347–60. doi: 10.1016/0092-8674(93)90234-H.

Lundblad, V. and Szostak, J. W. (1989) 'A mutant with a defect in telomere elongation leads to senescence in yeast.', *Cell*. Elsevier, 57(4), pp. 633–43. doi: 10.1016/0092-8674(89)90132-3.

Lustig, A. J., Kurtz, S. and Shore, D. (1990) 'Involvement of the silencer and UAS binding protein RAP1 in regulation of telomere length', *Science*, 250(4980), pp. 549–553. doi: 10.1126/science.2237406.

Lydeard, John R. *et al.* (2007) 'Break-induced replication and telomerase-independent telomere maintenance require Pol32', *Nature*, 448(7155), pp. 820–823. doi: 10.1038/nature06047.

Lydeard, John R *et al.* (2007) 'Break-induced replication and telomerase-independent telomere maintenance require Pol32', *Nature*. Nature Publishing Group, 448, p. 820. Available at: https://doi.org/10.1038/nature06047.

Ma, W. K., Cloutier, S. C. and Tran, E. J. (2013) 'The DEAD-box Protein Dbp2 Functions with the RNA-

Binding Protein Yra1 to Promote mRNP Assembly', *Journal of Molecular Biology*, 425(20), pp. 3824–3838. doi: https://doi.org/10.1016/j.jmb.2013.05.016.

Maciejowski, J. and De Lange, T. (2017) 'Telomeres in cancer: Tumour suppression and genome instability', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 18(3), pp. 175–186. doi: 10.1038/nrm.2016.171.

Maestroni, L., Matmati, S. and Coulon, S. (2017a) 'Solving the telomere replication problem', *Genes*, 8(2), pp. 1–16. doi: 10.3390/genes8020055.

Maestroni, L., Matmati, S. and Coulon, S. (2017b) 'Solving the telomere replication problem', *Genes*, 8(2), pp. 1–16. doi: 10.3390/genes8020055.

Maicher, A. *et al.* (2012) 'Deregulated telomere transcription causes replication-dependent telomere shortening and promotes cellular senescence', *Nucleic Acids Research*, 40(14), pp. 6649–6659. doi: 10.1093/nar/gks358.

Marcand, S. *et al.* (2000) 'Cell cycle restriction of telomere elongation', *Current Biology*, 10(8), pp. 487–490. doi: https://doi.org/10.1016/S0960-9822(00)00450-4.

Marcand, S. *et al.* (2008) 'Multiple pathways inhibit NHEJ at telomeres', *Genes & Development* , 22(9), pp. 1153–1158. doi: 10.1101/gad.455108.

Marcand, S., Brevet, V. and Gilson, E. (1999) 'Progressive cis-inhibition of telomerase upon telomere elongation', *The EMBO journal*, 18(12), pp. 3509–3519. doi: 10.1093/emboj/18.12.3509.

Marcand, S., Gilson, E. and Shore, D. (1997) 'A Protein-Counting Mechanism for Telomere Length Regulation in Yeast', *Science*, 275(5302), pp. 986–990. doi: 10.1126/science.275.5302.986.

Margalef, P. *et al.* (2018) 'Stabilization of Reversed Replication Forks by Telomerase Drives Telomere Catastrophe', *Cell*. Elsevier Inc., 172(3), pp. 439-453.e14. doi: 10.1016/j.cell.2017.11.047.

Martínez, P. *et al.* (2009) 'Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice', *Genes & Development* , 23(17), pp. 2060–2075. doi: 10.1101/gad.543509.

Mattarocci, S. *et al.* (2014) 'Rif1 Controls DNA replication timing in yeast through the PP1 Phosphatase Glc7', *Cell Reports*, 7(1), pp. 62–69. doi: 10.1016/j.celrep.2014.03.010.

Mazina, O. M. *et al.* (2017) 'Rad52 Inverse Strand Exchange Drives RNA-Templated DNA Double-Strand Break Repair', *Molecular Cell*. Elsevier Inc., 67(1), pp. 19-29.e3. doi: 10.1016/j.molcel.2017.05.019.

Mazzolini, R. *et al.* (2017) 'Snail1 transcription factor controls telomere transcription and integrity', *Nucleic Acids Research*, 46(1), pp. 146–158. doi: 10.1093/nar/gkx958.

McCarroll, R. M. and Fangman, W. L. (1988) 'Time of replication of yeast centromeres and telomeres', *Cell*, 54(4), pp. 505–513. doi: 10.1016/0092-8674(88)90072-4.

McClintock, B. (1941) 'The Stability of Broken Ends of Chromosomes in Zea Mays', *Genetics*, 26(2), pp. 234–282. Available at: https://www.ncbi.nlm.nih.gov/pubmed/17247004.

McEachern, M. J. and Haber, J. E. (2006) 'Break-Induced Replication and Recombinational Telomere Elongation in Yeast', *Annual Review of Biochemistry*, 75(1), pp. 111–135. doi: 10.1146/annurev.biochem.74.082803.133234.

McElhinny, S. A. N. *et al.* (2010) 'Genome instability due to ribonucleotide incorporation into DNA', *Nature Chemical Biology*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 6, p. 774. Available at: https://doi.org/10.1038/nchembio.424.

McGee, J. S. *et al.* (2010) 'Reduced Rif2 and lack of Mec1 target short telomeres for elongation rather

than double-strand break repair', *Nature Structural &Amp; Molecular Biology*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 17, p. 1438. Available at: https://doi.org/10.1038/nsmb.1947.

Mersaoui, S. Y. *et al.* (2019) 'Arginine methylation of the DDX5 helicase RGG/RG motif by PRMT5 regulates resolution of RNA:DNA hybrids', *The EMBO Journal*. John Wiley & Sons, Ltd, 38(15), p. e100986. doi: 10.15252/embj.2018100986.

Michelini, F. *et al.* (2017) 'Damage-induced lncRNAs control the DNA damage response through interaction with DDRNAs at individual double-strand breaks', *Nature Cell Biology*, 19(12), pp. 1400–1411. doi: 10.1038/ncb3643.

Miller, K. M., Rog, O. and Cooper, J. P. (2006) 'Semi-conservative DNA replication through telomeres requires Taz1', *Nature*, 440(7085), pp. 824–828. doi: 10.1038/nature04638.

Min, J., Wright, W. E. and Shay, J. W. (2017) 'Alternative Lengthening of Telomeres Mediated by Mitotic DNA Synthesis Engages Break-Induced Replication Processes', *Molecular and Cellular Biology*, 37(20), pp. 1–16. doi: 10.1128/MCB.00226-17.

Min, J., Wright, W. E. and Shay, J. W. (2019) 'Clustered telomeres in phase-separated nuclear condensates engage mitotic DNA synthesis through BLM and RAD52', *Genes & Development*, 33(13–14), pp. 814–827. doi: 10.1101/gad.324905.119.

Mischo, H. E. *et al.* (2011) 'Yeast Sen1 helicase protects the genome from transcription-associated instability', *Molecular Cell*, 41(1), pp. 21–32. doi: 10.1016/j.molcel.2010.12.007.

Misino, S. *et al.* (2018) 'Increased TERRA levels and RNase H sensitivity are conserved hallmarks of post-senescent survivors in budding yeast', *Differentiation*, 100, pp. 37–45. doi: https://doi.org/10.1016/j.diff.2018.02.002.

Montero, J. J. *et al.* (2016) 'Telomeric RNAs are essential to maintain telomeres', *Nature Communications*, 7, pp. 1–13. doi: 10.1038/ncomms12534.

Montero, J. J. *et al.* (2018) 'TERRA recruitment of polycomb to telomeres is essential for histone trymethylation marks at telomeric heterochromatin', *Nature Communications*. Springer US, 9(1), pp. 1–11. doi: 10.1038/s41467-018-03916-3.

Moradi-Fard, S. *et al.* (2016) 'Smc5/6 Is a Telomere-Associated Complex that Regulates Sir4 Binding and TPE', *PLoS Genetics*, 12(8), pp. 1–22. doi: 10.1371/journal.pgen.1006268.

Morales, C. P. *et al.* (1999) 'Absence of cancer–associated changes in human fibroblasts immortalized with telomerase', *Nature Genetics*, 21(1), pp. 115–118. doi: 10.1038/5063.

Moran-Jones, K. *et al.* (2005) 'hnRNP A2, a potential ssDNA/RNA molecular adapter at the telomere', *Nucleic Acids Research*, 33(2), pp. 486–496. doi: 10.1093/nar/gki203.

Moravec, M. *et al.* (2016) 'TERRA promotes telomerase-mediated telomere elongation in Schizosaccharomyces pombe', *EMBO reports*, 17(7), pp. 1–14. doi: 10.15252/embr.201541708.

Moreira, M. C. *et al.* (2004) 'Senataxin, the ortholog of a yeast RNA helicase, is mutant in ataxia-ocular apraxia 2', *Nature Genetics*, 36(3), pp. 225–227. doi: 10.1038/ng1303.

Moretti, P. *et al.* (1994) 'Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1.', *Genes & Development* , 8(19), pp. 2257–2269. doi: 10.1101/gad.8.19.2257.

Morissette, G. and Flamand, L. (2010) 'Herpesviruses and Chromosomal Integration', *Journal of Virology*, 84(23), pp. 12100–12109. doi: 10.1128/jvi.01169-10.

MORTIMER, R. K. and JOHNSTON, J. R. (1959) 'Life Span of Individual Yeast Cells', *Nature*, 183(4677), pp. 1751–1752. doi: 10.1038/1831751a0.

Muller, H. J. (1927) 'ARTIFICIAL TRANSMUTATION OF THE GENE', *Science*, 66(1699), pp. 84–87. doi: 10.1126/science.66.1699.84.

Muramatsu, M. *et al.* (2000) 'Class Switch Recombination and Hypermutation Require Activation-Induced Cytidine Deaminase (AID), a Potential RNA Editing Enzyme', *Cell*. Elsevier, 102(5), pp. 553–563. doi: 10.1016/S0092-8674(00)00078-7.

Murante, R. S., Henricksen, L. A. and Bambara, R. A. (1998) 'Junction ribonuclease: An activity in Okazaki fragment processing', *Proceedings of the National Academy of Sciences*, 95(5), pp. 2244 LP – 2249. doi: 10.1073/pnas.95.5.2244.

Murnane, J. P. *et al.* (1994) 'Telomere dynamics in an immortal human cell line.', *The EMBO Journal*. John Wiley & Sons, Ltd, 13(20), pp. 4953–4962. doi: 10.1002/j.1460-2075.1994.tb06822.x.

Nabetani, A., Yokoyama, O. and Ishikawa, F. (2004) 'Localization of hRad9, hHus1, hRad1, and hRad17 and caffeine-sensitive DNA replication at the alternative lengthening of telomeres-associated promyelocytic leukemia body', *Journal of Biological Chemistry*, 279(24), pp. 25849–25857. doi: 10.1074/jbc.M312652200.

Nakama, M. *et al.* (2012) 'DNA-RNA hybrid formation mediates RNAi-directed heterochromatin formation', *Genes to Cells*, 17(3), pp. 218–233. doi: 10.1111/j.1365-2443.2012.01583.x.

Nakamura, T. M., Cooper, J. P. and Cech, T. R. (1998) 'Two Modes of Survival of Fission Yeast Without Telomerase', *Science*, 282(5388), pp. 493–496. doi: 10.1126/science.282.5388.493.

Nanavaty, V. *et al.* (2017) 'Trypanosoma brucei RAP1 maintains telomere and subtelomere integrity by suppressing TERRA and telomeric RNA: DNA hybrids', *Nucleic Acids Research*, 45(10), pp. 5785–5796. doi: 10.1093/nar/gkx184.

Narita, Masashi *et al.* (2003) 'Rb-Mediated Heterochromatin Formation and Silencing of E2F Target Genes during Cellular Senescence', *Cell*, 113(6), pp. 703–716. doi: https://doi.org/10.1016/S0092-8674(03)00401-X.

Nautiyal, S., DeRisi, J. L. and Blackburn, E. H. (2002) 'The genome-wide expression response to telomerase deletion in Saccharomyces cerevisiae', *Proceedings of the National Academy of Sciences*, 99(14), pp. 9316–9321. doi: 10.1073/pnas.142162499.

Neil, A. J. *et al.* (2018) 'RNA–DNA hybrids promote the expansion of Friedreich's ataxia (GAA)n repeats via break-induced replication', *Nucleic Acids Research*. Oxford University Press, 46(7), pp. 3487–3497. doi: 10.1093/nar/gky099.

Nergadze, S. G. *et al.* (2009) 'CpG-island promoters drive transcription of human telomeres', *Rna*, 15(12), pp. 2186–2194. doi: 10.1261/rna.1748309.

Neurohr, G. E. *et al.* (2019) 'Excessive Cell Growth Causes Cytoplasm Dilution And Contributes to Senescence.', *Cell*. The Author(s), 176(5), pp. 1083-1097.e18. doi: 10.1016/j.cell.2019.01.018.

Ng, L. J. *et al.* (2009) 'Telomerase activity is associated with an increase in DNA methylation at the proximal subtelomere and a reduction in telomeric transcription', *Nucleic Acids Research*, 37(4), pp. 1152–1159. doi: 10.1093/nar/gkn1030.

Nguyen, D. T. *et al.* (2017) 'The chromatin remodelling factor ATRX suppresses R-loops in transcribed telomeric repeats', *EMBO reports*, 18(6), pp. 914–928. doi: 10.15252/embr.201643078.

Nguyen, H. D. *et al.* (2017) 'Functions of Replication Protein A as a Sensor of R Loops and a Regulator of RNaseH1', *Molecular Cell*. Elsevier Inc., 65(5), pp. 832-847.e4. doi: 10.1016/j.molcel.2017.01.029.

Nguyen, T. A. *et al.* (2011) 'Analysis of subunit assembly and function of the Saccharomyces cerevisiae RNase H2 complex', *The FEBS Journal*. John Wiley & Sons, Ltd (10.1111), 278(24), pp. 4927–4942. doi: 10.1111/j.1742-4658.2011.08394.x.

Nick McElhinny, S. A. *et al.* (2010) 'Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases', *Proceedings of the National Academy of Sciences of the United States of America*, 107(11), pp. 4949–4954. doi: 10.1073/pnas.0914857107.

Nowotny, M. *et al.* (2008) 'Specific recognition of RNA/DNA hybrid and enhancement of human RNase H1 activity by HBD', *The EMBO Journal*. John Wiley & Sons, Ltd, 27(7), pp. 1172–1181. doi: 10.1038/emboj.2008.44.

O'Connell, K., Jinks-Robertson, S. and Petes, T. D. (2015) 'Elevated Genome-Wide Instability in Yeast Mutants Lacking RNase H Activity', *Genetics*, 201(3), pp. 963–975. doi: 10.1534/genetics.115.182725.

O'Sullivan, R. J. *et al.* (2014) 'Rapid induction of alternative lengthening of telomeres by depletion of the histone chaperone ASF1', *Nature Structural &Amp; Molecular Biology*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 21, p. 167. Available at: https://doi.org/10.1038/nsmb.2754.

Ofir, R. *et al.* (1999) 'Position effect of human telomeric repeats on replication timing', *Proceedings of the National Academy of Sciences*, 96(20), pp. 11434–11439. doi: 10.1073/pnas.96.20.11434.

Ohle, C. *et al.* (2016) 'Transient RNA-DNA Hybrids Are Required for Efficient Double-Strand Break Repair', *Cell*, 167(4), pp. 1001-1013.e7. doi: 10.1016/j.cell.2016.10.001.

Olivier, M. *et al.* (2018) 'RAD51 and RTEL1 compensate telomere loss in the absence of telomerase', *Nucleic Acids Research*. Oxford University Press, 46(5), pp. 2432–2445. doi: 10.1093/nar/gkx1322.

Olovnikov, A. M. (1973) 'A theory of marginotomy: The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon', *Journal of Theoretical Biology*, 41(1), pp. 181–190. doi: https://doi.org/10.1016/0022-5193(73)90198-7.

Opresko, P. L. *et al.* (2005) 'POT1 stimulates RecQ helicases WRN and BLM to unwind telomeric DNA substrates', *Journal of Biological Chemistry*, 280(37), pp. 32069–32080. doi: 10.1074/jbc.M505211200.

Osterwald, S. *et al.* (2015) 'PML induces compaction, TRF2 depletion and DNA damage signaling at telomeres and promotes their alternative lengthening', *Journal of Cell Science*, 128(10), pp. 1887–1900. doi: 10.1242/jcs.148296.

Özer, Ö. and Hickson, I. D. (2018) 'Pathways for maintenance of telomeres and common fragile sites during DNA replication stress', *Open Biology*, 8(4), p. 180018. doi: 10.1098/rsob.180018.

Palm, W. and de Lange, T. (2008) 'How Shelterin Protects Mammalian Telomeres', *Annual Review of Genetics*, 42(1), pp. 301–334. doi: 10.1146/annurev.genet.41.110306.130350.

Pan, X. *et al.* (2017) 'FANCM, BRCA1, and BLM cooperatively resolve the replication stress at the ALT telomeres', *Proceedings of the National Academy of Sciences*, 114(29), pp. E5940–E5949. doi: 10.1073/pnas.1708065114.

Pardue, M. Lou and Debaryshe, P. G. (2008) 'Drosophila telomeres: A variation on the telomerase theme', *Fly*, 2(3), pp. 101–110. doi: 10.4161/fly.6393.

Perez-Romero, C. A. *et al.* (2018) 'Induction and relocalization of telomeric repeat-containing RNAs during diauxic shift in budding yeast', *Current Genetics*. Springer Berlin Heidelberg, 64(5), pp. 1117–1127. doi: 10.1007/s00294-018-0829-5.

Petersen-Mahrt, S. K., Harris, R. S. and Neuberger, M. S. (2015) 'Pillars Article: AID Mutates E. coli Suggesting a DNA Deamination Mechanism for Antibody Diversification. Nature. 2002. 418: 99-103.',

Journal of immunology (Baltimore, Md. : 1950), 194(5), pp. 2043–7. doi: 10.1038/nature00849.1.

Petreaca, R. C. *et al.* (2006) 'Chromosome end protection plasticity revealed by Stn1p and Ten1p bypass of Cdc13p', *Nature Cell Biology*, 8(7), pp. 748–755. doi: 10.1038/ncb1430.

Petreaca, R. C., Chiu, H.-C. and Nugent, C. I. (2007) 'The Role of Stn1p in Saccharomyces cerevisiae Telomere Capping Can Be Separated From Its Interaction With Cdc13p', *Genetics*, 177(3), pp. 1459–1474. doi: 10.1534/genetics.107.078840.

Pfeiffer, V. *et al.* (2013) 'The THO complex component Thp2 counteracts telomeric R-loops and telomere shortening', *EMBO Journal*, 32(21), pp. 2861–2871. doi: 10.1038/emboj.2013.217.

Pfeiffer, V. and Lingner, J. (2012) 'TERRA promotes telomere shortening through exonuclease 1-mediated resection of chromosome ends', *PLoS Genetics*, 8(6), p. e1002747. doi: 10.1371/journal.pgen.1002747.

Pfeiffer, V. and Lingner, J. (2013) 'Replication of telomeres and the regulation of telomerase', *Cold Spring Harbor perspectives in biology*, 5(5), p. a010405. doi: 10.1101/cshperspect.a010405.

Pickett, H. A. and Reddel, R. R. (2015) 'Molecular mechanisms of activity and derepression of alternative lengthening of telomeres', *Nature Structural and Molecular Biology*, 22(11), pp. 875–880. doi: 10.1038/nsmb.3106.

Platt, J. M. *et al.* (2013) 'Rap1 relocalization contributes to the chromatin-mediated gene expression profile and pace of cell senescence', *Genes and Development*, 27(12), pp. 1406–1420. doi: 10.1101/gad.218776.113.

Pohl, T. J. and Zakian, V. A. (2019) *Pif1 family DNA helicases: a helpmate to RNase H?*, *DNA Repair*. Elsevier B.V. doi: 10.1016/j.dnarep.2019.06.004.

Poole, L. A. and Cortez, D. (2016) 'SMARCAL1 and telomeres: Replicating the troublesome ends', *Nucleus*. Taylor & Francis, 7(3), pp. 270–274. doi: 10.1080/19491034.2016.1179413.

Porro, A. *et al.* (2010) 'Molecular Dissection of Telomeric Repeat-Containing RNA Biogenesis Unveils the Presence of Distinct and Multiple Regulatory Pathways', *Molecular and Cellular Biology*, 30(20), pp. 4808–4817. doi: 10.1128/MCB.00460-10.

Porro, A. *et al.* (2014) 'Functional characterization of the TERRA transcriptome at damaged telomeres', *Nature Communications*. Nature Publishing Group, 5, pp. 1–13. doi: 10.1038/ncomms6379.

Porro, A., Feuerhahn, S. and Lingner, J. (2014) 'TERRA-Reinforced Association of LSD1 with MRE11 Promotes Processing of Uncapped Telomeres', *Cell Reports*. The Authors, 6(4), pp. 765–776. doi: 10.1016/j.celrep.2014.01.022.

Porter, S. E. *et al.* (1996) 'The DNA-binding protein Hdf1p (a putative Ku homologue) is required for maintaining normal telomere length in Saccharomyces cerevisiae', *Nucleic acids research*, 24(4), pp. 582–585. doi: 10.1093/nar/24.4.582.

Poschke, H. *et al.* (2012) 'Rif2 Promotes a Telomere Fold-Back Structure through Rpd3L Recruitment in Budding Yeast', *PLOS Genetics*. Public Library of Science, 8(9), p. e1002960. Available at: https://doi.org/10.1371/journal.pgen.1002960.

Preti, M. *et al.* (2010) 'The Telomere-Binding Protein Tbf1 Demarcates snoRNA Gene Promoters in Saccharomyces cerevisiae', *Molecular Cell*. Elsevier, 38(4), pp. 614–620. doi: 10.1016/j.molcel.2010.04.016.

Qi, H. and Zakian, V. A. (2000) 'The Saccharomyces telomere-binding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase α and the telomerase-associated Est1 protein', *Genes & Development* , 14(14), pp. 1777–1788. doi: 10.1101/gad.14.14.1777.

Raghuraman, M. K. *et al.* (2001) 'Replication dynamics of the yeast Genome', 294(October), pp. 115–121.

Rai, R. *et al.* (2016) 'TRF2-RAP1 is required to protect telomeres from engaging in homologous recombination-mediated deletions and fusions', *Nature Communications*. The Author(s), 7, p. 10881. Available at: https://doi.org/10.1038/ncomms10881.

Ramamoorthy, M. and Smith, S. (2015) 'Loss of ATRX Suppresses Resolution of Telomere Cohesion to Control Recombination in ALT Cancer Cells', *Cancer Cell*. Elsevier Inc., 28(3), pp. 357–369. doi: 10.1016/j.ccell.2015.08.003.

Rappsilber, J., Mann, M. and Ishihama, Y. (2007) 'Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips', *Nature Protocols*. Nature Publishing Group, 2(8), pp. 1896–1906. Available at: https://doi.org/10.1038/nprot.2007.261.

Redon, S., Reichenbach, P. and Lingner, J. (2010) 'The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase', *Nucleic Acids Research*, 38(17), pp. 5797–5806. doi: 10.1093/nar/gkq296.

Redon, S., Zemp, I. and Lingner, J. (2013) 'A three-state model for the regulation of telomerase by TERRA and hnRNPA1', *Nucleic Acids Research*, 41(19), pp. 9117–9128. doi: 10.1093/nar/gkt695.

Rendeková, J. *et al.* (2016) 'Mgm101: A double-duty Rad52-like protein', *Cell Cycle*, 15(23), pp. 3169–3176. doi: 10.1080/15384101.2016.1231288.

Rhie, B. *et al.* (2013) 'Biochemical and Biophysical Research Communications Cellular aging is associated with increased ubiquitylation of histone H2B in yeast telomeric heterochromatin', *Biochemical and Biophysical Research Communications*. Elsevier Inc., 439(4), pp. 570–575. doi: 10.1016/j.bbrc.2013.09.017.

Rice, G. *et al.* (2007) 'Clinical and Molecular Phenotype of Aicardi-Goutières Syndrome', *The American Journal of Human Genetics*. Elsevier, 81(4), pp. 713–725. doi: 10.1086/521373.

Roberts, R. W. and Crothers, D. M. (1992) 'Stability and properties of double and triple helices: Dramatic effects of RNA or DNA backbone composition', *Science*, 258(5087), pp. 1463–1466. doi: 10.1126/science.1279808.

Robin, J. D. *et al.* (2014) 'Telomere position effect: Regulation of gene expression with progressive telomere shortening over long distances', *Genes and Development*, 28(22), pp. 2464–2476. doi: 10.1101/gad.251041.114.

Robyr, D. *et al.* (2002) 'Microarray Deacetylation Maps Determine Genome-Wide Functions for Yeast Histone Deacetylases', *Cell*. Elsevier, 109(4), pp. 437–446. doi: 10.1016/S0092-8674(02)00746-8.

Rog, O. *et al.* (2009) 'Sumoylation of RecQ Helicase Controls the Fate of Dysfunctional Telomeres', *Molecular Cell*. Elsevier, 33(5), pp. 559–569. doi: 10.1016/j.molcel.2009.01.027.

Roumelioti, F. *et al.* (2016a) ' Alternative lengthening of human telomeres is a conservative DNA replication process with features of break-induced replication ', *EMBO reports*, 17(12), pp. 1731–1737. doi: 10.15252/embr.201643169.

Roumelioti, F. *et al.* (2016b) 'Alternative lengthening of human telomeres is a conservative DNA replication process with features of break-induced replication', *EMBO reports*, 17(12), pp. 1731–1737. doi: 10.15252/embr.201643169.

Roux, K. J., Kim, D. I. and Burke, B. (2013) 'BioID: A Screen for Protein-Protein Interactions', *Current Protocols in Protein Science*. John Wiley & Sons, Ltd, 74(1), pp. 19.23.1-19.23.14. doi: 10.1002/0471140864.ps1923s74.

Roy, D. *et al.* (2010) 'Competition between the RNA Transcript and the Nontemplate DNA Strand during R-Loop Formation In Vitro: a Nick Can Serve as a Strong R-Loop Initiation Site', *Molecular and Cellular Biology*, 30(1), pp. 146–159. doi: 10.1128/mcb.00897-09.

Roy, D. and Lieber, M. R. (2009) 'G Clustering Is Important for the Initiation of Transcription-Induced R-Loops In Vitro, whereas High G Density without Clustering Is Sufficient Thereafter', *Molecular and Cellular Biology*, 29(11), pp. 3124–3133. doi: 10.1128/mcb.00139-09.

Roy, D., Yu, K. and Lieber, M. R. (2008) 'Mechanism of R-Loop Formation at Immunoglobulin Class Switch Sequences', *Molecular and Cellular Biology*, 28(1), pp. 50–60. doi: 10.1128/mcb.01251-07.

Roy, R. *et al.* (2004) 'Separation-of-function Mutants of Yeast Ku80 Reveal a Yku80p-Sir4p Interaction Involved in Telomeric Silencing', *Journal of Biological Chemistry*, 279(1), pp. 86–94. doi: 10.1074/jbc.M306841200.

Rydberg, B. and Game, J. (2002) 'Excision of misincorporated ribonucleotides in DNA by RNase H (type 2) and FEN-1 in cell-free extracts', *Proceedings of the National Academy of Sciences of the United States of America*, 99(26), pp. 16654–16659. doi: 10.1073/pnas.262591699.

Sabourin, M., Tuzon, C. T. and Zakian, V. A. (2007) 'Telomerase and Tel1p Preferentially Associate with Short Telomeres in S. cerevisiae', *Molecular Cell*, 27(4), pp. 550–561. doi: https://doi.org/10.1016/j.molcel.2007.07.016.

Salas-Armenteros, I. *et al.* (2017) 'Human THO–Sin3A interaction reveals new mechanisms to prevent R-loops that cause genome instability', *The EMBO Journal*, 36(23), p. e201797208. doi: 10.15252/embj.201797208.

Sandell, L. L. and Zakian, V. A. (1993) 'Loss of a yeast telomere: Arrest, recovery, and chromosome loss', *Cell*, 75(4), pp. 729–739. doi: 10.1016/0092-8674(93)90493-A.

Santos-Pereira, J. M. *et al.* (2013) 'The Npl3 hnRNP prevents R-loop- mediated transcription – replication conflicts and genome instability', 27(22), pp. 2445–2458. doi: 10.1101/gad.229880.113.associated.

Santos-Pereira, J. M. *et al.* (2014) 'Npl3, a new link between RNA-binding proteins and the maintenance of genome integrity', *Cell Cycle*, 13(10), pp. 1524–1529. doi: 10.4161/cc.28708.

Santos-Pereira, José M. and Aguilera, A. (2015) 'R loops: New modulators of genome dynamics and function', *Nature Reviews Genetics*. Nature Publishing Group, 16(10), pp. 583–597. doi: 10.1038/nrg3961.

Santos-Pereira, José M and Aguilera, A. (2015) 'R loops: New modulators of genome dynamics and function', *Nature Reviews Genetics*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., pp. 583–597. doi: 10.1038/nrg3961.

Sarek, G. *et al.* (2015) 'TRF2 recruits RTEL1 to telomeres in s phase to promote t-loop unwinding', *Molecular Cell*. Elsevier, 57(4), pp. 622–635. doi: 10.1016/j.molcel.2014.12.024.

Sarthy, J. *et al.* (2009) 'Human RAP1 inhibits non-homologous end joining at telomeres', *The EMBO Journal*. John Wiley & Sons, Ltd, 28(21), pp. 3390–3399. doi: 10.1038/emboj.2009.275.

Scheibe, M. *et al.* (2013) 'Quantitative interaction screen of telomeric repeatcontaining RNA reveals novel TERRA regulators', *Genome Research*, 23(12), pp. 2149–2157. doi: 10.1101/gr.151878.112.

Schoeftner, S. and Blasco, M. A. (2008) 'Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II', *Nature Cell Biology*, 10(2), pp. 228–236. doi: 10.1038/ncb1685.

Schoeftner, S. and Blasco, M. A. (2010) 'Chromatin regulation and non-coding RNAs at mammalian

telomeres', *Seminars in Cell and Developmental Biology*. Elsevier Ltd, 21(2), pp. 186–193. doi: 10.1016/j.semcdb.2009.09.015.

Schulz, V. P. and Zakian, V. A. (1994) 'The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation', *Cell*, 76(1), pp. 145–155. doi: 10.1016/0092-8674(94)90179-1.

Sfeir, A. *et al.* (2009) 'Mammalian Telomeres Resemble Fragile Sites and Require TRF1 for Efficient Replication', *Cell*. Elsevier Ltd, 138(1), pp. 90–103. doi: 10.1016/j.cell.2009.06.021.

Shevchenko, A. *et al.* (2006) 'In-gel digestion for mass spectrometric characterization of proteins and proteomes', *Nature Protocols*, 1(6), pp. 2856–2860. doi: 10.1038/nprot.2006.468.

De Silanes, I. L., D'Alcontres, M. S. and Blasco, M. A. (2010) 'TERRA transcripts are bound by a complex array of RNA-binding proteins', *Nature Communications*, 1(3), pp. 1–10. doi: 10.1038/ncomms1032.

Silva, B. *et al.* (2019) 'FANCM limits ALT activity by restricting telomeric replication stress induced by deregulated BLM and R-loops', *Nature Communications*. Springer US, 10(1), p. 2253. doi: 10.1038/s41467-019-10179-z.

Silva, S. *et al.* (2016) 'Mte1 interacts with Mph1 and promotes crossover recombination and telomere maintenance', *Genes and Development*, 30(6), pp. 700–717. doi: 10.1101/gad.276204.115.

Simon, M. N., Churikov, D. and Géli, V. (2016) 'Replication stress as a source of telomere recombination during replicative senescence in Saccharomyces cerevisiae', *FEMS Yeast Research*, 16(7), pp. 1–11. doi: 10.1093/femsyr/fow085.

Singer, M. S. and Gottschling, D. E. (1994) 'TLC1: template RNA component of Saccharomyces cerevisiae telomerase', *Science*, 266(5184), pp. 404–409. doi: 10.1126/science.7545955.

Skourti-Stathaki, K., Kamieniarz-Gdula, K. and Proudfoot, N. J. (2014) 'R-loops induce repressive chromatin marks over mammalian gene terminators', *Nature*. Nature Publishing Group, 516(7531), pp. 436–439. doi: 10.1038/nature13787.

Skourti-Stathaki, K. and Proudfoot, N. (2013) 'Histone 3 S10 Phosphorylation: "Caught in the R Loop!"', *Molecular Cell*. Elsevier Inc., 52(4), pp. 470–472. doi: 10.1016/j.molcel.2013.11.006.

Skourti-stathaki, K. and Proudfoot, N. J. (2014) 'A double-edged sword : R loops as t h reat s t o gen om e in t egrit y an d pow erfu l regu lat ors of gen e expression', *Genes & Development*, 28(13), pp. 1384–1396. doi: 10.1101/gad.242990.114.Freely.

Skourti-Stathaki, K., Proudfoot, N. J. and Gromak, N. (2011) 'Human Senataxin Resolves RNA/DNA Hybrids Formed at Transcriptional Pause Sites to Promote Xrn2-Dependent Termination', *Molecular Cell*. Elsevier Inc., 42(6), pp. 794–805. doi: 10.1016/j.molcel.2011.04.026.

Smith, J. S. *et al.* (2011) 'Rudimentary G-quadruplex – based telomere capping in Saccharomyces cerevisiae', 18(4), pp. 478–485. doi: 10.1038/nsmb.2033.Rudimentary.

Smogorzewska, A. *et al.* (2000) 'Control of Human Telomere Length by TRF1 and TRF2', *Molecular and Cellular Biology*, 20(5), pp. 1659–1668. doi: 10.1128/MCB.20.5.1659-1668.2000.

Smolka, M. B. *et al.* (2007) 'Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases', *Proceedings of the National Academy of Sciences of the United States of America*. 2007/06/11. National Academy of Sciences, 104(25), pp. 10364–10369. doi: 10.1073/pnas.0701622104.

Sobinoff, A. P. and Pickett, H. A. (2017) 'Alternative Lengthening of Telomeres: DNA Repair Pathways Converge', *Trends in Genetics*. Elsevier Ltd, 33(12), pp. 921–932. doi: 10.1016/j.tig.2017.09.003.

Song, C. *et al.* (2017) 'SIRT7 and the DEAD-box helicase DDX21 cooperate to resolve genomic R loops

and safeguard genome stability', *Genes and Development*, 31(13), pp. 1370–1381. doi: 10.1101/gad.300624.117.

Soudet, J., Jolivet, P. and Teixeira, M. T. (2014) 'Elucidation of the DNA end-replication problem in saccharomyces cerevisiae', *Molecular Cell*. Elsevier Inc., 53(6), pp. 954–964. doi: 10.1016/j.molcel.2014.02.030.

Sparks, J. L. *et al.* (2012) 'RNase H2-Initiated Ribonucleotide Excision Repair', *Molecular Cell*. Elsevier, 47(6), pp. 980–986. doi: 10.1016/j.molcel.2012.06.035.

van Steensel, B. and de Lange, T. (1997) 'Control of telomere length by the human telomeric protein TRF1', *Nature*, 385(6618), pp. 740–743. doi: 10.1038/385740a0.

Stein, H. and Hausen, P. (1969) 'Enzyme from Calf Thymus Degrading the RNA Moiety of DNA-RNA Hybrids: Effect on DNA-Dependent RNA Polymerase', *Science*, 166(3903), pp. 393–395. doi: 10.1126/science.166.3903.393.

Stevenson, J. B. and Gottschling, D. E. (1999) 'Telomeric chromatin modulates replication timing near chromosome ends', *Genes and Development*, 13(2), pp. 146–151. doi: 10.1101/gad.13.2.146.

Subramanian, L., Moser, B. A. and Nakamura, T. M. (2008) 'Recombination-Based Telomere Maintenance Is Dependent on Tel1-MRN and Rap1 and Inhibited by Telomerase, Taz1, and Ku in Fission Yeast', *Molecular and Cellular Biology*, 28(5), pp. 1443–1455. doi: 10.1128/MCB.01614-07.

Sui, J. *et al.* (2015) 'DNA-PKcs phosphorylates hnRNP-A1 to facilitate the RPA-to-POT1 switch and telomere capping after replication', *Nucleic Acids Research*, 43(12), pp. 5971–5983. doi: 10.1093/nar/gkv539.

Sun, Q. *et al.* (2013) 'Europe PMC Funders Group R-Loop Stabilization Represses Antisense Transcription at the Arabidopsis FLC Locus', 340(6132), pp. 619–621. doi: 10.1126/science.1234848.R-Loop.

Sun, Z.-W. and Allis, C. D. (2002) 'Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast', *Nature*, 418(6893), pp. 104–108. doi: 10.1038/nature00883.

Taddei, A. and Gasser, S. M. (2006) 'Repairing subtelomeric DSBs at the nuclear periphery', *Trends in Cell Biology*, 16(5), pp. 225–228. doi: 10.1016/j.tcb.2006.03.005.

Takahama, K. *et al.* (2013) 'Regulation of telomere length by G-quadruplex telomere DNA- and TERRA-binding protein TLS/FUS', *Chemistry and Biology*. Elsevier Ltd, 20(3), pp. 341–350. doi: 10.1016/j.chembiol.2013.02.013.

Takahashi, T. *et al.* (2011) 'Topoisomerase 1 provokes the formation of short deletions in repeated sequences upon high transcription in Saccharomyces cerevisiae', *Proceedings of the National Academy of Sciences*, 108(2), pp. 692–697. doi: 10.1073/pnas.1012582108.

Takai, H., Smogorzewska, A. and de Lange, T. (2003) 'DNA Damage Foci at Dysfunctional Telomeres', *Current Biology*, 13(17), pp. 1549–1556. doi: https://doi.org/10.1016/S0960-9822(03)00542-6.

Tardat, M. and Déjardin, J. (2018) 'Telomere chromatin establishment and its maintenance during mammalian development', *Chromosoma*. Chromosoma, 127(1), pp. 3–18. doi: 10.1007/s00412-017-0656-3.

Tardiff, D. F., Abruzzi, K. C. and Rosbash, M. (2007) 'Protein characterization of Saccharomyces cerevisiae RNA polymerase II after in vivo cross-linking', *Proceedings of the National Academy of Sciences*, 104(50), pp. 19948–19953. doi: 10.1073/pnas.0710179104.

Teasley, D. C. *et al.* (2015) 'Flap endonuclease 1 limits telomere fragility on the leading strand', *Journal of Biological Chemistry*, 290(24), pp. 15133–15145. doi: 10.1074/jbc.M115.647388.

Tedeschi, F. A. *et al.* (2018) 'The DEAD-box protein Dbp2p is linked to noncoding RNAs, the helicase Sen1p, and R-loops', *RNA*, 24(12), pp. 1693–1705. doi: 10.1261/rna.067249.118.

Teixeira, M. T. *et al.* (2004) 'Telomere Length Homeostasis Is Achieved via a Switch between Telomerase- Extendible and -Nonextendible States', *Cell*, 117(3), pp. 323–335. doi: https://doi.org/10.1016/S0092-8674(04)00334-4.

Teloni, F. *et al.* (2019) 'Efficient Pre-mRNA Cleavage Prevents Replication-Stress-Associated Genome Instability', *Molecular Cell*. Elsevier Inc., 73(4), pp. 670–683. doi: 10.1016/j.molcel.2018.11.036.

Teng, S.-C. *et al.* (2000) 'Telomerase-Independent Lengthening of Yeast Telomeres Occurs by an Abrupt Rad50p-Dependent, Rif-Inhibited Recombinational Process', *Molecular Cell*, 6(4), pp. 947–952. doi: https://doi.org/10.1016/S1097-2765(05)00094-8.

Teng, S.-C. and Zakian, V. A. (1999) 'Telomere-Telomere Recombination Is an Efficient Bypass Pathway for Telomere Maintenance in Saccharomyces cerevisiae', *Molecular and Cellular Biology*, 19(12), pp. 8083–8093. doi: 10.1128/MCB.19.12.8083.

Teng, Y. *et al.* (2018) 'ROS-induced R loops trigger a transcription-coupled but BRCA1/2-independent homologous recombination pathway through CSB', *Nature Communications*. Springer US, 9(1). doi: 10.1038/s41467-018-06586-3.

Therizols, P. *et al.* (2006) 'Telomere tethering at the nuclear periphery is essential for efficient DNA double strand break repair in subtelomeric region', *Journal of Cell Biology*, 172(2), pp. 189–199. doi: 10.1083/jcb.200505159.

Tomlinson, R. L. *et al.* (2005) 'Cell Cycle-regulated Trafficking of Human Telomerase to Telomeres', *Molecular Biology of the Cell*. American Society for Cell Biology (mboc), 17(2), pp. 955–965. doi: 10.1091/mbc.e05-09-0903.

Tsai, H. J. *et al.* (2006) 'Involvement of topoisomerase III in telomere-telomere recombination', *Journal of Biological Chemistry*, 281(19), pp. 13717–13723. doi: 10.1074/jbc.M600649200.

Tuduri, S. *et al.* (2009) 'Topoisomerase I suppresses genomic instability by preventing interference between replication and transcription', *Nature Cell Biology*. Nature Publishing Group, 11(11), pp. 1315–1324. doi: 10.1038/ncb1984.

Tutton, S. *et al.* (2016) ' Subtelomeric p53 binding prevents accumulation of DNA damage at human telomeres ', *The EMBO Journal*, 35(2), pp. 193–207. doi: 10.15252/embj.201490880.

Ungar, L. *et al.* (2009) 'A genome-wide screen for essential yeast genes that affect telomere length maintenance', *Nucleic Acids Research*, 37(12), pp. 3840–3849. doi: 10.1093/nar/gkp259.

Uringa, E. J. *et al.* (2011) 'RTEL1: An essential helicase for telomere maintenance and the regulation of homologous recombination', *Nucleic Acids Research*, 39(5), pp. 1647–1655. doi: 10.1093/nar/gkq1045.

Vannier, J.-B. *et al.* (2013) 'RTEL1 Is a Replisome-Associated Helicase That Promotes Telomere and Genome-Wide Replication', *Science*, 342(6155), pp. 239–242. doi: 10.1126/science.1241779.

Vannier, J. *et al.* (2012) 'RTEL1 Dismantles T Loops and Counteracts Telomeric G4-DNA to Maintain Telomere Integrity', *Cell*. Elsevier Inc., 149(4), pp. 795–806. doi: 10.1016/j.cell.2012.03.030.

Verdun, R. E. and Karlseder, J. (2006) 'The DNA Damage Machinery and Homologous Recombination Pathway Act Consecutively to Protect Human Telomeres', *Cell*, 127(4), pp. 709–720. doi: 10.1016/j.cell.2006.09.034.

Vodenicharov, M. D., Laterreur, N. and Wellinger, R. J. (2010) 'Telomere capping in non-dividing yeast cells requires Yku and Rap1', *The EMBO Journal*. John Wiley & Sons, Ltd, 29(17), pp. 3007–3019. doi: 10.1038/emboj.2010.155.

Vodenicharov, M. D. and Wellinger, R. J. (2006) 'DNA Degradation at Unprotected Telomeres in Yeast Is Regulated by the CDK1 (Cdc28/Clb) Cell-Cycle Kinase', *Molecular Cell*. Elsevier, 24(1), pp. 127–137. doi: 10.1016/j.molcel.2006.07.035.

Vodenicharov, M. D. and Wellinger, R. J. (2007) 'The Cell Division Cycle Puts Up with Unprotected Telomeres: Cell Cycle Regulated Telomere Uncapping as a Means to Achieve Telomere Homeostasis', *Cell Cycle*. Taylor & Francis, 6(10), pp. 1161–1167. doi: 10.4161/cc.6.10.4224.

Wahba, L. *et al.* (2011) 'RNase H and Multiple RNA Biogenesis Factors Cooperate to Prevent RNA:DNA Hybrids from Generating Genome Instability', *Molecular Cell*. Elsevier, 44(6), pp. 978–988. doi: 10.1016/j.molcel.2011.10.017.

Wahba, L., Gore, S. K. and Koshland, D. (2013) 'The homologous recombination machinery modulates the formation of RNA-DNA hybrids and associated chromosome instability', *eLife*, (2), p. e00505. doi: 10.7554/eLife.00505.

Wanat, J. J. *et al.* (2018) 'TERRA and the histone methyltransferase Dot1 cooperate to regulate senescence in budding yeast', *PLoS ONE*, 13(4), p. e0195698. doi: 10.1371/journal.pone.0195698.

Wang, H. *et al.* (2019) 'Cohesin SA2 and EWSR1 in R-Loop Regulation', *Biophysical Journal*. Elsevier, 116(3), p. 505a. doi: 10.1016/j.bpj.2018.11.2723.

Wang, Z. *et al.* (2015) 'Telomeric repeat-containing RNA (TERRA) constitutes a nucleoprotein component of extracellular inflammatory exosomes', *Proceedings of the National Academy of Sciences*, 112(46), pp. E6293–E6300. doi: 10.1073/pnas.1505962112.

Wang, Z. and Lieberman, P. M. (2016) 'The crosstalk of telomere dysfunction and inflammation through cell-free TERRA containing exosomes', *RNA Biology*. Taylor & Francis, 13(8), pp. 690–695. doi: 10.1080/15476286.2016.1203503.

Watson, J. D. (1972) 'Origin of Concatemeric T7DNA', *Nature New Biology*, 239(94), pp. 197–201. doi: 10.1038/newbio239197a0.

Wellinger, R. J. *et al.* (1996) 'Evidence for a new step in telomere maintenance', *Cell*, 85(3), pp. 423–433. doi: 10.1016/S0092-8674(00)81120-4.

Wellinger, R. J., Wolf, A. J. and Zakian, V. A. (1993) 'Origin activation and formation of single-strand TG1-3 tails occur sequentially in late S phase on a yeast linear plasmid', *Molecular and cellular biology*, 13(7), pp. 4057–4065. doi: 10.1128/mcb.13.7.4057.

Wellinger, R. J. and Zakian, V. A. (2012) 'Everything you ever wanted to know about Saccharomyces cerevisiae telomeres: Beginning to end', *Genetics*, 191(4), pp. 1073–1105. doi: 10.1534/genetics.111.137851.

Winston, F., Dollard, C. and Ricupero-Hovasse, S. L. (1995) 'Construction of a set of convenient saccharomyces cerevisiae strains that are isogenic to S288C', *Yeast*. John Wiley & Sons, Ltd, 11(1), pp. 53–55. doi: 10.1002/yea.320110107.

Wotton, D. and Shore, D. (1997) 'A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in Saccharomyces cerevisiae.', *Genes & Development* , 11(6), pp. 748–760. doi: 10.1101/gad.11.6.748.

Wright, J. H., Gottschling, D. E. and Zakian, V. A. (1992) 'Saccharomyces telomeres assume a non-nucleosomal chromatin structure.', *Genes & Development* , 6(2), pp. 197–210. doi: 10.1101/gad.6.2.197.

Wright, J. H. and Zakian, V. A. (1995) 'Protein-DNA interactions in soluble telosomes from Saccharomyces cerevisiae', *Nucleic acids research*, 23(9), pp. 1454–1460. doi: 10.1093/nar/23.9.1454.

Wright, W. E. *et al.* (1999) 'Normal Human Telomeres Are Not Late Replicating', *Experimental Cell Research*, 251(2), pp. 492–499. doi: https://doi.org/10.1006/excr.1999.4602.

Wu, G., Lee, W. H. and Chen, P. L. (2000) 'NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G2 phases in immortalized telomerase-negative cells. Implication of NBS1 in alternative lengthening of telomeres', *Journal of Biological Chemistry*, 275(39), pp. 30618–30622. doi: 10.1074/jbc.C000390200.

Wu, L. *et al.* (2006) 'Pot1 Deficiency Initiates DNA Damage Checkpoint Activation and Aberrant Homologous Recombination at Telomeres', *Cell*. Elsevier, 126(1), pp. 49–62. doi: 10.1016/j.cell.2006.05.037.

Wu, P. *et al.* (2010) 'Apollo Contributes to G Overhang Maintenance and Protects Leading-End Telomeres', *Molecular Cell*. Elsevier, 39(4), pp. 606–617. doi: 10.1016/j.molcel.2010.06.031.

Wu, P., Takai, H. and de Lange, T. (2012) 'Telomeric 3' overhangs derive from resection by Exo1 and Apollo and fill-in by POT1b-associated CST', *Cell*. 2012/06/28, 150(1), pp. 39–52. doi: 10.1016/j.cell.2012.05.026.

Wu, Y. and Zakian, V. A. (2011) 'The telomeric Cdc13 protein interacts directly with the telomerase subunit Est1 to bring it to telomeric DNA ends in vitro', *Proceedings of the National Academy of Sciences*, 108(51), pp. 20362–20369. doi: 10.1073/pnas.1100281108.

Wu, Z. *et al.* (2017) 'Rad6–Bre1-mediated H2B ubiquitination regulates telomere replication by promoting telomere-end resection', *Nucleic Acids Research*, 45(6), pp. 3308–3322. doi: 10.1093/nar/gkx101.

Wu, Z. *et al.* (2018) 'Rad6-Bre1 mediated histone H2Bub1 protects uncapped telomeres from exonuclease Exo1 in Saccharomyces cerevisiae', *DNA Repair*, 72, pp. 64–76. doi: https://doi.org/10.1016/j.dnarep.2018.09.007.

Xu, B. and Clayton, D. A. (1996) 'RNA-DNA hybrid formation at the human mitochondrial heavy-strand origin ceases at replication start sites: an implication for RNA-DNA hybrids serving as primers.', *The EMBO journal*, 15(12), pp. 3135–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8670814%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC450256.

Xu, L. *et al.* (2009) 'TEN Is Essential for CDC13-Mediated Telomere Capping', *Genetics*, 183(3), pp. 793–810. doi: 10.1534/genetics.109.108894.

Xu, Z. *et al.* (2015) 'Two routes to senescence revealed by real-time analysis of telomerase-negative single lineages', *Nature Communications*. Nature Publishing Group, 6(May), pp. 1–10. doi: 10.1038/ncomms8680.

Yasuhara, T. *et al.* (2018) 'Human Rad52 Promotes XPG-Mediated R-loop Processing to Initiate Transcription-Associated Homologous Recombination Repair', *Cell*. Elsevier Inc., 175(2), pp. 558-570.e11. doi: 10.1016/j.cell.2018.08.056.

Ye, J. *et al.* (2010) 'TRF2 and Apollo Cooperate with Topoisomerase 2α to Protect Human Telomeres from Replicative Damage', *Cell*, 142(2), pp. 230–242. doi: 10.1016/j.cell.2010.05.032.

Yoo, H. H. and Chung, I. K. (2011) 'Requirement of DDX39 DEAD box RNA helicase for genome integrity and telomere protection', *Aging Cell*, 10(4), pp. 557–571. doi: 10.1111/j.1474-9726.2011.00696.x.

Yu, T.-Y., Kao, Y. and Lin, J.-J. (2014) 'Telomeric transcripts stimulate telomere recombination to suppress senescence in cells lacking telomerase', *Proceedings of the National Academy of Sciences*, 111(9), pp. 3377–3382. doi: 10.1073/pnas.1307415111.

Zaitsev, E. N. and Kowalczykowski, S. C. (2000) 'A novel pairing process promoted by Escherichia coli RecA protein: Inverse DNA and RNA strand exchange', *Genes and Development*, 14(6), pp. 740–749. doi: 10.1101/gad.14.6.740.

Zhang, J. *et al.* (2019) 'Alternative Lengthening of Telomeres through Two Distinct Break-Induced Replication Pathways Article Alternative Lengthening of Telomeres through Two Distinct Break-Induced Replication Pathways', *CellReports*. ElsevierCompany., 26(4), pp. 955-968.e3. doi: 10.1016/j.celrep.2018.12.102.

Zhang, Q. *et al.* (2006) 'hnRNP A1 associates with telomere ends and stimulates telomerase activity hnRNP A1 associates with telomere ends and stimulates telomerase activity', 12(6), pp. 1116–1128. doi: 10.1261/rna.58806.proteinaceous.

Zhao, H. *et al.* (2018) 'RNase H eliminates R-loops that disrupt DNA replication but is nonessential for efficient DSB repair', *EMBO reports*, p. e45335. doi: 10.15252/embr.201745335.

Zhou, B. O. *et al.* (2011) 'Histone H4 Lysine 12 Acetylation Regulates Telomeric Heterochromatin Plasticity in Saccharomyces cerevisiae', *PLOS Genetics*. Public Library of Science, 7(1), p. e1001272. Available at: https://doi.org/10.1371/journal.pgen.1001272.

Zhu, X. and Gustafsson, C. M. (2009) 'Distinct differences in chromatin structure at subtelomeric X and Y' elements in budding yeast', *PloS one*. Public Library of Science, 4(7), pp. e6363–e6363. doi: 10.1371/journal.pone.0006363.

Zimmer, A. D. and Koshland, D. (2016) 'Differential roles of the RNases H in preventing chromosome instability', *Proceedings of the National Academy of Sciences of the United States of America*, 113(43), pp. 12220–12225. doi: 10.1073/pnas.1613448113.

Acknowledgements

I would like to thank everyone who participated directly or indirectly in this project.